Journal of Cellular Biochemistry 28:229–239 (1985)

# Cell Surface Galactosyltransferase:

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A cell surface UDP-galactose:N-acetylglucosamine galactosyltransferase (GT) has been directly localized on bovine cells in tissue culture by immunohistochemical techniques. A conventional rabbit heteroantiserum was prepared against an affinity-purified soluble form of GT from bovine milk, and a monospecific IgG fraction was isolated by affinity chromatography on a GT adsorbent. As demonstrated by indirect immunofluorescence, antigen to this antibody is present on the surface of all three bovine cell lines tested. It was uniformly distributed over the exposed membrane surface of fixed cells. Exposure of living cells to the anti-GT antibody resulted in its time-dependent aggregation in the plane of the membrane. Antigen (GT) was released from the membrane surface by trypsin digestion, and its reappearance required protein synthesis, since cycloheximide effectively prevented repopulation of the cell surface.

**Key words: cell surface, galactosyltransferase, immunochemical localization**

The glycosyltransferases are a family of membrane-bound enzymes that participate coordinately in the biosynthesis of the carbohydrate moieties of glycoproteins and glycolipids. Specific glycosyltransferases have been demonstrated at two distinct intracellular membrane sites, the rough endoplasmic reticulum and the Golgi apparatus, where assembly of the mannose/N-acetylglucosamine core and terminal glycosylation take place, respectively [17].

An additional functional role of glycosyltransferases in intercellular adhesion has been postulated. As envisioned, adjacent cells are bound together by enzyme-substrate interactions between glycosyltransferases and their corresponding oligosaccharide acceptors [15,22]. As a prerequisite, this hypothesis requires glycosyltransferases to be externally oriented plasma-membrane constitutents. Consequently a variety of methods have been employed in an attempt to detect such a distribution. These methods include autoradiography of sugars transferred from exogenously added

Received September 21, 1984; revised and accepted January 11, 1985.

nucleotide sugars, as well as glycosylation (by intact cells) of exogenously added glycoprotein or glycosaminoglycan acceptors, endogenous cell surface constituents, monosaccharides covalently linked to agarose beads, and glycolipids covalently attached to glass beads (comprehensively reviewed in Pierce et al [13] and Shur [19]). Although these methods clearly detect glycosyltransferase activity, because of their indirect nature, the localization of glycosyltransferase activity to the cell surface has been questioned [5,11].

In this study, we report the development of a sensitive and specific antiserum directed against an affinity-purified bovine UDP-galactose:N-acetylglucosamine galactosyltransferase (GT). Using this antiserum as our analytical probe, we have directly demonstrated a cell surface GT on bovine cells in tissue culture by indirect immunofluorescence. Part of this work has appeared in abstract form [18].

## MATERIALS AND METHODS

### Purification of Bovine UDP-galactosyltransferase

Soluble bovine GT was purified from raw skim milk by sequential affinity chromatography on UDP-Sepharose-4B and α-lactalbumin-Sepharose-4B. Preparation of the affinity adsorbents, enzyme assays, and characterization of the purified enzyme were performed as described previously [3]. Purified GT was routinely stored at 4°C, bound to α-lactalbumin-Sepharose-4B. Prior to use, the enzyme was eluted from the adsorbent, immediately concentrated to 1.0–2.0 mg/ml protein in a collodion bag apparatus (Schleicher and Schuell, Keene, NH, 25-kD cutoff), and dialyzed at 4°C against $Ca^{2+}$, $Mg^{2+}$-free phosphate-buffered saline (PBS) containing 1.0 mg/ml N-acetylglucosamine.

