RELATIONSHIP OF TRANSFORMATION, CELL DENSITY,
AND GROWTH CONTROL TO THE CELLULAR DISTRIBUTION
OF NEWLY SYNTHESIZED GLYCOSAMINOGLYCAN

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ABSTRACT

Mouse 3T3 cells and their Simian Virus 40-transformed derivatives (3T3SV) were
used to assess the relationship of transformation, cell density, and growth control
to the cellular distribution of newly synthesized glycosaminoglycan (GAG). Glu-
cosamine- and galactosamine-containing GAG were labeled equivalently by [^{3}H]-
glucose regardless of culture type, allowing incorporation into the various GAG
to be compared under all conditions studied. Three components of each culture
type were examined: the cells, which contain the bulk of newly synthesized GAG
and are enriched in chondroitin sulfate and heparan sulfate; cell surface materials
released by trypsin, which contain predominantly hyaluronic acid; and the media,
which contain predominantly hyaluronic acid and undersulfated chondroitin
sulfate.

Increased cell density and viral transformation reduce incorporation into GAG
relative to the incorporation into other polysaccharides. Transformation, how-
ever, does not substantially alter the type or distribution of newly synthesized
GAG; the relative amounts and cellular distributions were very similar in 3T3 and
3T3SV cultures growing at similar rates at low densities. On the other hand,
increased cell density as well as density-dependent growth inhibition modified the
type and distribution of newly synthesized GAG. At high cell densities both cell
types showed reduced incorporation into hyaluronate and an increase in cellular
GAG due to enhanced labeling of chondroitin sulfate and heparan sulfate. These
changes were more marked in confluent 3T3 cultures which also differed in
showing substantially more GAG label in the medium and in chondroitin-6-sulfate
and heparan sulfate at the cell surface.

Since cell density and possibly density-dependent inhibition of growth but not
viral transformation are major factors controlling the cellular distribution and type
of newly synthesized GAG, differences due to GAGs in the culture behavior of
normal and transformed cells may occur only at high cell density. The density-
induced GAG alterations most likely involved are increased chondroitin-6-sulfate
and heparan sulfate and decreased hyaluronic acid at the cell surface.

Glycosaminoglycans are major components of the carbohydrate at the surfaces of animal cells (20). Cell surface glycosaminoglycan (GAG) has been implicated in a variety of phenomena including cell recognition and intercellular adhesion (32), cell mobility (49), embryonic induction (27), and maintenance of embryonic organ morphology (1). Several studies implicate cell surface GAG in the control of fibroblast growth in vitro. The growth rate of cells in suspension cultures is inhibited by addition of several types of sulfated GAG (24) and of dextran sulfate, a GAG-like polyanion (13, 28), which also reversibly reduces saturation densities of cells in monolayer culture (6). Viral transformation of cells to the neoplastic state alters their in vitro growth behavior, and transformation has repeatedly been shown to decrease sulfated GAG synthesis (12, 34, 36, 48), but there are conflicting reports of its effect on hyaluronic acid synthesis (15, 39).

Compared to normal cells, the surfaces of transformed cells show greater heterogeneity of glucosamine-labeled GAG (52) and a thicker layer of ruthenium red-staining material, presumably representing GAG (25). After removal of cells from the substratum with chelators, a greater proportion of sulfate-labeled GAG remains bound to the substratum in cultures with low saturation densities than in transformed cell cultures (34), although studies of the GAG remaining bound to the substratum using glucosamine labeling revealed few quantitative differences between the cell types (34). Heparan sulfate at the cell surface is selectively lost to the medium just before mitosis (22) and is more susceptible to removal by trypsin on normal cells than on transformed cells (4), suggesting that it may play a role in controlling cell growth. A possible mechanism for this control has recently been proposed (34, 48) which suggests that increased GAG at the cell surface may be involved in enhancing cell-substratum adhesion, thereby reducing saturation densities.

While such studies have suggested that surface GAG influences or reflects the growth behavior of cells in culture, most of these studies have used radiosulfate as the GAG precursor, preventing assessment of hyaluronic acid and undersulfated proteoglycan production, and in no instance to our knowledge has the cellular distribution of sulfated and nonsulfated GAG been systematically examined in normal and transformed cells under varying growing conditions. In the present study, the cellular distribution of newly synthe-

sized glucose-labeled GAG was assessed in mouse 3T3 and SV40-transformed 3T3 cells at low cell densities where the cells were growing at equivalent rates and at high cell densities where the 3T3 cells were growth inhibited. Under the labeling conditions used, GAG precursors were at the same specific activities regardless of culture type. Three components were isolated from each culture type (Fig. 1): (*a*) the medium, which contains GAG secreted during the labeling period; (*b*) the cell surface, containing the GAG removed from the cells and the substratum with crystalline trypsin; and (*c*) the cells, which were free of trypsin-sensitive surface materials. For each fraction, [^{3}H]glucose incorporation into the following GAG was measured: the galactosaminoglycans, chondroitin-6-sulfate, dermatan and chondroitin-4-sulfate, and chondroitin; and the glucosaminoglycans, hyaluronic acid, and heparan sulfate. This experimental design allowed an assessment of the relationship of transformation, cell density, and density-dependent growth inhibition to the cellular distribution of newly synthesized GAG.

MATERIALS AND METHODS

Reagents

Trypsin (3× crystalline) was obtained from Worthington Biochemical Corp. (Freehold, N. J.) and pronase, B grade, from Calbiochem (San Diego, Calif.). Chondroitinase ABC (lot 7201), chondro-4-sulfatase, and chondro-6-sulfatase from *Proteus vulgaris*, and the unsaturated disaccharides, Δdi-4S,[1] Δdi-6S, and Δdi-0S were obtained from Miles Laboratories, Inc. (Elkhart, Ind.). Hyaluronic acid, potassium salt (grade III-P), and crude chondroitin sulfate were obtained from Sigma Chemical Co. (St. Louis, Mo.), and NCS scintillation solubilizer from Amersham-Searle Corp. (Arlington Heights, Ill.). [3-^{3}H]Glucose (6.7 Ci per mmol) was obtained from New England Nuclear (Boston, Mass.). Dulbecco's modified Eagle's Medium (DME) and Eagle's Basal Medium (BME) were obtained from Gibco (Chagrin Falls, Ohio). Heparin, heparan sulfate, and dermatan

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[1] *Abbreviations used in this paper*: GlcNAc, N-acetylglucosamine (2-acetamido-2-deoxy-D-glucose); GalNAc, N-acetylgalactosamine (2-acetamido-2-deoxy-D-galactose); and for the unsaturated disaccharides: Δdi-4S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose; Δdi-6S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose; Δdi-0S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; Δdi-OHA, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose.

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LOW DENSITY        HIGH DENSITY
   3T3                 3T3
  3T3SV               3T3SV
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CELLS
SURFACE
MEDIUM

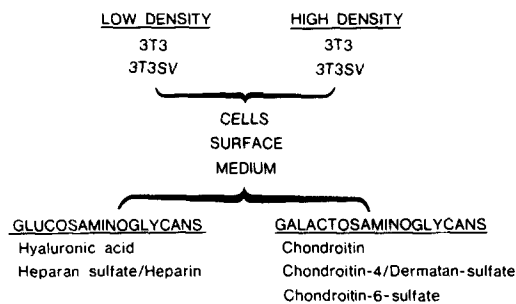| GLUCOSAMINOGLYCANS	GALACTOSAMINOGLYCANS
Hyaluronic acid	Chondroitin
Heparan sulfate/Heparin	Chondroitin-4/Dermatan-sulfate
	Chondroitin-6-sulfate

FIGURE 1 Schematization of parameters examined. Cells, trypsin-released cell-surface materials, and media of both low and high density cultures of the two cell types (3T3 and 3T3SV) were assessed for labeled glucosaminoglycans and galactosaminoglycans.

sulfate were kindly supplied by Dr. J. Cifonelli (University of Chicago).

