CELL CONTACT DEPENDENCE OF SURFACE GALACTOSYLTRANSFERASE ACTIVITY AS A FUNCTION OF THE CELL CYCLE

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ABSTRACT

Mitotic, nonmalignant Balb/c 3T3 cells exhibit endogenous, surface galactosyltransferase activity that does not require intercellular contact throughout the assay period. In this respect, mitotic 3T3 cells resemble malignant Balb/c 3T12 cells which similarly show no contact requirement for optimum transferase activity in any phase of their cell cycle.

Previously, it was shown that randomly growing populations of 3T3 cells have lower galactosyltransferase activity when assayed under conditions which decreased cell contact. This led to the conclusion that these normal (3T3) and malignant (3T12) cells differed in that intercellular contact is required for optimum activity of surface galactosyltransferases on the normal cell type.

The present data indicate that mitotic 3T3 cells may be capable of expressing enzyme activities exhibited at all times by malignant cells. That is, mitotic 3T3 cells and randomly growing 3T12 cells may readily catalyze galactosyltransferase reactions between enzymes and acceptors on the same cell. Interphase 3T3 cells, on the other hand, might require that enzymes glycosylate acceptors on adjacent cells. A model is proposed that suggests that changes in the spatial arrangement of surface enzymes and acceptors or variations in the fluidity of the cell membrane can account for this contact-related glycosylation.

Recent reports have suggested the presence of glycosyltransferases on the external surfaces of cell plasma membranes in five different systems. In the chick neural retina (14), these transferases have been implicated in the process of cell recognition via enzyme-acceptor interactions. Jamieson et al. (8) and Bosmann (2) have postulated that, in blood platelets, surface transferases may bind to collagen-linked glycosides and thereby initiate hemostasis. In early chick embryos (17), Shur and Roth have found a spatial and temporal correlation between surface glycosylation potential and known inductive events. Along the same lines, Weiser (18) has reported transferase activities in rat intestine that reflect the developmental state of the intestinal epithelial cells. Finally, a number of glycosyltransferases and their acceptors have been located on a variety of cultured cell lines (3, 15).

The glycosyltransferases catalyze the following general reaction:

\[ \text{nucleotide-X} + \text{acceptor} \rightarrow \text{nucleotide} + \text{acceptor-X} \]
cells, when in mitosis, display lectin agglutinability dispersed and random on both normal and malignant cells show no difference in the incorporation of the cell cycle. The results suggest that mitotic 3T3 in normal 3T3 cells was tested at various stages of 3T3 cell, if the enzymes and acceptors were not equally capable of this proximity, glycosylation would require intercellular contact; that is, enzymes would utilize acceptors on adjacent cells (trans-glycosylation).

Mobility differences between surface components on normal and malignant cells have previously been proposed (13). Using hemocyanin as a marker for the surface distribution of concanavalin A (Con A), Rosenblith et al. (13) showed that the inherent distribution of Con A-binding sites was dispersed and random on both normal and malignant cells. However, these molecules may be capable of greater mobility within the membrane of malignant cells. This increased mobility has been cited to explain the relative ease of agglutination of malignant cells by plant lectins and to account for the clustering of sites on malignant cells reported earlier (9). It has also been demonstrated that normal mouse (5, 16) and hamster (16) cells, when in mitosis, display lectin agglutinability similar to malignant variants.

In the present study, the contact requirement for endogenous surface galactosyltransferase activity in normal 3T3 cells was tested at various stages of the cell cycle. The results suggest that mitotic 3T3 cells show no difference in the incorporation of labeled galactose when assayed under conditions allowing either greater or lesser cell contact. Interphase 3T3 cells maintain the contact-dependent incorporation of galactose previously demonstrated for randomly growing 3T3 cells. Thus, galactosylation of normal, mitotic cells seems to have no contact requirement. This is similar to the result for galactosylation of 3T12 cells at all phases of their cell cycle. These data imply, as do others (5, 16), that cell transformation may be characterized by permanent cell surface alterations which resemble normal cells only in mitosis.