### Preparation and Characterization of Antisera to Bovine UDP-galactosyltransferase

Affinity-purified GT (1.0 mg protein) in 1.0 ml of PBS was emulsified with 1.0 ml of Freund's complete adjuvant and injected intradermally at multiple sites on the backs of New Zealand white female rabbits (3–4 months old). This procedure was repeated three times at 2-week intervals using 0.5 mg of protein in 1.0 ml of PBS emulsified in 1.0 ml Freund's complete adjuvant. The animals were bled 14 days following the last injection and subsequently at 7-day intervals. Booster injections containing 0.1–0.2 mg protein in 1.0 ml PBS emulsified with 1.0 ml of Freund's incomplete adjuvant were given intramuscularly at multiple sites when required to raise or restore antiserum titers.

All serial bleedings from the immunized rabbits were screened for anti-GT antibody by the Ouchterlony double-diffusion technique. Preimmune sera from the same animals served as controls. Sera that were positive in the double-diffusion assay were further characterized by immunoelectrophoresis and immunoprecipitation of affinity-purified bovine GT enzymatic activity either directly or after the addition of a suspension of Pansorbin (Calbiochem, La Jolla, CA). Sera with comparable anti-GT titers were pooled and monospecific anti-GT antibody was isolated by chromotography at 4°C on a resin of AffiGel-10 (BioRad, Richmond, CA) to which affinity-purified GT (0.5 mg protein per 1 ml of resin) was covalently linked. Bound antibody was recovered by elution with 3 M NaSCN, pH 7.0, concentrated, and dialyzed against multiple changes of PBS in a collodion bag apparatus (75-kD cutoff). For

control experiments a partially purified IgG fraction was prepared from preimmune sera by ammonium sulfate precipitation followed by dialysis against 0.01 M sodium phosphate buffer, pH 7.0, and chromatography on DEAE cellulose. Preimmune and immune sera were stored at −20°C. The affinity-purified anti-GT antibody was stored at either 4°C or −20°C.

### Cell Cultures

The bovine tissue culture lines used in this study included the following: adult kidney epithelial cells (MDBK; ATCC No. CCL22), embryonic trachea fibroblasts (EBTr, ATCC No. CCL 44), and turbinate fibroblasts (BT, ATCC No. 1390). MDBK and EBTr cells were maintained in complete medium consisting of Eagle's minimal essential medium with Earles' salts (MEM) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY), nonessential amino acids (1.0%), penicillin (100 U/ml), and streptomycin (100 μg/ml). The BT cell line was maintained in the same medium except that 10% horse serum was substituted for fetal calf serum. All cells were grown at 37°C in a humidified atmosphere of 95% air/5% $CO_2$.

### Fluorescent Labeling of Cells

Cells were plated onto nitric acid-cleaned, ethanol-sterilized coverslips in 35-mm plastic dishes at approximately $1 \times 10^4$ cells per dish 2 days prior to antibody treatment. Each dish contained 2 ml of the appropriate growth medium plus 5 mM MOPS (pH 7.1).

Cells were fixed and then stained to determine the normal surface membrane distribution of GT as follows: Subconfluent coverslip cultures were rinsed and fixed with 3.7% formaldehyde/5% sucrose in PBS for 15 min at room temperature. They were then rinsed and treated for 30 min at 37°C with the anti-GT antibody (primary antibody) diluted in PBS containing 1 mg/ml rabbit serum albumin. The cells were again rinsed and counterstained for 15 min at 37°C with fluorescein- or rhodamine-conjugated goat anti-rabbit antibody (secondary antibody) (Cappel Labs, Cochranville, PA). After this incubation, the cells were rinsed, mounted on slides in PBS:glycerol (1:1), and sealed with nail polish.

Live cells were stained to determine the effects of antibody on the surface redistribution of GT as follows: Subconfluent coverslip cultures were rinsed and incubated with the primary antibody for 30 min at 37°C. After this incubation the cells were rinsed and treated with the secondary antibody for 15 min at 37°C. They were again rinsed, fixed, and mounted as described above. Trypan blue exclusion was used to estimate cell viability.