Preparation of Δ di-OHA from Hyaluronic Acid

Hyaluronic acid (15 mg) was dissolved in 2 ml of enriched Tris buffer (37) at pH 6.8, and 2.5 U of chondroitinase ABC (in 0.5 ml) was added. The reaction mixture was incubated for 17 h at 37°C, applied to Whatman 3 MM paper (Whatman Inc., Clifton, N.J.) and chromatographed for 19 h in solvent A (1-butanol-acetic acid-1 N NH$_4$OH[2:3:1, by volume] [37]). Products were visualized as bands under ultraviolet light, and the band migrating faster than the Δdi-OS standard was cut out and eluted with water. This material was further purified by paper chromatography for 72 h in solvent B (1-butanol-ethanol-water[52:32:16, by volume] [51]). The single major band visualized was eluted with water. The material migrated as a single spot in solvents A and B and had an absorption maximum at 232 nm (0.04 N HCl). Assuming an E_M^{232} of 5.7 × 10³ M⁻¹ cm⁻¹ (23, 51), it had an E_M^{585} of 16.0 × 10³ M⁻¹ cm⁻¹ in the Morgan-Elson reaction (46). The overall yield was 48%.

Cells and Culture Conditions

The established mouse embryo BALB/c cell line 3T3 and its SV40-transformed derivative (3T3SV) were gifts from Dr. P. Berg, Stanford University. Cells were cultured in 60-mm tissue culture dishes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.; 21.9 cm²) containing bicarbonate-buffered DME (supplemented with 2 mM glutamine, 100 U penicillin and 5 μg streptomycin per ml, and 10% calf serum) and incubated at 37°C in an atmosphere of 5% CO$_2$, 95% air, and 100% humidity. Cells were routinely passaged after reaching confluent densities by trypsinization (0.05% Difco trypsin [1:250] [Difco Laboratories, Detroit, Mich.] in 0.02% (EDTA), 0.02 M Tris-buffered saline, pH 7.4).

No attempt was made to maintain low saturation densities by routine passaging at low cell densities. Both cell types were found to be free of Mycoplasma contamination by culture (courtesy of Dr. L. Hayflick, Stanford University) and by [³H]thymidine autoradiography (8).

Data from growth curves determined the plating densities which would provide cells at the proper growth phase and cell densities for labeling. Cells were plated as follows (in cells per square centimeter): 3T3, 9.5 × 10³ and 6.6 × 10⁴; 3T3SV, 4.7 × 10³ and 3.1 × 10⁴. The medium was changed 24 h after plating and the cells were cultured for an additional 24 h before labeling. Cell densities (average of four replicate dishes for each cell type) in cells per square centimeter at the time of labeling were as follows: logarithmically growing low density 3T3 cells, 3.3 × 10⁴; growth-inhibited confluent 3T3 cells, 7.4 × 10⁴; logarithmically growing low density 3T3SV cells, 1.7 × 10⁴; and logarithmically growing crowded 3T3SV cells, 13.4 × 10⁴. Protein determinations (31) gave the following protein content per 10⁶ trypsinized cells: low density 3T3 cells, 233 μg; confluent 3T3 cells, 258 μg; low density 3T3SV, 101 μg; crowded 3T3SV cells, 173 μg. Differences in protein content per cell between normal and transformed cells, and between cells at high and low density have been previously reported (30, 31).

Labeling and Preparation of Culture Components

Labeling medium was serum- and glucose-free bicarbonate-buffered BME containing 2 mM glutamine and 10 mM sodium pyruvate. Serum was omitted to avoid possible differential effects of fresh serum in the different culture types. Control studies using glucose-free serum showed that the absence of serum during labeling did not alter the incorporation of glucose into GAG or total polysaccharide of low density 3T3 cells. Duplicate culture dishes were washed three times with labeling medium, and 1.5 ml labeling medium containing 100 μCi per ml [3-³H]glucose was added to each dish and the cells were incubated for 4.5 h. The labeling medium was removed and each dish rinsed with 0.5 ml Earle's balanced salt solution (BSS) which was pooled with the medium. The pooled solution was centrifuged at 1,000g for 10 min at 4°C. This cell-free supernate constituted the medium fraction.

To prepare surface and cell fractions, the cells were further rinsed five times with BSS and the rinses discarded. Trypsin (1.0 ml of 0.05% 3× crystalline trypsin in Tris-buffered saline, pH 7.5) was added to each dish and the cells were incubated at 37°C for 10 min to suspend the cells. More than 97% of the cells removed were viable by trypan blue exclusion. The cells and trypsin solution were removed from the dishes at room temperature and placed on ice. The dishes were rinsed with 0.5 ml of BSS which was added to the cell suspension. The cells were centrifuged at 400 g for 10 min at

4°C, washed with BSS, and centrifuged again. The combined supernate constituted the surface fraction, which included any GAG removed from the dish by trypsin. The cell fraction was obtained by washing the trypsinized cells three times as before, discarding the wash solutions, and suspending the cells in 0.5 ml H_2O. The labeled components were quick frozen and stored at -20°C.

Purification of Polysaccharides for GAG Analysis

Labeled medium, surface, and cell components from each duplicate dish were handled identically, except as indicated. The cells were disrupted by sonication at 0°C. Carrier hyaluronic acid and crude chondroitin sulfate (12.5 μg each) were added to each sample and the solutions adjusted to contain 150 mM Tris (pH 8.3), 1.5 mM $CaCl_2$, and 0.2 mM glucose. The samples were boiled for 10 min, cooled to room temperature, and 2 mg of pronase was added to each. After incubation at 55°C for 8 h, the samples were mixed, an equal amount of fresh pronase added, and incubation continued for 16 h. The digests were boiled for 10 min and clarified by centrifugation (20,000 g for 15 min). 3 vol of ice cold 95% ethanol (containing 1% potassium acetate) was added to each supernate and the samples were stored at -20°C overnight. Samples were centrifuged (30,000 g for 20 min) and the precipitate obtained was dried in vacuo. The precipitates were dissolved in 0.5 ml of water and reprecipitated twice with 3 vol of 95% ethanol containing 1% potassium acetate to remove any remaining free isotope. The medium fractions were reprecipitated one more time. Precipitates were collected by centrifugation and dried in vacuo.

Determination of Chondroitinase-Susceptible GAG

The methodology used was similar to that described by Saito et al. (37), except that more enzyme was used and the pH of the digestion was 7.4 rather than 8.0. Aliquots of the dried samples dissolved in 0.05 ml of Tris-enriched buffer (pH 7.4) received 0.05 ml (0.25 U) of chondroitinase ABC solution (5 U per ml in the same buffer). After incubation at 37°C for 3 h, carrier Δdi-OS, Δdi-OHA, Δdi-6S, and Δdi-4S (25 μg each) in a total volume of 0.01 ml were added to each digestion mixture. Adequacy of chondroitinase digestion was assessed as described by Yamagata et al. (51). Digestion of 250 μg of hyaluronic acid or chondroitin sulfate (amounts that are 10-fold the amount of carrier used in experimental reactions) by 0.15 U of chondroitinase ABC was complete within 2 h (Fig. 2). The plateau in absorbance at limiting enzyme concentration and after completion of the reactions demonstrates the absence of possible contaminating activities which cleave the disaccharides. Incubation of the enzyme with Δdi-4S and Δdi-6S as well as with chondroitin-4-sulfate, chondro-
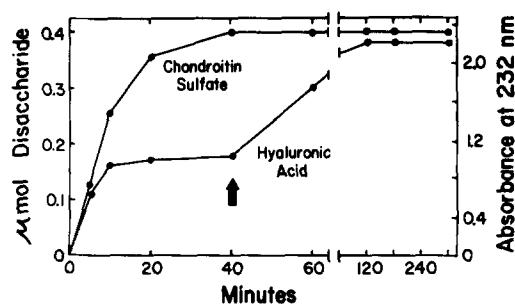

FIGURE 2 Adequacy of chondroitinase ABC digestion. Chondroitin sulfate or hyaluronic acid (250 μg) was incubated with 0.01 U of chondroitinase ABC in 0.1 ml of Tris-enriched buffer (pH 7.4) at 37°C. At 40 min (arrow) an additional 0.15 U of enzyme was added.

itin-6-sulfate, and dermatan sulfate followed by chromatography failed to demonstrate contaminating sulfatase activity.