MATERIALS AND METHODS

Cells

Normal, nonmalignant 3T3 and spontaneously transformed, highly malignant 3T12 mouse cells were grown on 150-mm glass Petri plates in Dulbecco's modified Eagle medium supplemented with 10% calf serum (DECS) (1). For subculturing, cells were harvested (15) with 0.1% trypsin (Difco Laboratories, Detroit, Mich. 1:250) in Ca++-, Mg++-, and glucose-free Hanks' basic salt solution (HBSS). All 3T3 cells were collected from cultures containing less than 10^4 cells/cm^2. All 3T12 cells were collected from cultures containing greater than 5 x 10^4 cells/cm^2. It has previously been shown (15) that both of these cell types have highest endogenous galactosyltransferase activities when collected from cultures in the stated density ranges.

Cell Cycle Designation

The cells were grouped into three categories with regard to their position in the cell cycle. Mitotic Populations: These cells were obtained by treating 2- to 3-day old cell cultures with 0.1 μg/ml of Colcemid (Grand Island Biological Co., Grand Island, N. Y.) in DECS for 4 h to increase the number of mitotic cells. The cultures were gently rinsed with DECS using a Pasteur pipette to dislodge the loosely attached mitotic cells. The efficiency of this collection procedure was tested by determining the percentage of cells showing mitotic figures in stained slide preparations of the cells (Table I). Mitotic figure determination is a conservative estimate of mitosis, since it fails to detect very early and very late stages of mitosis.

Colcemid-Treated, Interphase Populations: The cells remaining on the tissue culture plates after collection of mitotic cells as described above were presumed to be in interphase. They were harvested by trypsinization after removal of mitotic cells. The percentage of these cells showing mitotic figures is given in Table I.

Randomly Growing Populations: These cells were collected by trypsinizing 2- to 3-day old
The cell suspension was divided equally to give a final concentration of 5.27 ~m at 250 °C. [~4C]UDP-Gal (New England Nuclear, Boston, Mass.) were added to a 3-ml conical centrifuge tube containing assays, 600 ~l of a solution of intact cells in medium “J” under conditions previously described (15). For these assays, 600 ~l of a solution of intact cells in medium “J” were added to a 3-ml conical centrifuge tube containing [~4C]UDP-Gal (New England Nuclear, Boston, Mass.) to give a final concentration of 5.27 ~m at 250 mCi/mm. The cell suspension was then divided equally between another 3-ml conical centrifuge tube and a 2-ml flat-bottomed shell vial with a magnetic stirring bar externally attached to its bottom. The cells in the conical centrifuge tube (stationary incubation) fell to the bottom of the tube within 5-10 min. These cells remained in a loose pellet throughout the reaction except when they were resuspended for sampling. The shell vial, with an identical aliquot of the cell suspension (spinning incubation), was spun at approximately 200 rpm on an immersible magnetic stirrer (15). In this case the cells remained in suspension throughout the reaction period. The two reaction tubes were incubated at 37°C water bath for 2 h. At 30-min intervals, 25-~l samples were taken from each incubation and the reaction stopped by adding 5 ~l 0.3 M EDTA, pH 6.5, to each aliquot. Incorporation of galactose onto endogenous acceptor was determined by subjecting 25 ~l of each aliquot to high voltage paper electrophoresis in borate buffer (14). The reaction products remained at the origin and were counted in a liquid scintillation spectrometer.

**Washes and Preincubation**

After collection, all cells were washed twice and diluted to the appropriate cell concentration with medium “J” (glucose-, phosphate-, and bicarbonate-free HBSS with 10 mM MnCl₂ and 10 mM NaN₃, buffered with 10 mM HEPES [Calbiochem, San Diego, Calif.]). To avoid a precipitate, the medium was adjusted to pH 7.2 before the addition of the MnCl₂. Before being washed and suspended in medium “J”, 3T3 cells that were harvested with trypsin were preincubated by resuspending the cells in 10 ml DECS and incubating them for 30 min in a 25-ml DeLong flask on a gyratory shaker (180 rpm) at 37°C. Cell concentrations during this incubation did not exceed 10⁶ cells/ml. These cells were then washed and suspended in medium “J” as described above. This treatment increased incorporation of galactose by 3T3 cells when assaying surface galactosyltransferase activity (15). Although preincubation enhanced 3T12 cell galactosyltransferase activity towards exogenously added N-acetylglucosamine, it did not significantly alter endogenous activity and was therefore omitted (15).

