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Decreased biosynthesis of Forssman glycolipid after retinoic acid-induced differentiation of mouse F9 teratocarcinoma cells. Lectin-affinity chromatography of the glycolipid-derived oligosaccharide*,†

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ABSTRACT

Glycolipids synthesized by the mouse teratocarcinoma F9 cells and F9 cells (RA/F9 cells) induced to differentiate by a 3-day treatment with 0.1 μM all-*trans*-retinoic acid were analyzed. Both F9 cells and RA/F9 cells were incubated in media containing either D-[6-^{3}H]galactose or D-[6-^{3}H]glucosamine; the metabolically-radiolabeled glycolipids were isolated and the oligosaccharides were released from the glycolipids by ozonolysis and alkali fragmentation. From both cells, a single major pentasaccharide was isolated from the mixture of neutral [^{3}H]oligosaccharides by affinity chromatography on a column of immobilized *Helix pomatia* agglutinin. The structure of this oligosaccharide was analyzed by methylation analysis and specific exoglycosidase treatments and identified as the Forssman pentasaccharide α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→4)-α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-D-Glc. There was a 3–4-fold decreased amount of the Forssman pentasaccharide from RA/F9 cells relative to F9 cells. In contrast, there were no major differences between these cells in the levels of globoside, the precursor to Forssman glycolipid. To investigate the basis for the decline in Forssman glycolipid synthesis upon differentiation, the activity of UDP-D-GalNAc:GbOse$_4$Cer α-(1→3)-*N*-acetyl-D-galactosaminyltransferase (Forssman synthase) was determined in extracts of both the F9 and RA/F9 cells. The specific activity of Forssman synthase was approximately 70% lower in differentiated relative to the nondifferentiated cells. These data demonstrated that F9 cells synthesize authentic Forssman glycolipid, and that its expression and the activity of Forssman synthase were decreased following induced cellular differentiation.

INTRODUCTION

A number of teratocarcinoma cell lines display morphological and biochemical properties similar to the discrete populations of cells that arise during early embryogenesis[1–3]. The murine teratocarcinoma cell line F9 expresses many of the surface carbo-

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hydrate antigens found on the pluripotent stem cells of the developing mouse embryo[4-10]. When grown in culture in the presence of retinoic acid, F9 cells differentiate into parietal endoderm-like cells and initiate synthesis of the basement membrane components, laminin and Type IV collagen[11-15]. Because of these discrete biochemical changes, F9 cells are a useful model for studying certain events accompanying, or associated with, embryonic development.

Immunological techniques have suggested that retinoic acid-induced differentiation of F9 cells results in altered expression of some glycoconjugates. For example, based on immunological studies, F9 cells contain both stage specific embryonic antigen-1 (SSEA-1)[4,16,17] and Forssman antigen[18-20]. After being cultured in the presence of retinoic acid for three days, they showed decreased levels of SSEA-1 antigen, as demonstrated by indirect immunofluorescence techniques and the lack of agglutinability of the cells in the presence of specific antisera[13,21]. The radioimmune-overlay technique indicated that there is a reduction of Forssman active glycolipids on established, differentiated F9 cell lines[20].

Although immunological approaches are useful for studying developmentally regulated changes in the expression of cell surface oligosaccharides[6,7,22], they provide little quantitative information and are limited to the detection of those structures for which antibodies are available. It is also possible that different glycoconjugates may share the same antigenic determinant. For example, the terminal trisaccharide sequence, α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→, which is Forssman reactive, can be present on different glycolipids[23-25]. Finally, immunological detection *per se* does not address the question of what metabolic changes lead to the altered expression of an antigen.

Because the Forssman glycolipid is expressed usually in small amounts, we have used the technique of metabolic radiolabeling. This approach has been especially successful in analyzing the structures of glycoprotein oligosaccharides and the lipid-linked oligosaccharide involved in glycoprotein biosynthesis (for example, see refs. 26 and 27). This report describes the application of these methods to the detection and structural analysis of the Forssman glycolipid in F9 teratocarcinoma cells and the decreased synthesis of this component after retinoic acid-induced differentiation of these cells.