The samples were quantitatively spotted on Whatman 3MM paper, dried in a stream of warm air, and chromatographed for 19-24 h in solvent A. The UV-absorbing spots were cut out, divided into pieces, placed in scintillation vials containing 0.3 ml of H_2O and 2.0 ml of NCS, and incubated at 50°C for 2 h. After allowing the vials to cool, 15 ml of toluene-based scintillant was added to each. Counting efficiency for paper containing ^3H-samples was 48% in a Beckman LS-233 refrigerated liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Radioactivity detected on chromatograms (disaccharides plus resistant material) averaged 95.5% of that applied.

The following disaccharides represent their parent GAGs: Δdi-6S is derived from chondroitin-6-sulfate; Δdi-4S is derived from chondroitin-4-sulfate and dermatan-4-sulfate (23); Δdi-OS is derived from chondroitin and nonsulfated residues of the chondroitin sulfates; Δdi-OHA is derived from hyaluronic acid. The relative mobilities (mean ± SE, n = 12) for Δdi-OS, Δdi-OHA, Δdi-4S, and Δdi-6S were 1.00, 1.36±0.006, 0.66±0.0036, and 0.37±0.0085. The spots were clearly separated from each other and from any possible [^3H]glucose (relative mobility of 1.61).

Determination of Heparan Sulfate

Heparin and heparan sulfate are closely related molecules resistant to chondroitinase ABC digestion and containing unsubstituted and O-sulfated uronic acid, and O-sulfated N-acetylglucosamine, as well as N-sulfated glucosamine (20). Heparin is produced almost exclusively by mucus-producing epithelia and mast cells and was not differentiated from heparan sulfate in this report. Nitrous acid cleaves heparan sulfate at the glycosidic bond adjacent to N-sulfate group while bonds adjacent to N-acetylhexosamines are resistant (5). Thus, quantitation of sugar-labeled heparan sulfate by measuring the

amount of label resistant to nitrous acid degradation by subtraction procedures may underestimate the amount of label by the proportion of hexosamines which are not sulfated (estimated to be approx. 40% for lung heparan sulfate [5]). Heparan sulfate is precipitable by cetylpyridinium chloride (CPC) at 0.6 M NaCl, whereas other chondroitinase-resistant polysaccharides potentially labeled with glucose, such as nucleic acids, keratan sulfate, and glycogen are not (35).

High molecular weight heparan sulfate was determined by selective CPC precipitation of chondroitinase ABC-treated mixtures. Carrier heparin and heparan sulfate (20 μg each) were added to aliquots of [^{3}H]glucose-labeled polysaccharide fractions and the samples exhaustively digested with chondroitinase ABC as described above. The mixture was made 0.6 M NaCl, and heparan sulfate was precipitated by incubation in 0.05% CPC at 37°C for 1 h. The precipitates were collected on glass fiber filters, washed with 0.005% CPC in 0.6 M NaCl and then with water. The filters were dried and counted in toluene scintillant at an efficiency of 38%.

Precipitation with CPC was compared with HNO$_2$ degradation by measuring the apparent yield of both methods. Aliquots of chondroitinase ABC-resistant labeled polysaccharides from crowded 3T3SV cells were CPC precipitated as above and were treated with HNO$_2$ (19). The degree of HNO$_2$ degradation was determined after chromatography (42) of the reaction mixture for 120 h. About 30% more chondroitinase-resistant polysaccharide, presumably heparan sulfate, was precipitated by CPC in 0.6 M NaCl than was degraded by HNO$_2$.

Determination of the Fraction of Disaccharide Label Due to N-Acetylhexosamine

Labeled polysaccharides derived from low density 3T3 and crowded 3T3SV cells (approx. 20,000 dpm each) were converted to unsulfated disaccharides by incubation with chondroitinase ABC (0.25 U), chondro-4-sulfatase, and chondro-6-sulfatase (0.08 U each) in Tris-enriched buffer (pH 7.4) for 3 h at 37°C. Control studies showed that under these conditions one-half of these levels of sulfatase completely and specifically converted 50 μg of Δdi-6S to Δdi-OS. After incubation, 50 μg of carrier Δdi-OS and Δdi-OHA were added and the digests chromatographed on 3 MM paper for 19 h in solvent A. After elution of the UV-absorbing spots, an additional 100 μg of Δdi-OS and Δdi-OHA were added to the eluant to minimize losses of radioactivity during the subsequent procedures. Aliquots were taken from each disaccharide fraction for determination of radioactivity and measurement of disaccharide content with the Morgan-Elson reaction (46). Comparison of the absorbancies of the disaccharides at 232 nm and of the Morgan-Elson disaccharide chromophore (585 nm) yielded E$_M^{85}$ of 13.8 × 10^3 M^{-1} cm^{-1} for Δdi-OS and 16.0 × 10^3 M^{-1} cm^{-1} for Δdi-OHA. The disaccharides

were hydrolyzed (0.04 N HCl in sealed vials at 100°C for 1 h [47]) and the hydrolysates were chromatographed in solvent A for 17 h alongside N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc) standards which were visualized with a silver dip reagent (45). Areas from the samples corresponding to the standards were eluted with water. Eluants were dried in vacuo and redissolved in water. Aliquots were taken for determination of radioactivity and to estimate recoveries by measurement of N-acetylhexosamine content. The E$_M^{85}$ of the Morgan-Elson chromophore was determined to be 7.2 × 10^3 M^{-1} cm^{-1} for GalNAc and 11.3 × 10^3 M^{-1} cm^{-1} for GlcNAc.

RESULTS

Comparison of [^{3}H]glucose Utilization for GAG Synthesis by 3T3 and 3T3SV Cells

Since the relative proportions of various glucose-labeled GAG were being compared in this study, it was necessary to know whether 3T3 and 3T3SV cells incorporate [^{3}H]glucose equivalently into the various polysaccharides. Unsulfated disaccharides were prepared from GAG isolated from low density 3T3 and crowded 3T3SV cells labeled with [^{3}H]glucose as described in Materials and Methods. It was assumed that the uronic acid moiety of the disaccharide was equivalently labeled regardless of GAG type (16), and the proportion of the label derived from hexosamine was determined in the disaccharide derived from hyaluronate (Δdi-OHA, which contains GlcNAc) and in the disaccharide derived from the chondroitin sulfates (Δdi-OS, which contains GalNAc). These proportions were equivalent for both low density 3T3 and crowded 3T3SV cells (Table I), demonstrating that glucose labels GlcNAc- and GalNAc-containing GAG equivalently regardless of culture type. Since approximately 50% of the label in the disaccharides was found in the amino sugar, this result also indicates that under the conditions of labeling, the specific activities of the precursor nucleotide sugars were approximately equal. Glucose incorporation into the various GAG types, therefore, can be compared in transformed and untransformed cells.