**Transferase Assay**

Surface galactosyltransferase activity was determined under conditions previously described (15). For these assays, 600 ~l of a solution of intact cells in medium “J” were added to a 3-ml conical centrifuge tube containing [~4C]UDP-Gal to give a final concentration of 5.27 ~m at 250 mCi/mm. The cell suspension was then divided equally into 3T3 interphase population 500 58 11.4

| Cell type* | Total cells counted | Mitotic figures | Mitotic figures % |
|------------|---------------------|----------------|-----------------|
| 3T3 mitotic population | 419 | 245 | 58.4 |
| 3T3 interphase population | 500 | 42 | 8.4 |
| 3T12 mitotic population | 500 | 296 | 59.2 |
| 3T12 interphase population | 506 | 58 | 11.4 |

* After collection, the cells were washed, fixed, smeared on slides, and stained with hematoxylin. Cell counts were made to determine the percentage of mitotic figures.

**Transferase Activity toward N-Acetyl-Glucosamine**

Cellular Enzyme Activity: Incubations were carried out as described above except that, along with [~4C]UDP-Gal, N-acetyl-D-glucosamine was added to a final concentration of 1 mM in 800 ~l of cell suspension. The cell suspension was divided equally between spinning and stationary incubations. At 30-min intervals, 50-~l samples were taken from the stationary and spinning incubations, and these were centrifuged at 500 g for 10 min to pellet the cells. 25-~l samples were decanted from each supernate, and the reaction stopped by adding 5 ~l 0.3 M EDTA, pH 6.5 to each sample. Production of N-acetyl-lactosamine was determined by spotting 25 ~l of each sample for borate electrophoresis and scintillation counting as described above. The pelleted cells were washed and resuspended in medium “J” to determine the degree of galactose transfer to endogenous acceptors.

 Supernatant Control: To test for galactosyltransferase activity in the supernate, 800 ~l of cell suspension, as prepared above, were divided equally.

**Autoradiography**

For autoradiographic analysis of the transferase reaction on 3T3 monolayer cultures, the cells were grown to a density of 10⁶ cells/cm² on 35-mm Falcon tissue culture plates. The cultures were washed with HBSS and 0.5 ml of medium “J” containing [3H]UDP-Gal (2.6 ~m; 1.54 Ci/mmol) was added to each plate. The plates were incubated at 37°C for 3 h and then washed with HBSS and fixed with Bouin’s fluid. After several distilled water washes, the plates were dipped in Kodak NTB-2 Nuclear Track Emulsion, dried, and exposed for 3 wk at 4°C. The plates were then developed and stained with hematoxylin.

**Table 1**

| Cell type*                                                                 | Total cells counted | Mitotic figures | Mitotic figures % |
|--------------------------------------------------------------------------|---------------------|----------------|-----------------|
| 3T3 mitotic population                                                   | 419                 | 245            | 58.4            |
| 3T3 interphase population                                               | 500                 | 42             | 8.4             |
| 3T12 mitotic population                                                 | 500                 | 296            | 59.2            |
| 3T12 interphase population                                              | 506                 | 58             | 11.4            |

* After collection, the cells were washed, fixed, smeared on slides, and stained with hematoxylin. Cell counts were made to determine the percentage of mitotic figures.
between spinning and stationary incubation tubes containing neither sugar nucleotide nor exogenous acceptor. Both tubes were incubated at 37°C for 1 h. After this incubation period, 50-μl samples were removed from each, and the cells were pelleted by centrifugation at 550 g for 10 min. 25 μl of the supernate from spinning and stationary assays were added to incubation tubes (Pyrex culture tubes, size 6 × 50 mm) containing [14C]UDP-Gal and N-acetyl-D-glucosamine to final concentrations of 5.27 μM and 1 mM, respectively. The tubes were incubated under stationary conditions for 2 h. At the end of this incubation period 5 μl 0.3 M EDTA, pH 6.5, were added to each tube. 25 μl of each reaction mixture were spotted for borate electrophoresis and scintillation counting.