EXPERIMENTAL

Materials. — *Helix pomatia* lectin and *Escherichia coli* β-D-galactosidase were purchased from Sigma Chemical Co. (St. Louis, MO). *Ricinus communis* agglutinin-1 (RCA-1), immobilized on agarose beads, was purchased from E-Y Laboratories (San Mateo, CA). *N*-Acetyl-α-D-galactosaminidase from *Patella vulgata* and *N*-acetyl-β-D-hexosaminidase from jack bean were obtained from V-Labs (Covington, LA). Coffee bean α-D-galactosidase was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Bio-Gel P-2 and P-6 (−400 mesh), Dowex AG 50W-X4 (H^+), and AG 1-X8 (AcO^-) ion-exchange resins were purchased from Bio-Rad Laboratories (Richmond,

CA). D-[6-^{3}H]Galactose and D-[6-^{3}H]glucosamine (spec. act., 1.1 T Bq/mmol) were purchased from ICN Radiochemicals (Irvine, CA). UDP-*N*-acetyl-[1-^{14}C]galactosamine (1.8 GBq/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Tissue culture reagents were obtained from Flow Laboratories, Inc. (McClean, VA). All-*trans*-retinoic acid was purchased from Eastman Kodak Co. (Rochester, NY) and globoside from Supelco Inc. (Bellefonte, PA). All other chemicals used were of the highest grade available.

Cell culture. — F9 mouse teratocarcinoma cells were cultured on gelatin-coated plates in Dulbecco's modified Eagle's medium containing 15% fetal calf serum as described by Strickland and Mahdavi[11]. Cells were induced to differentiate by adding all-*trans*-retinoic acid from a mM solution in ethanol to a final concentration of 0.1 μM. Cells were cultured with the inducer for three days before being used in these studies. Differentiation of these cells (RA/F9 cells) was confirmed by immunofluorescent staining with mouse IgM antibodies to SSEA-1, or visible agglutination of the cells in the presence of the same antibodies as described previously[21].

Metabolic labeling of F9 and RA/F9 cells. — Four, 60-mm Petri dishes were seeded with 1×10^5 F9 cells. Two plates were cultured under standard conditions and the other two were incubated under identical conditions for three days in the presence of a medium containing retinoic acid. After removal of the medium, fresh medium containing 1.1 MBq/mL of D-[^{3}H]galactose was added to one plate of F9 cells and RA/F9 cells. Other plates of cells were labeled under similar conditions with a medium containing 1.1 MBq/mL of D-[^{3}H]glucosamine. After incubation for 24 h, the medium was removed and the cells were washed three times with phosphate-buffered saline solution (PBS). Cells were harvested with a rubber policeman, transferred to a 2-mL Eppendorf centrifuge tube, and centrifuged. The supernatant was removed and the resulting cell pellets were lyophilized.

Extraction of ^{3}H-labeled glycolipids and glycopeptides. — The lyophilized cell pellets were suspended in cold water and treated in a bath sonicator for 15 min at 4°. The homogenate was quantitatively transferred with water to a glass tube in a final volume of 1.0 mL. Glycolipids were extracted twice in a final solvent ratio of 4:8:3 chloroform–methanol–water as described previously[28]. Insoluble material was removed by centrifugation, washed twice with absolute ethanol, and subjected to exhaustive protease digestion to obtain glycopeptides[29]. The labeled glycopeptides were separated from the protease digest as previously described[30].