[^{3}H]Glucose Incorporation by Transformed and Untransformed Cells

Low density and confluent 3T3 cells and low density and crowded 3T3SV cells were labeled

with [³H]glucose for 4.5 h. As indicated above, this period is sufficient for the GAG precursors to reach the same specific activity. The growth rate of these cells is shown in Fig. 3 and the

TABLE I

[³H]Glucose Labeling of GAGs by 3T3 and 3T3SV Cells

	Percent of label in N-acetylhexosamine	
Disaccharide	Low density 3T3	Crowded 3T3SV
Experiment 1		
Δdi-OHA	42.1	49.7
Δdi-OS	44.6	50.0
Experiment 2		
Δdi-OHA	45.2	46.7
Δdi-OS	50.7	51.1

Labeled disaccharides were prepared from GAG isolated from [³H]glucose-labeled cells by chondroitinase treatment. Sulfated disaccharides were converted to Δdi-OS with specific sulfatases. The percent of disaccharide label in N-acetylhexosamine was determined for Δdi-OHA and Δdi-OS by hydrolysis of the disaccharides and correcting for recoveries. For experimental details, see Materials and Methods.

degree of cell contact in Fig. 4. The generation time of the 3T3 cells was 18.3 h and that of the 3T3SV cells was 20.1 h. Crowded 3T3SV cells were dividing at the same rate as low density 3T3SV cells and were not detaching from culture dishes. Low density 3T3 cells were at about 45% confluence.

The incorporation of [³H]glucose into macromolecules resistant to pronase digestion and precipitable with ethanol, operationally defined as polysaccharide, is shown in Fig. 5. In the absence of any alterations in utilization, differences between culture types in total incorporation into polysaccharide should reflect differences in the transport and intracellular pools of glucose. At low densities, 3T3SV cells incorporate about five times more total label than 3T3 cells, a difference consistent with the 2.5- to 4-fold difference in their rate of hexose transport per milligram protein (17, 41). With increased cell densities, glucose incorporation into polysaccharide was markedly inhibited in both transformed and untransformed cells. The 11-fold reduction in incorporation as the 3T3 cells become confluent is consistent with the 8- to 10-fold decrease in hex-
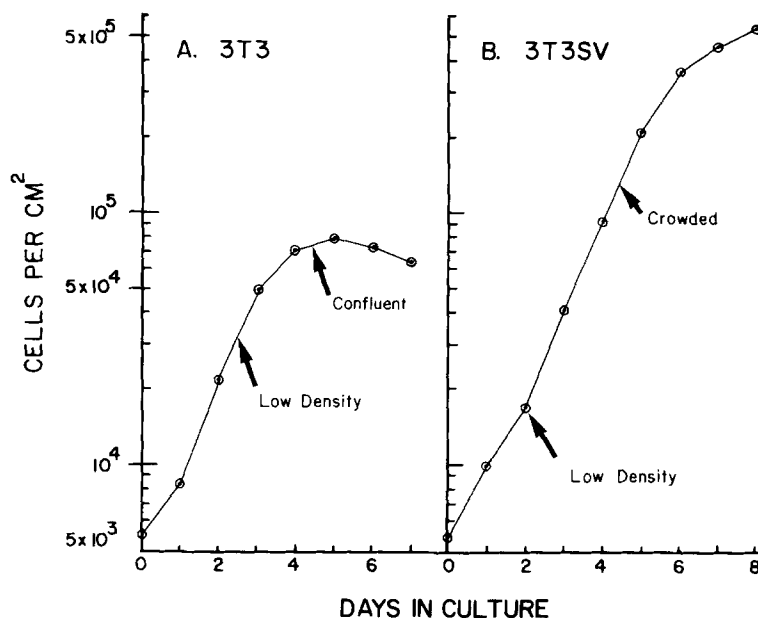

FIGURE 3 Growth curves of 3T3 and 3T3SV cells. Cells were plated into 35-mm dishes (8.8 cm²) at a density of approx. 5 × 10³ cells per cm² and the medium was changed 24 h after plating. Cell numbers were determined on duplicate dishes every 24 h with a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) after removal of the cells with 0.05% Difco trypsin (1:250) in 0.02% EDTA, 0.02 M Tris-buffered saline, pH 7.4. (A) 3T3 cells; (B) 3T3SV cells. Arrows point to cell densities at which experiments proper were performed.
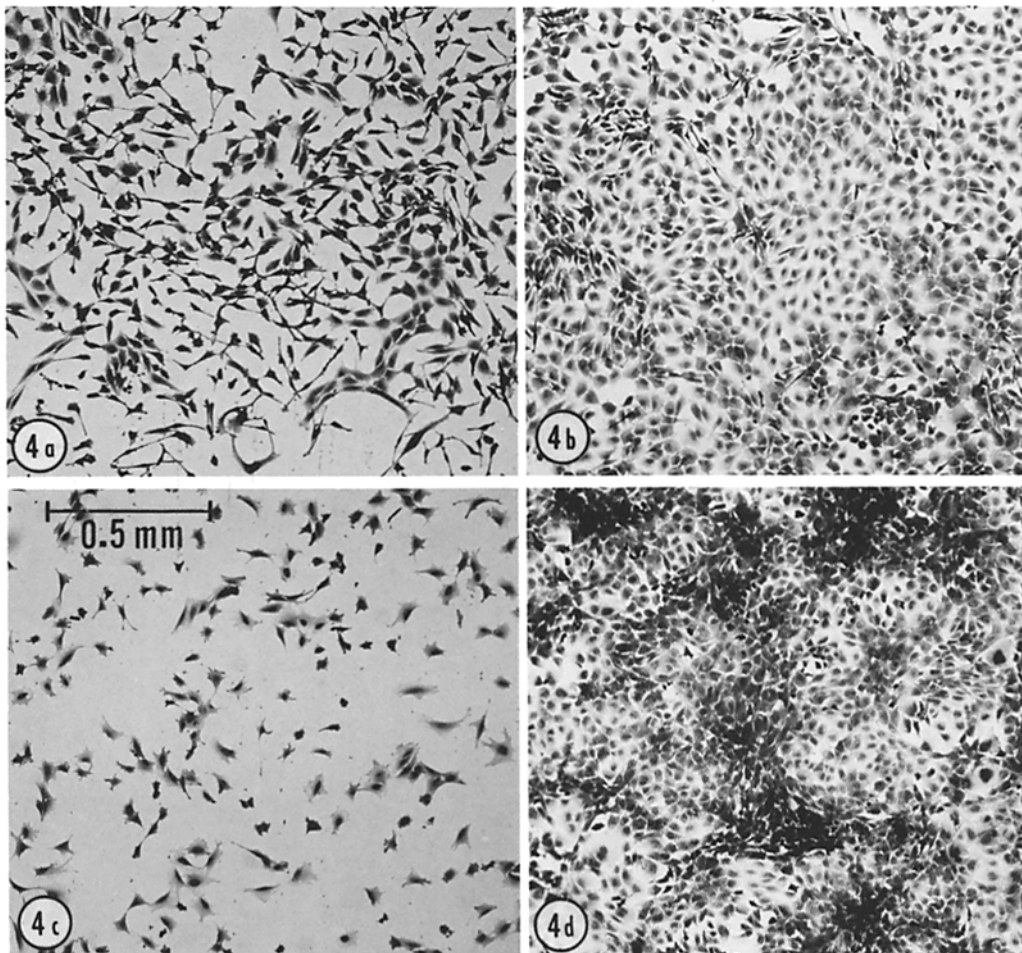
FIGURE 4   Photomicrographs of fixed and stained 3T3 and 3T3SV cells at low and high density. Cultures were plated at approx. 5×10^3 cells per cm² in 35-mm dishes and allowed to grow to densities (determined by counting two replicate dishes) similar to those used in the experiments proper. Cultures were washed three times with BSS, fixed in 2% glutaraldehyde (buffered with 75 mM PO₄ at pH 7.2) for 1 h, and stored in the cold in 0.18 M sucrose in the same buffer. For photography, the cultures were rinsed with BSS, stained with 0.5% toluidine blue in 1% sodium borate for 10 min, and then rinsed three times with water. Magnification ×45. (4a) Low-density, logarithmically growing 3T3 cells at a density of 4.0×10^4 cells per cm², a density slightly greater than that used in the experiments, showing minimal intimate cell contact. (4b) Confluent, nongrowing 3T3 cells at a density of 7.4×10^4 cells per cm² showing a nearly complete monolayer of cells in intimate contact. (4c) Low-density, logarithmically growing 3T3SV cells at a density of 1.7×10^4 cells per cm². At this density, there is very little cell contact. (4d) Crowded, logarithmically growing 3T3SV cells at a density of 1.3×10^5 cells per cm². These cells are in intimate contact and have formed heavily stained, multilayered cell arrays.

ose transport rate occurring under similar circumstances (2, 41). The threefold reduction in total incorporation with 3T3SV cells is not accounted for by differences in transport since low- and high-density 3T3SV cells have similar rates of hexose uptake (41).