For the studies in which metabolic inhibitors were used, the coverslip cultures were first incubated with 10 mM each of 2-deoxyglucose (Sigma Chemical Co., St. Louis, MO) and sodium azide in complete medium for 30 min at 37°C. They were then incubated sequentially with the primary antibody plus inhibitors (10 mM each) and then the secondary antibody and fixed as described above. For experiments in which the proteolytic release of GT from the cell surface was investigated, a more adherent line of MDBK cells was selected by passaging the cells several times and, at each passage, discarding the cells that initially came off after trypsin treatment and only subculturing those that remained. Subconfluent coverslip cultures of these cells were first preincubated with or without 20 μg/ml cycloheximide (Sigma) for 30 min at 37°C, then exposed to trypsin/EDTA (0.125% Trypsin–0.05% EDTA diluted in

PBS) for approximately 20 min at room temperature (to the point where cells were just beginning to lift off the coverslip). The coverslips were then returned to complete medium (with or without cycloheximide) and allowed to incubate 90 min at 37°C. The coverslip cultures were then reacted with the primary and secondary antibody and fixed as described above.

To visualize F-actin fibers, NBD-Phallocidin (NBD-Ph, Molecular Probes, Inc., Junction City, OR) was used following the protocol recommended by the supplier. Briefly, the coverslip cultures were stained with the anti-GT antibody, counterstained with rhodamine-conjugated goat anti-rabbit IgG, incubated in −20°C acetone for 5 min, washed twice in PBS, and stained with NBD-Ph (165 ng/ml) in PBS for 20 min at room temperature. The coverslips were washed and mounted as described above.

### Photomicroscopy

Slides were viewed with a Zeiss Universal microscope equipped with epifluorescent illumination and appropriate filters for fluorescein or rhodamine fluorescence. Photographs were taken with either a 25× or 40× objective. Color fluorescence was recorded on Kodak Ektachrome ASA 400 film. An identical exposure time was used for antisera with positive fluorescence and the corresponding negative control serum.

## RESULTS

### Antiserum Characterization

A soluble form of bovine GT found in milk was purified to constant specific activity by sequential affinity chromatography on UDP-agarose and α-lactalbumin agarose. The bovine GT is a glycoprotein and exists in three different molecular weight forms of 43, 49, and 54 kD [3]. These three molecular weight variants apparently arise from the initial proteolytic event that releases the membrane-bound form of the enzyme [23]. The higher-molecular-weight forms can be converted to the lower by controlled trypsin digestion [14].

Individual affinity-purified bovine GT preparations, consisting of the 47-kD and 43-kD size variants, were used to elicit antibody production. The purity of the GT preparations was established by SDS-PAGE, determination of specific activity, and amino acid composition. Results were comparable to those previously described [3,25].

Figure 1 (upper panel) shows a typical Ouchterlony double-diffusion pattern for antisera from three animals versus affinity-purified bovine GT. A single precipitin band is observed, consistent with the presence of a single antigen-antibody complex. The antisera from the three individual animals are apparently homologous in their ability to precipitate the bovine GT as judged by the absence of spurs in the Ouchterlony plate. The immunoelectrophoresis pattern (data not shown) also indicated a single precipitating antibody. The crude antisera, partially purified IgG fractions, and monospecific antisera were also examined for their ability to precipitate GT enzymatic activity from solution. As shown in Figure 1 (lower panel), greater than 90% precipitation of GT activity was observed in the presence of appropriate amounts of antiserum. In contrast, normal rabbit serum controls showed no precipitation of the GT enzymatic activity at comparable IgG concentrations.