The total lipid extract was partitioned twice by addition of water, and subsequently water and methanol, to obtain upper- and lower-phase glycolipid fractions[28]. Metabolically radiolabeled glycolipids were separated from other radiolabeled contaminants in the upper phase by reverse-phase chromatography on Sep-Pak cartridges[31]. Briefly, the upper-phase glycolipids were dried under N_2 and dissolved in 1:1 methanol–water (3 mL) containing 0.1M sodium acetate. After the salts and polar contaminants were separated from radiolabeled glycolipids by use of the Sep-Pak cartridges[31], these were washed additionally with 4:8:3 chloroform–methanol–water to ensure complete removal of more polar, higher-molecular-weight glycolipids. The organic washes were pooled and dried under reduced pressure.

The radiolabeled upper-phase glycolipids were dissolved in 4:8:3 chloroform–methanol–water (1 mL) and separated into acidic and neutral glycolipid fractions by chromatography, on a column (0.5 × 4 cm) of DEAE-Sepharose, as described previously[32], using 4:8:3 chloroform–methanol–water as the eluent. Unbound, neutral glycolipids were eluted with the equilibration solvent, and the acidic glycolipids were subsequently eluted with the same solvent containing 0.15M ammonium acetate. The lower-phase, radiolabeled glycolipids were combined with the neutral upper-phase fraction and designated neutral glycolipid fraction.

Ozonolysis–alkali fragmentation of labeled glycolipids. — A modification[33] of the ozonolysis–alkali fragmentation procedure[34,35] was employed to release oligosaccharides from the metabolically labeled, neutral glycolipid fraction. To insure solubilization of the more polar glycolipids during ozonolysis, 1:7:2 chloroform–methanol–water rather than methanol was used to dissolve glycolipids prior to the addition of ozone to a solution in dichloromethane. After being dried under an N_2 stream, the ozone-treated glycolipids were dissolved in 0.1M sodium methoxide in methanol (1 mL) and the solution was kept at room temperature for 30 min. After the addition of water (1 mL), the mixture was mixed (Vortex) and then kept for 30 min, and the base neutralized with 2M acetic acid (100 μL).

Unfragmented glycolipids were separated by reverse-phase chromatography from free oligosaccharides by use of Sep-Pak cartridges as described for the preparation of upper-phase glycolipids. Free oligosaccharides were obtained in the 1:1 methanol–water and water washes. Ozone-resistant and unfragmented glycolipids were eluted with the organic solvent washes. The aqueous fractions were combined and dried under reduced pressure. Neutral oligosaccharides were desalted by passage through small, mixed-bed columns containing alternating layers of AG 50W (H^+) and AG 1 (AcO^-) ion-exchange resins.

Chromatographic methods. — Gel filtration was carried out on columns of Bio-Gel P-6 (0.9 × 95 cm) or Bio-Gel P-2 (1.0 × 45 cm), equilibrated in 0.1M pyridinium acetate buffer, pH 5.4 The columns were calibrated for estimating the size of oligosaccharides as previously described[36]. Lectin-affinity chromatography on a column of immobilized *Helix pomatia* lectin, equilibrated in PBS, was carried out as previously described[37]. Descending paper chromatography for the identification of monosaccharides was accomplished on Whatman No. 1 paper with (*A*) 12:5:4 ethyl acetate–pyridine–water or (*B*) 6:4:3 butanol–glacial acetic acid–water. Radiolabeled monosaccharides were located on 2.5 × 50-cm chromatograms by eluting 1-cm segments with water and counting the radioactivity of the eluates. Partially methylated [^{3}H]monosaccharides were separated by t.l.c. using 500:6:3 acetone–water–ammonium hydroxide (solvent *C*), and detected by scraping 0.5-cm segments on the silica gel into vials and counting the radioactivity as previously described[30,38].