The proportion of [³H]glucose incorporated into GAG should not be altered by transport or pool size differences. In both transformed and untransformed cells, however, the proportion of label incorporated into GAG decreased at high cell densities, and this decrease was more pronounced in transformed cells (Fig. 5). These results suggest that high degrees of cell contact

reduce the incorporation of glucose into GAG. These findings are consistent with the decrease in radiosulfate incorporation into GAG observed after long-term labeling of analogous cultures (12, 34, 36).
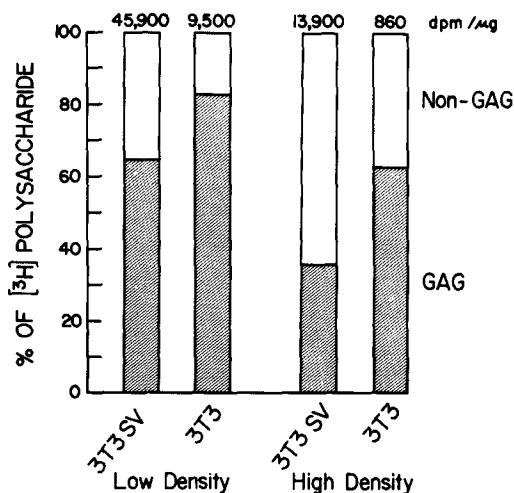
The distribution of glucose-labeled polysac-

FIGURE 5 Incorporation of [³H]glucose into polysaccharide. Materials which are resistant to pronase digestion and precipitable with ethanol are operationally defined as polysaccharide (see Materials and Methods). GAG represents chondroitinase ABC susceptible polysaccharide plus heparan sulfate, while non-GAG represents total polysaccharide less GAG. Results are the average of analyses of duplicate cultures and represent the sum of all culture components. Above the bars is indicated the disintegrations per minute [³H]glucose incorporated into total polysaccharide per microgram cell protein.

charide among the culture components is shown in Table II. At low cell densities, each culture component contained a similar proportion of total polysaccharide label in GAG. At high cell densities, GAG labeling relative to other polysaccharides decreases due to a greater decrease of GAG label in the cell and surface fractions than in the medium.

Incorporation into GAG of Various Culture Components

Whereas differences between culture types in incorporation into total polysaccharide and in the proportion of this label in GAG may be due to differences in [³H]glucose utilization, the distribution of labeled GAG among the culture components should be independent of this variable. The distribution of labeled GAG among the cells, surface materials, and medium was similar for 3T3 and 3T3SV cells growing at low densities (Fig. 6). The proportion of labeled GAG associated with the cells was nearly equivalent to that removed by trypsin and about twice that found in the medium. In crowded 3T3SV cultures, however, the proportion of newly synthesized GAG in the cells increased while that in the surface fraction decreased. A more marked density-induced decrease in surface GAG was seen in 3T3 cultures, and, in addition, confluent 3T3 cells showed a greater proportion of labeled GAG in the medium. These data indicate that the cellular distribution of newly synthesized GAG varies with the density of the culture, and possibly with growth control, but not with viral transformation.

TABLE II

Distribution of Labeled Polysaccharide among Culture Components

	Cells		Surface		Medium	
	Total polysaccharide	GAG	Total polysaccharide	GAG	Total polysaccharide	GAG
	dpm/μg	%	*dpm/μg*	%	*dpm/μg*	%
Low density 3T3SV	18,600	60.9	18,700	63.6	8,580	75.6
Low density 3T3	4,190	78.7	3,300	86.5	1,980	83.3
Crowded 3T3SV	9,420	29.8	2,650	44.4	1,790	53.6
Confluent 3T3	511	55.7	125	59.2	225	80.7

Three components were prepared from the cultures: the medium, containing newly synthesized polysaccharides secreted by the cells during incubation; the cell surface, containing labeled polysaccharides released from the cells by treatment with crystalline trypsin; and the cells, which are free of trypsin-susceptible surface polysaccharides. Total polysaccharide and GAG are defined in Fig. 5. Results are the average of analyses of duplicate cultures. Incorporation data are disintegrations per minute [³H]glucose per microgram cell protein.

Relative Incorporation into Various Types of GAG

Table III shows the proportions of the various types of labeled GAG according to culture type. At low cell density, 3T3 and 3T3SV cultures showed similar proportions of the various types of newly synthesized GAG. Most of the label was incorporated into hyaluronate, while among the galactosaminoglycans the lowest incorporation was that into chondroitin-6-sulfate. With increased cell density, both transformed and untransformed cell cultures incorporated a greater proportion of label into sulfated GAG, and this change was more marked in 3T3 cultures. As indicated above, less labeled GAG is produced at
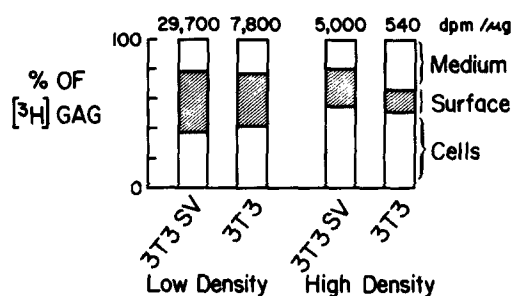

FIGURE 6 Incorporation of [³H]glucose into GAG. Culture components are as in Table II, and GAG is defined in Fig. 5. Above the bars is indicated the disintegrations per minute [³H]glucose incorporated into total GAG per microgram cell protein. Results are the average of separate analyses of duplicate cultures.

high density, but this inhibition was least marked for heparan sulfate and chondroitin-6-sulfate and most marked for hyaluronic acid.

Distribution of Labeled GAGs among Culture Components

The distribution of various types of newly synthesized GAG among the medium, cell surface, and cell components was examined to assess whether specific types of polysaccharides accumulated at distinct sites under different growth conditions. Data are presented for labeled GAG according to type (Fig. 7) and within each culture component (Table IV).

TYPES OF LABELED GAG

The various polysacharides have distinct distributions among the culture components (Fig. 7). At low densities, the surface and cell components contained the bulk of newly synthesized hyaluronate. At high cell densities, the proportion of labeled hyaluronate decreased at cell surfaces and increased in the media. Nearly all of the newly synthesized heparan sulfate was associated with the cells regardless of growth condition or transformation. At low densities, the major proportion of galactosaminoglycan label was associated with the cells, and this proportion increased in cultures labeled at high densities.