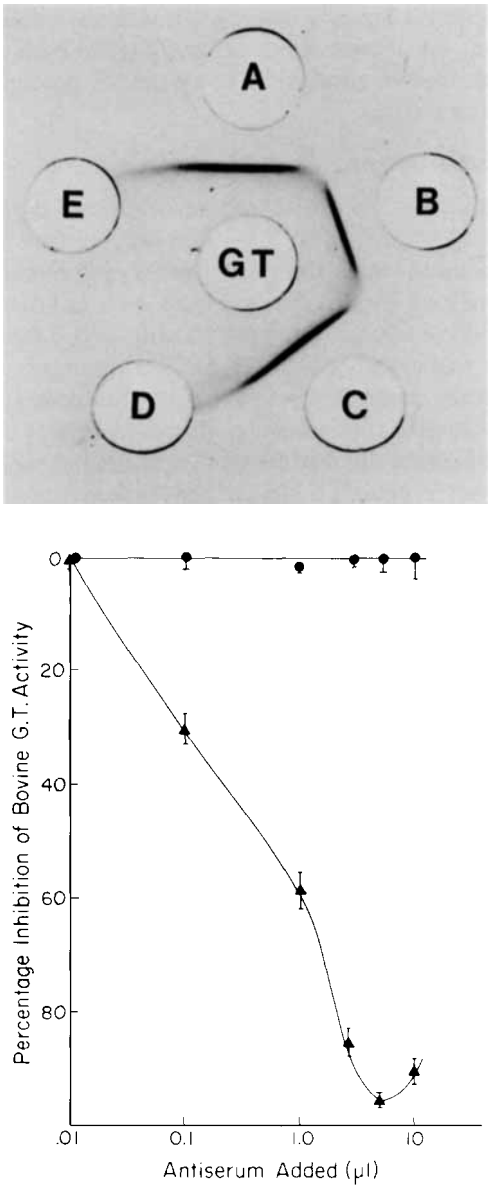


Fig. 1. Upper panel) Double-immunodiffusion analysis of rabbit antisera to affinity-purified bovine GT. GT and rabbit antisera were prepared as described in Materials and Methods. The precipitin bands were stained with Coomassie blue. Center well: UDP-galactosyltransferase (0.06 μg protein); peripheral wells: rabbit antisera (10 μl) from three individual animals (A,B,C) or equal volume of preimmune serum (D,E). Lower panel) Precipitation of bovine UDP-galactosyltransferase activity with rabbit anti-bovine GT antibody. Affinity-purified bovine GT was incubated for 60 min at 22°C with 25 μl of rabbit antiserum (▲—▲) or preimmune serum (●—●) at various dilutions in PBS containing 1% rabbit serum albumin. After incubation, the mixture was centrifuged at 10,000g for 2 min. Aliquots (25 μl) were assayed for GT activity by measuring the transfer of $^{3}$H-galactose to the acceptor sugar N-acetylglucosamine. The neutral enzymatic product $^{3}$H-lactosamine was separated from the reaction mixture by chromatography on Dowex-1-X8 ($Cl^-$ form) and quantitated in a liquid scintillation spectrophotometer. The verticle bars indicate the mean value ± SD.

Lastly, this antiserum is highly cross-reactive with the soluble form of GT found in the serum of human, rat, mouse, hamster, and dog. In each case, titration of the serum GT from these species resulted in a maximum precipitation of enzymatic activity of 90% (data not shown).

### Detection of Cell Surface-Associated GT

Our initial experiments were designed to determine if GT is a cell surface membrane component of MDBK cells. To ensure only surface detection, cells were prefixed under conditions in which the plasma membrane remained impermeable to macromolecules as analyzed by exposure of fixed cells to an anti-nuclear envelope antiserum and trypan blue exclusion (data not shown). When these cells were stained with the monospecific rabbit anti-bovine GT antibody (primary antibody) and counterstained with fluorescein-conjugated goat anti-rabbit antibody (secondary antibody), a weak but clearly discernible microgranular fluorescence was observed, suggesting that the GT antigen was uniformly distributed over the entire surface of the cell (Fig. 2B,C). Anti-GT antibody produced a similar cell surface staining pattern on bovine turbinate (BT) and bovine embryonic trachae (EBTr) fibroblast cells. Preimmune serum did not generate significant fluorescent staining (Fig. 2H).