**Viability:** Cell viability was determined on the basis of trypan blue exclusion (10).

**RESULTS**

**Contact Dependence of Galactosyltransferase Activity by Intact, Random 3T3 Cells**

Randomly growing 3T3 cells incubated under spinning conditions incorporated two to three times less galactose onto surface acceptor than cells incubated under stationary conditions (Fig. 1).

**Contact Dependence of Galactosyltransferase Activity by Intact, Colcemid-Treated 3T3 Cells**

In direct contrast to the data shown in Fig. 1, Colcemid-treated, mitotic 3T3 cells (Fig. 2) gave similar reaction kinetics when assayed under spinning or stationary conditions. In order to determine whether this apparent lack of contact dependence was caused by Colcemid treatment alone, interphase 3T3 cells which had been treated with Colcemid were assayed for galactosyltransferase activity. The cells were harvested using trypsin and preincubated in DECS containing Colcemid (0.1 μg/ml). The data in Fig. 3 show that these interphase 3T3 cells had decreased transferase activity when assayed in a spinning reaction tube despite the prior 4-h treatment with Colcemid. This would suggest that the data obtained with mitotic cells were characteristic of cells in that phase of the cell cycle and not an artifact of Colcemid treatment.

Another difference in assay procedure which could affect the data obtained with mitotic 3T3 cells was the collection of mitotic cells without trypsin treatment and preincubation. Therefore, after their collection, mitotic cells were trypsinized for 15 min at 37°C with 0.1% trypsin containing Colcemid (0.1 μg/ml) and then preincubated for 30 min in DECS containing Colcemid at the same
concentration. Fig. 4 shows that after trypsin treatment and preincubation, mitotic cells were still able to catalyze the transferase reaction to the same extent whether incubated under spinning or stationary conditions.

**Contact Dependence of Galactosyltransferase Activity by Intact 3T12 Cells**

The data presented in Fig. 5 show that intact, randomly growing 3T12 cells exhibited no difference in the incorporation of galactose onto surface acceptors when assayed under spinning and stationary conditions. Further, Figs. 6 and 7 show, respectively, that mitotic and interphase 3T12 cells collected after a 4-h Colcemid treatment, exhibited no difference in the rate or extent of incorporation under the above assay conditions.

**Galactosyltransferase Activity by 3T3 Cells in Monolayer Cultures**

Fig. 8 shows the result of autoradiographs of 3T3 cells in monolayer cultures labeled with \[^{3}H\]UDP-Gal. Most of the cells show no labeling above background. However, about 5% of them are heavily labeled as seen in Fig. 8 a. Some of these are paired (Fig. 8 b) and all have the condensed nuclei characteristic of cells in mitosis.

**Linearity of Transferase Assay**

The transfer of galactose to intact mitotic, interphase, and random populations of both cell types was linear over all the cell concentrations used in the experiments reported.
Cell Viability

During the 2-h assay period, cell viability was determined by trypan blue exclusion by cells taken from spinning and stationary incubations. In both types of incubations, viability typically decreased from 95 to 70% during the assay, while the detectable decreases in cell concentration were small (<10%), and equal, under spinning and stationary conditions.

Galactosyltransferase Activity Released into the Medium by Cells

It is possible that the difference in endogenous galactosyltransferase activity in spinning and stationary incubations of randomly growing 3T3 cells was due to greater leakage of enzymes by cells in stationary tubes. Mitotic 3T3 cells may be generally "leaky," which would explain their high transferase activity under both assay conditions. In order to deal with this possibility, various cell supernates were assayed for enzyme activity. Randomly growing 3T3 cells were prepared for transferase assay as described in Materials and Methods. Cells were tested for their ability to transfer galactose to an exogenous acceptor by incubating them in the presence of [14C] UDP-Gal and N-acetyl-D-glucosamine, a galactose acceptor. The data in Table II show that there was a minimal difference in the ability of these cells to transfer galactose to an exogenous acceptor when assayed under spinning or stationary conditions.