These data show that the cellular distribution of various types of labeled GAG is similar for 3T3 and 3T3SV cultures growing at low densi-

TABLE III

Relative Incorporation into Various GAGs

	Percent of total GAG			
Type of GAG	Low density 3T3SV	Low density 3T3	Crowded 3T3SV	Confluent 3T3
Hyaluronic acid	76.6	77.9	62.2	39.8
Heparan sulfate	8.3	5.9	15.2	10.7
Galactosaminoglycan	15.2	16.2	22.6	49.5
Chondroitin-6-sulfate	1.9	1.8	4.7	13.4
Dermatan-4-sulfate Chrondroitin-4-sulfate }	6.6	8.0	10.5	21.2
Chrondroitin	6.7	6.4	7.4	14.9

Data are presented as the percent of the total GAG label in the various types of GAG by each culture type. The extent of incorporation by each cell type is shown in Fig. 6. Results are the average of analyses of duplicate cultures. The mean difference between duplicate cultures was 1.1%.
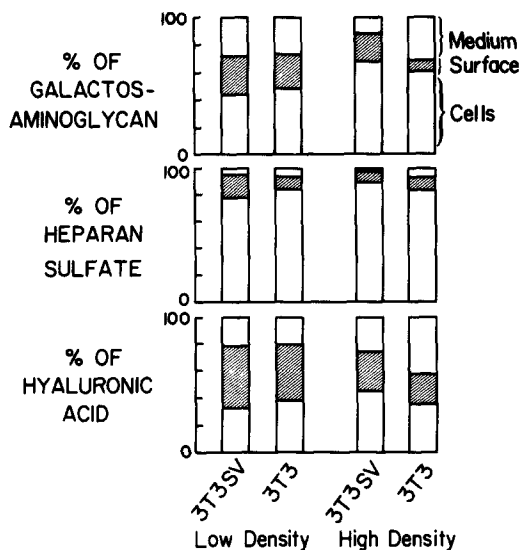
FIGURE 7 Distribution of labeled GAGs among culture components. Cellular components are as in Table II. Data are presented as the percent of each type of [³H]GAG in each cellular component. Results are the average of separate analyses of duplicate cultures.

ties. The change in labeled GAG distribution occurring with increased density of 3T3SV cultures is a shift of label into the cells; from the medium for galactosaminoglycans, and from the surface fraction for hyaluronate and heparan sulfate. On the other hand, in 3T3 cultures which have reached confluence, galactosaminoglycan label is shifted from surface components into the cells, and hyaluronate label from the surface into the medium.

Cells

The cells are enriched in galactosaminoglycan and contain the bulk of heparan sulfate (Table IV). Transformed cells label heparan sulfate more heavily than untransformed cells regardless of density. Except for this difference, low density 3T3 and 3T3SV cells were similar. Dense cultures showed a lower proportion of cellular GAG in hyaluronic acid than sparse cultures, a difference that was substantially greater in confluent 3T3 cells.

Surface

Hyaluronic acid is the predominant polysaccharide released by trypsinization (Table IV) even for cultures labeled at high densities, in which there is a lower proportion of total hyalu-

ronate and of surface label. Label in heparan sulfate was relatively resistant to removal from the cells by trypsin. Nevertheless, heparan sulfate accounted for a greater proportion of the surface label on high density cells.

Medium

The GAG label in the medium (Table IV) was predominantly in unsulfated polysaccharides, hyaluronic acid, and chondroitin, the latter presumably derived from undersulfated chondroitin sulfate (42). This low degree of sulfation resulted from at least half of the secreted galactosaminoglycan being unsulfated. Although the proportion of unsulfated polysaccharide label was remarkably similar for all culture types (ranging from a low of 80.6% for confluent 3T3 to a high of 91.0% for low density 3T3SV cultures), growth-inhibited cultures showed substantially more chondroitin. Compared to either the cells or the surface materials, the medium contained the lowest relative abundance of labeled heparan sulfate.

DISCUSSION

The intent of this study was to assess the cellular distribution of newly synthesized GAGs associated with viral transformation, with changes in cell density, and with growth control. The results reported here show that cell density and possibly density-dependent inhibition of growth but not viral transformation are major factors controlling the cellular distribution and type of newly synthesized GAG. Thus, there is no simple relationship between GAGs and the differences in culture behavior of normal and transformed cells.

Total GAG Produced

Glucose was used as the precursor to allow comparisons of incorporation into all types of GAG. Although the various GAGs were shown to be equivalently labeled by [³H]glucose under the conditions used, the incorporation of [³H]glucose does not represent synthesis only. For example, density-dependent differences in rates of hexose transport between the cell types are well known (4, 17, 41). By analyzing the relative proportion of total labeled polysaccharide in GAG, differences in transport are minimized. The analysis (Fig. 5 and Table II) shows that transformation and increased cell density reduce GAG production. This is in agreement with the findings that

TABLE IV

GAG Label Associated with Cells, Cell Surfaces, and Media

Type of GAG	Percent of cell-associated label				Percent of trypsin-released label (surface fraction)				Percent of medium label			
	Low density 3T3SV	Low density 3T3	Crowded 3T3SV	Confluent 3T3	Low density 3T3SV	Low density 3T3	Crowded 3T3SV	Confluent 3T3	Low density 3T3SV	Low density 3T3	Crowded 3T3SV	Confluent 3T3
Hyaluronic acid	65.9	70.4	49.3	26.5	86.0	87.1	75.9	64.6	78.0	77.4	83.2	50.5
Heparan sulfate	16.7	11.6	23.8	16.7	3.4	1.7	5.2	7.7	2.3	1.9	2.5	2.4
Galactosaminoglycan Chondroitin-6-sulfate	17.2	18.1	26.9	56.8	10.5	11.3	19.0	27.8	19.7	20.8	14.3	47.0
	2.8	2.7	7.0	21.0	1.0	0.9	1.5	6.7	1.7	1.7	1.7	4.3
Dermatan-4-sulfate Chondroitin-4-sulfate	9.0	10.1	12.3	28.4	5.0	6.1	10.3	14.4	5.0	7.1	5.5	12.6
Chondroitin	5.4	5.3	7.6	7.4	4.5	4.3	7.2	6.7	13.0	12.0	7.1	30.1

Cells, cell surface, and medium components are as in Table II. Results are presented as the percent of [^{3}H]glucose incorporation into GAG of the three components and are the average of separate analyses of duplicate cultures. The extent of incorporation into each culture component is shown in Table IV.

less radiosulfate is incorporated into GAG by transformed and by high density cells than by untransformed (12, 36, 48) and low density cells (34), and with the results of chemical measurements of the amount of GAG produced by primary fibroblast cultures growing at low and high density (24). The decrease in GAG production due to transformation observed here, however, is relatively small but is further reduced when transformed cells reach high density. Therefore, a reduction in GAG production at high density occurs even in cells not showing density-dependent inhibition.

Cellular Distribution of GAG

The major differences between cell types and growth conditions were in the distribution of labeled GAG in the three culture components. Each component had a distinct composition regardless of growth conditions or transformation, indicating that the components represent qualitatively distinct pools of GAG. The cells contained the majority of newly synthesized GAG (Fig. 6) and most of the sulfated galactosaminoglycan and heparan sulfate (Fig. 7). The bulk of this GAG represents GAG stored for eventual degradation (21, 29), and an unknown proportion is in not yet completed chains. The fraction released from cells and substratum by trypsin treatment showed nearly as much GAG label as the cells in low density cultures (Fig. 6), and was predominantly hyaluronic acid (Table IV). This fraction should be almost totally derived from GAG at cell surfaces, since after prolonged labeling of analogous cultures less than 4% of cell-associated glucosamine-labeled GAG remains with the substratum when cells are removed with EGTA (48). The labeled GAG secreted into the medium was predominantly unsulfated, consisting of hyaluronate and chondroitin, and likely contained some high molecular weight products of intracellular degradation.

Effect of Cell Density

At low cell densities, the distribution of label among the culture components and relative amounts of the various types of labeled GAG in each of these components were very similar in transformed and untransformed cultures, although transformed cells contained slightly more labeled heparan sulfate label at the cell surface. These data establish that viral transformation per se does not substantially alter the type or distribution of newly synthesized GAG.