The cell surface GT on MDBK, BT, and EBTr cell lines was aggregated or patched by exposure of living cells to either the primary antibody alone or sequentially to the primary and secondary antibody prior to fixation (Figs. 2E and 4A). The antibody-induced aggregation of GT effectively amplified the fluorescence signal, resulting in the appearance of bright antigen-antibody clusters in a stippled or punctate pattern over a large majority of the cell surface. While the nonmotile margins of individual cells in groups were clearly stained (Fig. 2D,E), the leading ruffled, apparently motile cell margins were conspicuously devoid of fluorescence, probably indicating a clearance of the antibody cross-linked antigen from the active cell edges [8]. The patching of the cell surface GT was not affected if cells were preincubated (30 min) with the metabolic inhibitors sodium azide and 2-deoxyglucose; however, the leading ruffled, motile cell margins were stained after such treatment, suggesting the inhibition of clearance of fluorescence from these membrane areas (Fig. 2F,G). In control experiments, both prefixed or living cells treated with preimmune serum or immune serum adsorbed with GT covalently linked to Sepharose-4B lacked significant fluorescence, resulting in a pattern similar to that shown in Figure 2H. In summary, these experiments demonstrate that GT is distributed on the cell surface of the MDBK, BT, and EBTr cells and is mobile in the plane of the membrane.

### Proteolytic Release of the Cell Surface GT

To establish that the visualized cell surface GT is an endogenous cellular component and not the result of spurious adsorption of exogenous enzyme from the growth medium, conditions were established to proteolytically release surface GT from living cells by treating the cells with trypsin under conditions in which they still remained attached to coverslips. The cellular origin of GT was established by the demonstration that, after treatment with trypsin, the reappearance of the cell surface GT required protein synthesis. While cells incubated in the absence of cycloheximide showed a repopulation of the cell surface with GT within 90 min (Fig. 3C,D), cycloheximide (20 $\mu$g/ml) prevented repopulation of the cell surface with the antigen (Fig. 3A,B). In addition, exogenously added affinity-purified GT was not adsorbed