![Figure 6](image-url)

**Figure 6** Incorporation of galactose onto Colcemid-treated, mitotic 3T12 cells as a function of cell contact. ○, stationary reaction; O-O, spinning reaction. Reaction mixtures were prepared as described in Fig. 1 and the text except that the cell concentration was $1 \times 10^6$ cells/ml.

![Figure 7](image-url)

**Figure 7** Incorporation of galactose onto Colcemid-treated, interphase 3T12 cells as a function of cell contact. ○, stationary reaction; O-O, spinning reaction. Reaction mixtures were prepared as described in Fig. 1 and the text except that the cell concentration was $9 \times 10^6$ cells/ml.

### Table II

| Enzyme source | Product cpm/2-h incubation |
|---------------|---------------------------|
|               | Stationary incubation | Spinning incubation |
| Intact 3T3 cells* | 14,300 | 11,290 |
| Supernate from intact 3T3 cells | 2,760 | 2,439 |
| 3T3 cells† | 2,760 | 2,439 |

* Cells ($7 \times 10^6$ cells/ml) were incubated at 37°C with 5.27 μM [14C] UDP-Gal at 250 mCi/mmol and 1 mM N-acetyl-D-glucosamine. 50-μl samples were removed from both stationary and spinning incubations and centrifuged at 550 g to pellet the cells. 25-μl samples from each supernate were tested for incorporation of radioactivity into N-acetyl-lactosamine as described in Materials and Methods.

† Cells ($7 \times 10^6$ cells/ml) were incubated under stationary and spinning conditions at 37°C for 1 h. Suspensions from each were centrifuged at 550 g to pellet the cells. 25-μl samples from each supernate were incubated at 37°C with 5.27 μM [14C] UDP-Gal at 250 mCi/mmol and 1 mM N-acetyl-D-glucosamine. Incorporation of radioactivity into N-acetyl-lactosamine was determined as described in Materials and Methods.
FIGURE 8  Autoradiographs of monolayer cultures of 3T3 cells incubated with [3H]UDP-Gal, at 37°C for 3 h. (Fig. 8 a) The single cell showing strong incorporation of label represents about 5% of the population. (Fig. 8 b) The two cells showing label appear to be early daughter cells just after completion of mitosis. Bar represents 15 \( \mu \)m. \( \times \) 1,200.
However, endogenous acceptor activity exhibited the normal spinning and stationary difference (data not shown). When supernates taken from cell suspensions which had been incubated under spinning or stationary conditions for 1 h were incubated in the presence of $[^{14}C]$ UDP-Gal and N-acetyl-d-glucosamine for 2 h (Materials and Methods) they had 10–20% of the cellular activity. More importantly, the amount of enzyme leakage always appeared to be the same under spinning and stationary conditions (Table II). Therefore, the difference in transferase activity found between spinning and stationary cultures of randomly growing 3T3 cells is probably not accounted for by differential leakage of cellular enzymes.

Presently, no native cell surface galactose acceptor has been isolated from 3T3 cells; however, these cells do show galactosyltransferase activity toward N-acetyl-d-glucosamine. Therefore, the absence of excess supernatant enzyme activity toward this exogenous acceptor in stationary incubations is taken to be representative of the leakage of other galactosyltransferases. The preferential release of different transferases cannot be excluded.

**Measurement of Substrate and Product Degradation**

Transferase differences between spinning and stationary incubations of randomly growing 3T3 cells could result from differential degradation of $[^{14}C]$UDP-Gal or galactoside product by hydrolytic enzymes, yielding galactose-1-phosphate and galactose. In order to rule out these possibilities, electrophoretograms from spinning and stationary assays of these cells were compared. The electrophoretograms were scanned with a Packard Radiograph Scanner (model 7200, Packard Instrument Co., Inc., Downers Grove, Ill.) using authentic galactose-1-phosphate and galactose as standards. After all incubations, radioactivity in both of these areas was always less than 5% of input counts. More significantly, there was no difference in the amount of these two products in spinning and stationary incubations.