Upon increased cell density, both transformed and normal cells showed substantially more GAG label within the cells and substantially less at the surface with corresponding changes in the predominant GAG type in these components. Possible explanations for the change in GAG distribution include decreased susceptibility of the surface material to release by trypsin, reduced deposition of GAG at the surface, increased GAG uptake from the medium, and a reduction in secretion. The latter two possibilities are unlikely, since an increase in density produced little change in the amount of GAG label in the medium in 3T3SV cultures, and an increase in the amount in 3T3 cultures (Fig. 6). The possibility that the GAG at the surface of crowded cells is less susceptible to trypsin release is consistent with the observations that trypsinization releases less glycopeptide from the surfaces of high density cells (3, 26, 38). The data are equally consistent, however, with a reduction in the deposition of newly synthesized GAG at the cell surface at high cell densities. Less deposition of newly synthesized GAG at the surface might, in the steady state, cause new GAG to accumulate intracellularly, thereby slowing the rate or reducing the synthesis of GAG. Thus, reduction in surface GAG deposition could account for the reduced incorporation observed in high density cultures.

The changes in the type and distribution of labeled GAG occurring at high density were more marked in 3T3 cultures, especially the increased labeling of chondroitin-6-sulfate and heparan sulfate at the cell surface and the reduction in hyaluronic acid production.

Relationship of Culture Behavior to GAG Distribution

Are the cellular distributions of newly synthesized GAG relevant to the differences in culture behavior shown by 3T3 and 3T3SV cells? Transformed cells show reduced adhesivity to the substratum regardless of density (11, 43) and, except at very low densities, the motility of transformed cells is greater than that of untransformed cells (10). In contrast to untransformed cells, transformed cells show high or indefinite saturation density and little density-dependent inhibition of movement (10) or growth (9). The behavioral differences evident at low density probably do not

involve differences in cell surface GAG because, although there is a small difference in amount, the types and relative proportions of labeled surface GAG on low density 3T3 and 3T3SV cells are very similar. This conclusion is consistent with the report that cell locomotion is not modified by inhibition of GAG synthesis (44).

Differences in culture behavior at high densities, however, may be related to differences in GAG distribution. Inhibition of growth appears to correlate with increased GAG in the medium. A shift of GAG label into the medium was seen when 3T3 cells reached confluence, but not when 3T3SV cells became crowded. GAG secretion is markedly increased by addition of several adenylate cyclase inhibitors to primary mouse fibroblasts (40) and of dibutyryl cyclic adenosine monophosphate and theophylline to normal and transformed cells (12). These treatments, which slow the growth rate and decrease the saturation density of untransformed cells (18), are analogous to those of adding dextran sulfate (6, 13) and sulfated GAG (28) to the medium. Thus, it is possible that GAG accumulation in the medium may contribute to growth inhibition.

Cultures with low saturation densities (3T3 and 3T3SV revertant) show more sulfated GAG remaining attached to the substratum than 3T3SV cultures, findings which have led to the proposal that increased cell surface GAG contributes to lowering of the saturation density (34). Substantial evidence, however, indicates that transformed cells have higher anionic surface charge than untransformed cells and that even after sialidase treatment, their surface charges are similar (50).

A possible resolution of these conflicting results is that low saturation densities are associated with changes in surface GAG which do not alter the surface charge. Compared with other culture types, confluent 3T3 cells show more surface labeling of the most anionic GAG, chondroitin-6-sulfate and heparan sulfate, and less surface labeling of the least anionic GAG, hyaluronic acid (Table IV). These changes might result in no net change in surface charge since the reduction in anionic charge due to decreased hyaluronate would be counter-balanced by the increase in the highly anionic GAGs. If these anionic GAGs were more firmly bound to the substratum, despite the lower surface GAG label on confluent cells, more sulfated GAG would remain with the substratum after cells were removed.

The relationship between low saturation density

and surface GAG is unclear. Enhanced cell-substratum adherence has been proposed (7, 34), based upon the idea that the GAG binds cells to the substratum by a calcium ion bridging mechanism. However, normal and transformed cells differ in substratum adherence regardless of density, but show major differences in surface GAG only at high density. Therefore, although changes in surface GAG at high density may contribute to enhanced adhesion, processes other than adhesion to the substratum must also be considered. The similarity in GAG distributions of normal and transformed cells at low density and the differences induced by high density suggest the possibility that GAG is involved in cell-cell interactions. The most striking density-induced change is in hyaluronic acid production and, although hyaluronate has been implicated in various cellular interactions (32, 33, 49), and knowledge of its molecular structure is emerging (14), very little is known about its biological effects.

REFERENCES

1. BERNFIELD, M. R., R. H. COHN, and S. D. BANERJEE. 1973. Basal lamina, glycosaminoglycan (acid mucopolysaccharide) and epithelial morphogenesis. Am. Zool. 13:1057–1083.
2. BOSE, S. K., and B. J. ZLOTNICK. 1973. Growth-and density-dependent inhibition of deoxyglucose transport in Balb 3T3 cells and its absence in cells transformed by murine sarcoma virus. Proc. Natl. Acad. Sci. U. S. A. 70:2374–2378.
3. BUCK, C. A., M. C. GLICK, and L. WARREN. 1971. Effect of growth on the glycoproteins from the surface of control and Rous sarcoma virus transformed hamster cells. Biochemistry. 10:2176–2180.
4. CHIARUGI, V. P., S. VANNUCCHI, and P. URBANO. 1974. Exposure of trypsin-removable sulphated polyanions on the surface of normal and virally transformed BHK 21/C13 cells. Biochim. Biophys. Acta. 345:283–293.
5. CIFONELLI, J. A. 1968. Reaction of heparitin sulfate with nitrous acid. Carbohydr. Res. 8:233–242.

6. CLARKE, G. D., and M. G. P. STOKER. 1971. Conditions affecting the response of cultured cells to serum. Growth Control in Cell Cultures. *Ciba Found. Symp.* 17–32.

7. CULP, L. A. 1974. Substrate-attached glycoproteins mediating adhesion of normal and virus-transformed mouse fibroblasts. *J. Cell Biol.* **63:**71–83.

8. CULP, L. A., and P. H. BLACK. 1972. Contact-inhibited revertant cell lines isolated from simian virus 40-transformed cells. III. Concanavalin A-selected revertant cells. *J. Virol.* **9:**611–620.

9. DULBECCO, R. 1970. Topoinhibition and serum requirement of transformed and untransformed cells. *Nature (Lond.).* **227:**802–804.

10. GAIL, M. H., and C. W. BOONE. 1971. Density inhibition of motility in 3T3 fibroblasts and their SV40 transformants. *Exp. Cell Res.* **64:**156–162.

11. GAIL, M. H., and C. W. BOONE. 1962. Cell-substrate adhesivity. A determinant of cell motility. *Exp. Cell Res.* **70:**33–40.

12. GOGGINS, J. F., G. S. JOHNSON, and I. PASTAN. 1972. The effect of dibutyryl cyclic adenosine monophosphate on synthesis of sulfated acid mucopolysaccharides by transformed fibroblasts. *J. Biol. Chem.* **247:**5759–5764.

13. GOTO, M., Y. KATAOKA, T. KIMURA, K. GOTA, and H. SATO. 1973. Decrease of saturation density of cells of hamster cell lines after treatment with dextran sulfate. *Exp. Cell Res.* **82:**367–374.

14. GUSS, J. M., D. W. L. HUKINS, P. J. C. SMITH, S. ARNOTT, R. MOORHOUSE, and D. A. REES. 1975. Hyaluronic acid: molecular conformations and interactions in two sodium salts. *J. Mol. Biol.* **95:**359–384.

15. HAMERMAN, D., G. J. TODARO, and H. GREEN. 1965. The production of hyaluronate by spontaneously established cell lines and viral transformed lines of fibroblastic origin. *Biochim. Biophys. Acta.* **101:**343–351.

16. HOOK, M., U. LINDAHL, G. BACKSTROM, A. MALMSTROM, and L. FRANSSON. 1974. Biosynthesis of heparin. III. Formation of iduronic acid residues. *J. Biol. Chem.* **249:**3908–3915.