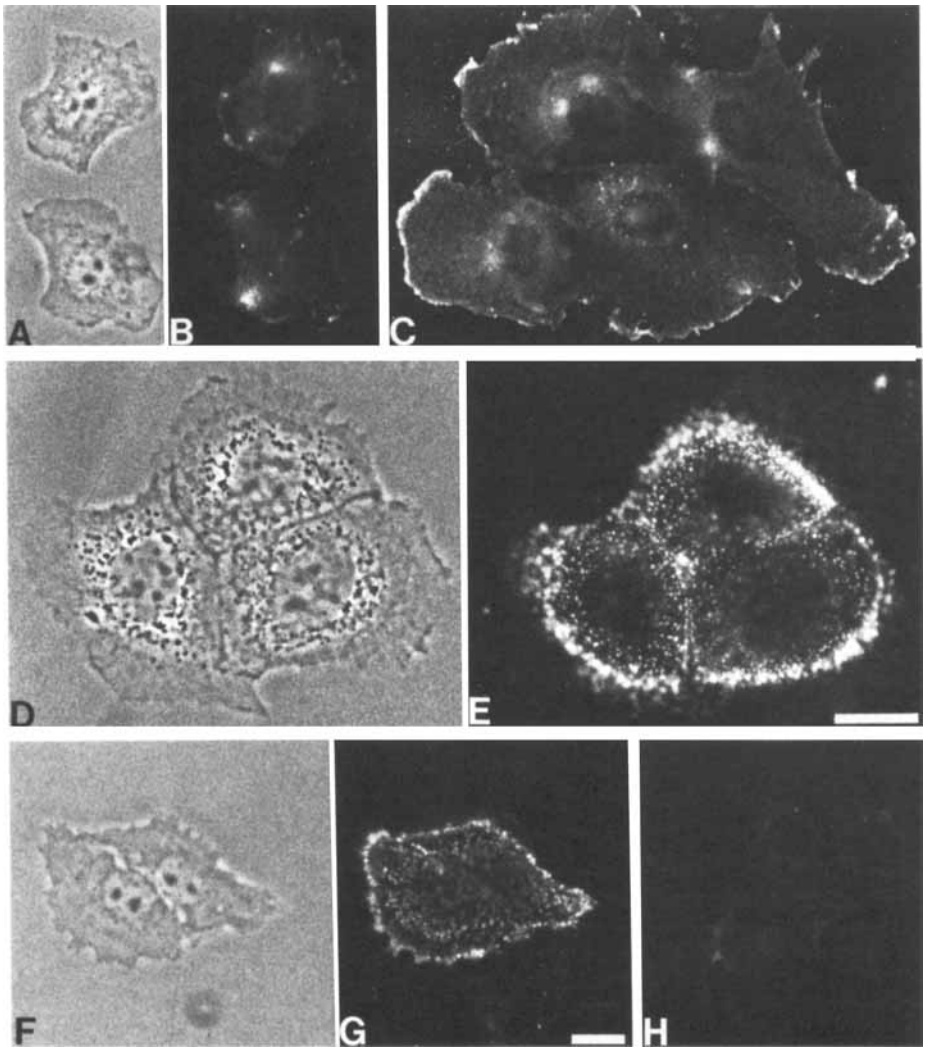


Fig. 2. Immunolocalization and antibody-induced redistribution of GT on the cell surface of MDBK cells. Cells were fixed in formaldehyde, under conditions in which they remained impermeable to macromolecules, and were stained by indirect immunofluorescence with monospecific anti-bovine GT antibody as described in Materials and Methods (panels A–C). For the antibody-induced redistribution of GT, living cells were sequentially incubated, in the absence (panels D,E) or presence (panels F,G) of 10 mM sodium azide and 2-deoxyglucose, with monospecific anti-bovine GT antibody, then with the secondary antibody, and then were fixed. Preimmune serum gave a staining pattern as shown in panel H. The plane of focus was on the perimeter of the cells. Bar = 5 $\mu$m.

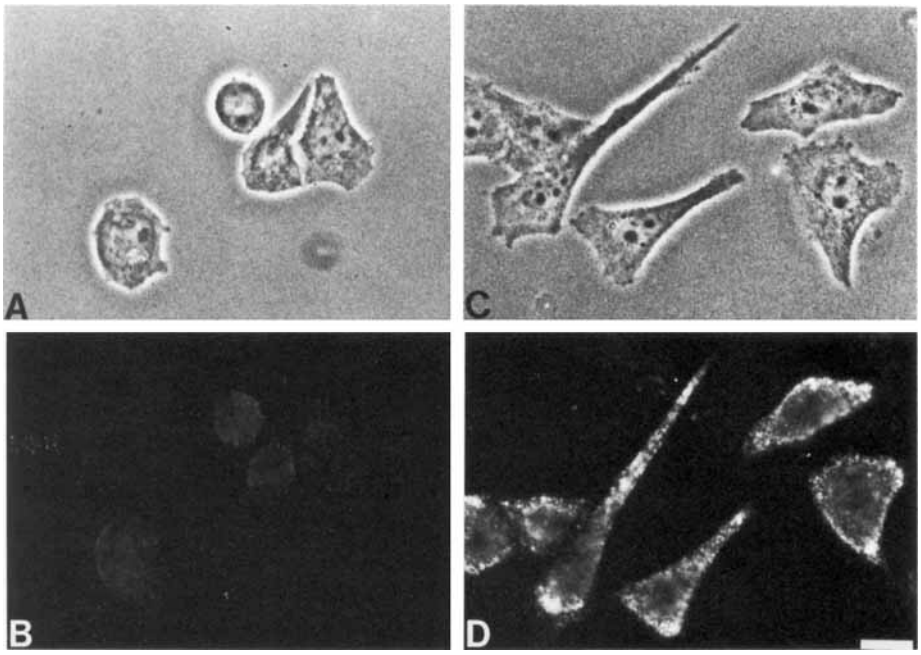


Fig. 3. Effects of trypsin treatment on the cell surface galactosyltransferase. Coverslip cultures of MDBK cells were treated with trypsin/EDTA at room temperature, washed, and incubated at 37°C for 90 min in complete medium in the presence (panels A,B) or absence (panels C,D) of cycloheximide (20 μg/ml). Cells were stained with monospecific anti-bovine GT antibody, counterstained, and fixed as described in Materials and Methods. Film exposure times were identical for panels B and D. In a parallel set of experiments, incorporation of $^{35}$S-methionine into TCA-precipitable material indicated that, under these conditions, protein synthesis was inhibited by aproximately 80%. Bar = 5 μm.