**DISCUSSION**

The data show that in the presence of UDP-Gal and cation, intact, mitotic 3T3 cells transfer galactose from the nucleotide sugar to cell surface acceptors at the same rate, regardless of the degree of cell contact. This result implies that, under these conditions, prolonged cell contact is not required for the endogenous galactosyltransferase reaction on these normal cells in mitosis.

It has been shown (15) that under the same conditions galactose transfer in random populations of intact 3T3 cells is two- to threefold less rapid when cell contact is diminished. However, a random population of the highly malignant 3T12 cells is capable of catalyzing this reaction regardless of the degree of cell contact. This difference in contact requirement could be explained on the basis of the average spatial distance of enzymes and acceptors. On a single 3T3 cell, enzyme and acceptor molecules may not come in close enough proximity to allow interaction. The endogenous reaction would require contact so that enzymes on one 3T3 cell could glycosylate acceptors on an adjacent cell (trans-glycosylation). On a 3T12 cell, these enzymes and acceptors might be able to come sufficiently close for completion of the reaction (cis-glycosylation).

The data reported show that, when in mitosis, 3T3 cells are capable of catalyzing the galactosyltransferase reaction in a manner characteristic of transformed 3T12 cells, i.e., they show no difference in reaction kinetics when assayed under conditions of varying cell contact. Control experiments indicate that this result could not be accounted for by the procedures used for collecting the mitotic populations of 3T3 cells. Further controls suggest that these data can neither be a result of differential cell viability, nor leakage of transferases or hydrolases. The simplest interpretation compatible with these and other observations is that, during mitosis, 3T3 cells undergo a rearrangement of their surface galactosyltransferases and acceptors that results in these molecules being close enough so that cis-glycosylation may occur. Autoradiographic analysis supports this interpretation since about 5% of the cells in a random 3T3 monolayer culture are heavily labeled and seem to have condensed nuclei characteristic of mitotic cells (Fig. 8). Alternatively, membrane fluidity (6, 13) may increase upon transformation and in mitotic normal cells. This would allow enzymes and acceptors on the same cell to approach one another more often, resulting in greater cis-glycosylation.

However, greater cis-glycosylation does not necessarily imply greater transferase activity but, rather, indicates increased interaction of existing enzymes and acceptors. Although 3T12 cells seem always capable of cis-glycosylation, they incorpo-
rate galactose to a lesser extent than do 3T3 cells. One possible explanation is that 3T12 cells have smaller amounts of surface acceptors. Other factors that would affect the extent of incorporation cannot be ruled out.

Malignant 3T12 cells may be “defective” in that they continuously display a cell surface phenomenon found only during mitosis in normal 3T3 cells (Fig. 9). From the data presented here, enzymes and acceptors on a single transformed cell seem to have increased ability to interact with each other (cis-glycosylation) under the incubation conditions described. However, in culture, transformed cells apparently do not complete the transferase reaction as evidenced by high endogenous acceptor activity (15) in cells harvested from sparse or confluent cultures. On the other hand, normal cells show decreased endogenous acceptor activity as cultures become more confluent (15). This could result from the glycosylation of acceptors as cells come in contact with each other (trans-glycosylation). Therefore, the consistently high endogenous activity of 3T12 cells implies a second defect that results in their inability to carry out the transferase reaction in spite of the increased interaction between enzymes and acceptors. This could explain the fact that transformed cells do not possess the more complex glycolipids found in confluent, normal cell cultures (4, 7, 11).

The significance of cis-glycosylation is not yet apparent. It is possible that it does not occur in culture. However, the fact that it can be forced to occur indicates a degree of interaction between surface components on mitotic normal cells and randomly growing malignant cells which does not exist between components on interphase normal cells.

Analogous to our transferase-acceptor model (Fig. 9), the difference in agglutinability of normal and transformed cells by plant lectins has been attributed to a differential arrangement of surface lectin-binding sites due to differential mobility (13). Accordingly, mitotic, normal cells show lectin-binding patterns and agglutination patterns that resemble transformed cells (5, 7). The data are indicative of a change in lectin-binding sites on mitotic and transformed cells similar to that proposed here for transferases and acceptors.

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