17. ISSELBACHER, K. J. 1972. Increased uptake of amino acids and 2-deoxy-D-glucose by virus-transformed cells in culture. *Proc. Natl. Acad. Sci. U. S. A.* **69:**585–589.

18. JOHNSON, G. S., and I. PASTAN. 1972. Role of 3', 5'-adenosine monophosphate in regulation of morphology and growth of transformed and normal fibroblasts. *J. Natl. Cancer Inst.* **48:**1377–1387.

19. KOSHER, R. A., and R. L. SEARLS. 1973. Sulfated mucopolysaccharide synthesis during the development of *Rana pipiens. Dev. Biol.* **32:**50–68.

20. DRAEMER, P. M. 1971. Complex carbohydrates of animal cells: biochemistry and physiology of the cell periphery. *In* Biomembranes. L. A. Manson, editor. Plenum Press, New York. **1:**67–190.

21. KRAEMER, P. M., and D. A. SMITH. 1974. High molecular-weight heparan sulfate from the cell surface. *Biochem. Biophys. Res. Commun.* **56:**423–430.

22. KRAEMER, P. M., and R. A. TOBEY. 1972. Cell-cycle dependent desquamation of heparan sulfate from the cell surface. *J. Cell Biol.* **55:**713–717.

23. LINKER, A., P. HOFFMAN, K. MEYER, P. SAMPSON, and E. D. KORN. 1960. The formation of unsaturated disaccharides from mucopolysaccharides and their cleavage to α-keto acid by bacterial enzymes. *J. Biol. Chem.* **235:**3061–3065.

24. LIPPMAN, M. 1968. Glycosaminoglycans and cell division. *In* Epithelial-Mesenchymal Interactions. P. Fleischmajer and R. Billingham, editors. The Williams and Wilkins Co., Baltimore, Md. 208–229.

25. MARTINEZ-PALOMO, A., C. BRAISLOVSKY, and W. BERNHARD. 1969. Ultrastructural modifications of the cell surface and intercellular contacts of some transformed cell strains. *Cancer Res.* **29:**925–937.

26. MEEZAN, E., H. C. WU, P. H. BLACK, and P. W. ROBBINS. 1969. Comparative studies on the carbohydrate-containing membrane components of normal and virus-transformed mouse fibroblasts. II. Separation of glycoproteins and glycopeptides by Sephadex chromatography. *Biochemistry.* **8:**2518–2524.

27. MEIER, S., and E. D. HAY. 1974. Stimulation of extracellular matrix synthesis in the developing cornea by glycosaminoglycans. *Proc. Natl. Acad. Sci. U. S. A.* **71:**2310–2313.

28. MONTAGNIER, L. 1971. Factors controlling the multiplication of untransformed and transformed BHK_{21} cells under various environmental conditions. Growth Control in Cell Cultures. *Ciba Found. Symp.*, 33–44.

29. NEUFELD, E. F., and M. CANTZ. 1973. The mucopolysaccharidoses studied in cell culture. *In* Lysosome and Storage Disease. H. G. Hirs and F. Van Hoof, editors. Academic Press, Inc., New York. 261–275.

30. NOONAN, K. D., and M. M. BURGER. 1973. Binding of [³H]concanavalin A to normal and transformed cells. *J. Biol. Chem.* **248:**4286–4292.

31. OYAMA, V. I., and H. EAGLE. 1956. Measurement of cell growth in tissue culture with a phenol reagent (Folin-Ciocalteau). *Proc. Soc. Exp. Biol. Med.* **91:**305–307.

32. PESSAC, B., and V. DEFENDI. 1972. Cell aggregation: role of acid mucopolysaccharides. *Science (Wash. D. C.),* **175:**898–900.

33. PRATT, R. M., M. A. LARSON, and M. C. JOHNSTON. 1975. Migration of cranial neural crest cells in a cell-free hyaluronic acid-rich matrix. *Dev. Biol.* **44:**298–304.

34. ROBLIN, R., S. O. ALBERT, N. A. GELB, and P. H. BLACK. 1975. Cell surface changes correlated with density-dependent growth inhibition. Glycosami-

noglycan metabolism in 3T3, SV3T3, and Con A-selected revertant cells. *Biochemistry*. **14**:347-357.

35. Roden, L., J. R. Baker, J. A. Cifonelli, and M. B. Mathews. 1972. Isolation and characterization of connective tissue polysaccharides. *Methods Enzymol.* **28**(Pt. B.):73-140.

36. Saito, H., and B. G. Uzman. 1971. Production and secretion of chondroitin sulfates and dermatan sulfate by established mammalian cell lines. *Biochem. Biophys. Res. Commun.* **43**:723-728.

37. Saito, H., T. Yamagata, and S. Suzuki. 1968. Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfates. *J. Biol. Chem.* **243**:1536-1542.

38. Sakiyama, H., and B. W. Burge. 1972. Comparative studies of the carbohydrate-containing components of 3T3 and simian virus 40-transformed 3T3 mouse fibroblasts. *Biochemistry*. **11**:1366-1376.

39. Satoh, C., R. Duff, R. Rapp, and E. A. Davidson. 1973. Production of mucopolysaccharides by normal and transformed cells. *Proc. Natl. Acad. Sci. U. S. A.* **70**:54-56.

40. Schonhofer, P. S., H. D. Peters, K. Karzel, V. Dinnendah, and P. Westhofen. 1974. Influence of antiphlogistic drugs on prostaglandin E₁ stimulated cyclic 3',5'-amp levels and glycosaminoglycan synthesis in fibroblast tissue cultures. *Pol. J. Pharmacol. Pharm.* **26**:51-60.

41. Schultz, A. R., and L. A. Culp. 1973. Contact-inhibited revertant cell lines isolated from SV40-transformed cells. *Exp. Cell Res.* **81**:95-103.

42. Shapiro, S. S., and M. I. Sherman. 1974. Sulfated mucopolysaccharides of midgestation embryonic and estraembryonic tissues of the mouse. *Arch. Biochem. Biophys.* **162**:272-280.

43. Shields, R., and K. Pollack. 1974. The adhesion of BHK and PyBHK cells to the substratum. *Cell.* **3**:31-38.

44. Spooner, B. S., and G. W. Conrad. 1975. The role of extracellular materials in cell movement. I. Inhibition of mucopolysaccharide synthesis does not stop ruffling membrane activity or cell movement. *J. Cell Biol.* **65**:286-297.

45. Strominger, J. O. 1962. Uridine and guanosine nucleotides of hen oviduct. *J. Biol. Chem.* **237**:1388-1392.

46. Strominger, J. O., J. T. Park, and R. E. Thompson. 1959. Composition of the cell wall of *Staphylococcus aureus*: its relation to the mechanism of action of penicillin. *J. Biol. Chem.* **234**:3263-3268.

47. Suzuki, S., H. Saito, T. Yamagata, K. Anno, N. Seno, Y. Kawai, and T. Furuhashi. 1968. Formation of three types of disulfated disaccharides from chondroitin sulfates by chondroitinase digestion. *J. Biol. Chem.* **243**:1543-1550.

48. Terry, A. H., and L. A. Culp. 1974. Substrate-attached glycoproteins from normal and virus-transformed cells. *Biochemistry*. **13**:414-424.

49. Toole, B. P., and R. L. Trelstad. 1971. Hyaluronate production and removal during corneal development in the chick. *Dev. Biol.* **26**:28-35.

50. Weiss, L. 1967. The Cell Periphery, Metastasis and Other Contact Phenomena. North Holland, Amsterdam. 1-388.

51. Yamagata, T., H. Saito, O. Habuchi, and S. Suzuki. 1968. Purification and properties of bacterial chondroitinases and chondrosulfatases. *J. Biol. Chem.* **243**:1523-1535.

52. Yamamoto, K., and H. Terayama. 1973. Comparison of cell coat acid mucopolysaccharides of normal liver and various ascites hepatoma cells. *Cancer Res.* **33**:2257-2264.