onto the cell surface of trypsin-treated cells in detectable quantities as monitored by indirect immunofluorescence (data not shown).

## Association of Cell Surface Antibody-Induced Clusters of GT With Intracellular Actin Filaments

Cell surface integral membrane proteins appear to have the common characteristic of forming transmembrane linkages to intracellular actomyosin-containing filaments subsequent to antibody-induced clustering [1,10]. To examine the distribution of antibody-induced GT clusters on the cell surface relative to these filaments, BT cells were exposed sequentially to primary and secondary antibody, fixed, permeabilized, and stained with nitrobenzoxadiazole (NBD)-phallacidin, which specifically decorates actin-containing fibers [2]. As seen in Figure 4, the GT clusters are arranged in linear arrays along the major axis of the cell and are coincident with the underlying stress fibers. When these images are projected together, the GT clusters are clearly seen to be primarily arranged directly over the actin-containing filaments. These results not only indicate that GT is a cell surface constituent, but also suggest that the cell surface GT is a transmembrane protein or is associated with transmembrane proteins.

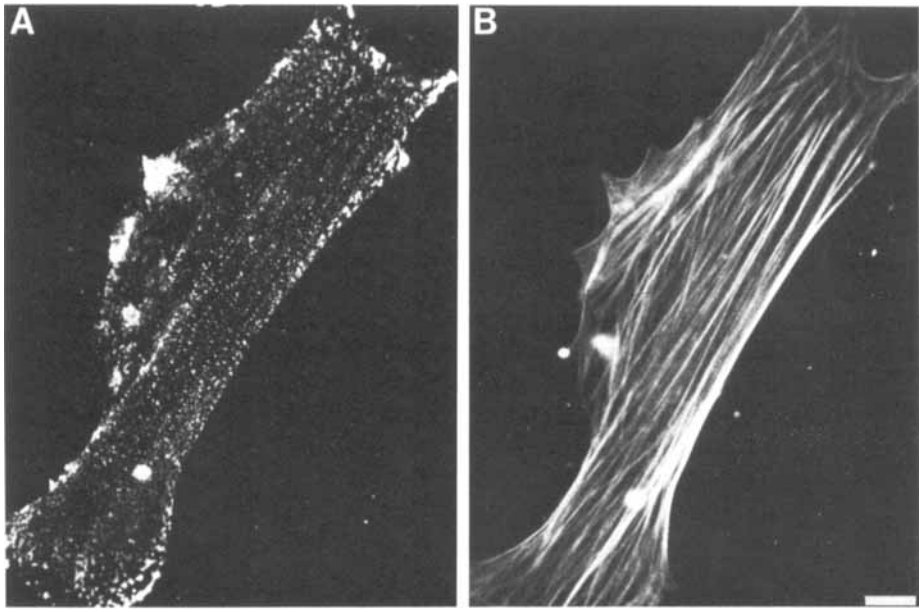


Fig. 4. Relationship of cell surface antibody-induced clusters of cell surface GT to intracellular actin filaments in BT cells. Living bovine turbinate cells were sequentially exposed to monspecific anti-bovine GT antibody and rhodamine goat anti-rabbit IgG (panel A). After fixation in formaldehyde, the cells were permeabilized with acetone at −20°C and treated with NBD-phallacidin to simultaneously visualize the actin-containing filaments (panel B). Bar = 5 μm.

## DISCUSSION

The immunohistochemical data in this paper strongly support the conclusion that galactosyltransferase is an endogenous cell surface component of the tissue culture cells examined. The use of immunohistochemical studies to localize a specific antigen rely for their interpretation on the specificity of the antibody and the ability to relate the observed immunohistochemical pattern to a specific cellular structure.

With regard to the specificity of the antiserum, we have used a soluble form of bovine GT, affinity-purified to apparent homogeneity, to elicit an antibody response. This antigen preparation was free from detectable bovine immunoglobins, the presence of which appears to have been a problem in the development of specific anti-GT antisera in at least one reported study [27]. Furthermore, we have shown that both immune serum and monospecific antibody, isolated by chromotography on an adsorbent prepared from the affinity-purified GT, can precipitate GT enzymatic activity from solution and can recognize denatured GT by immunoblot analysis (data not shown). Lastly, immune serum, partially purified IgG from immune serum, and monospecific antibody each gave similar immunohistochemical patterns on tissue culture cells.

Our conclusion that GT is localized to the plasma membrane is based on the following experimental considerations and observations. First, we have chosen fixation conditions in which the plasma membrane is not permeable to antibodies as

judged by trypan blue exclusion and the inability to decorate the nuclear envelope using an appropriate antiserum. Second, when living cells were used instead of prefixed cells, it was observed that the cell surface GT could be aggregated or patched in the plane of the membrane. The use of metabolic inhibitors did not prevent the initial patches from forming but did prevent the clearance of the antibody cross-linked antigen from the active cell edges. Finally, we have demonstrated that the antigen, after proteolytic release from the surface of living cells, requires protein synthesis for repopulation of the plasma membrane. These observations collectively support the conclusion that GT is a cell surface component actively synthesized by the cell lines examined.

Several groups have reported the development of conventional polyclonal antisera against a soluble form of native GT from human serum [7] and human milk [4], denatured GT from bovine milk [9] and monoclonal antisera against native GT from bovine milk [26]. Several of these antisera have been used to localize GT, at the light microscope level, to the Golgi region in interphase cells [4,9,24], mitotic cells [9], and tissue sections [12]. At the electron microscope level, GT distribution was observed to be limited to two or three trans cisternae of the Golgi apparatus indicating a compartmentalization of GT in the cisternal stack [16]. Restricted distribution in the Golgi complex of other enzymatic activities including thiamine pyrophosphatase and acid phosphatase has also been observed (for review, Farquhar and Palade [6]).

Immunohistochemical localization of galactosyltransferase to the cell surface of acetone-fixed human fibroblast and HeLa cells has been addressed, with negative results reported [4]. In contrast, with the same conventional antibody, positive histochemical staining for GT in tissue sections was demonstrated on the surface of epithelial cells in stomach and pancreas [12]. One explanation for these dissimilar results is the lack of preservation of the cell surface antigen during acetone fixation of the tissue culture cells.

Our demonstration of a cell surface galactosyltransferase raises the question of its functional significance. Does its presence on the plasma membrane reflect imperfect segregation of the enzyme from its intracellular Golgi membrane compartment during normal membrane cycling or, as originally suggested, is the galactosyltransferase involved in intercellular recognition and adhesion? In support of a functional role, several studies have shown that exposure of living cells to reagents that can potentially perturb cell surface galactosyltransferase activity has profound effects on the social behavior of the cells examined [19–21]. With the development of immunologic probes to directly demonstrate that the perturbed galactosyltransferase activities are in fact plasma membrane constituents, it should be possible to begin to analyze their functional role.

## ACKNOWLEDGMENTS

We wish to thank Drs. S. Kaufmann, R. Luftig, M. Satake, B. Shur, and R. Schnaar for many helpful suggestions. The technical assistance of Mr. Charles M. Ewing, the photographic assistance of Mr. Chester Reather, and the editorial assistance of Mrs. Sandy Smith is gratefully acknowledged. This study was supported in part by a grant from the American Cancer Society (Maryland Chapter) and a contract (CB-84260) and grant (CA-30471) from the National Cancer Institute.

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