Analytical methods. — The radiolabeled monosaccharide components of the affinity-purified [^{3}H]oligosaccharide were identified by descending paper chromatography of acid hydrolyzates. The [^{3}H]galactose-labeled oligosaccharide was hydrolyzed in 2M trifluoroacetic acid for 4 h at 100°. The hydrolyzate was dried under N_2 and

subjected to paper chromatography in solvent *A*. Free amino sugars, obtained by hydrolysis of the [^{3}H]glucosamine-labeled oligosaccharide in 4M HCl for 4 h at 100°, were dried under N_2, *N*-reacetylated, and subjected to chromatography on borate-impregnated paper using solvent *B*[30]. Monosaccharides were identified by comigration with authentic standards.

For methylation analyses, the [^{3}H]galactose-labeled oligosaccharide was reduced with $NaBH_4$ and permethylated by use of a modification[40] of the iodomethane–NaOH–dimethyl sulfoxide procedure[41]. The permethylated oligosaccharide was hydrolyzed in 2M trifluoroacetic acid for 4 h at 100°. The sample was cooled and the trifluoroacetic acid was evaporated under N_2 at 37°. The partially methylated, metabolically radiolabeled monosaccharides were separated by t.l.c. in solvent *C*[42] and identified by cochromatography with authentic standards as previously described[36].

Assay for N-*acetyl-α-*D-*galactosaminyltransferase activity*. — To prepare cell extracts, F9 and RA/F9 cells were scraped from dishes in PBS, and washed once in PBS and a second time in 100mM cacodylate buffer, pH 6.5. The cell pellets were disrupted by sonication in the cacodylate buffer and protein was determined by the method of Lowry *et al.*[43] using bovine serum albumin as a standard. Triton X-100 was added to a concentration of 1% and the suspension kept on ice for 30 min prior to use to ensure complete solubilization. The *N*-acetyl-α-D-galactosaminyltransferase activity was assayed with globoside as an acceptor. The reaction mixtures contained globoside (50 μg), 10mM $MnCl_2$, 0.3% Triton X-100, 0.084mM UDP-D-[1-^{14}C]GalNAc (109 500 c.p.m./nmol), and cell extract protein (3 mg). After being incubated for 4 h at 37°, the reaction mixture was diluted with water (1 mL) and boiled for 5 min. The mixture was then centrifuged and the supernatant was extracted with 2:1 chloroform–methanol. The aqueous phase was extracted again with 10:10:3 chloroform–methanol–water (1 mL). The organic phases from the two extractions were combined, evaporated, and then resuspended in 2:1 chloroform–methanol (20 μL). This material was deposited on an Anasil G plate (Analabs, Norwalk, CT) for t.l.c. in 60:35:8 chloroform–methanol–water. Segments (0.5 cm) of each lane were scraped into scintillation counting vials. Authentic globoside and Forssman glycolipid[37] standards were deposited separately on the same plate and detected with the orcinol spray reagent[44].

Exoglycosidase digestions. — Oligosaccharides were digested with *N*-acetyl-α-D-galactosaminidase[39] (0.05 units/mL) or *N*-acetyl-β-D-hexosaminidase[45] (0.1 unit/mL) in 20mM citrate buffer, pH 4.0, for 36 h. Digestions[46] with coffee bean α-D-galactosidase (0.1 unit/mL) were done in 0.1M KH_2PO_4, pH 6.5, for 16 h, and digestions with *E. coli* β-D-galactosidase (0.1 unit/mL) were carried out in 0.05M NaH_2PO_4, pH 7.2, for 24 h. All incubations were at 37° in an atmosphere of toluene and a final volume of 0.05 mL.

RESULTS

Metabolic-radiolabeling of F9 and RA/F9 cells. — Both F9 and RA/F9 cells were incubated in a normal medium containing either D-[^{3}H]galactose or D-[^{3}H]glucosamine for 24 h, and the metabolically radiolabeled glycoconjugates were isolated as described

TABLE I

Incorporation of ^{3}H-labeled monosaccharides into glycoconjugates from F9 and RA/F9 cells

Cells	*Precursor sugar*	*Radioactivity incorporated (c.p.m. × 10^{-6})*[a]		
		Neutral glycolipids	*Acidic glycolipds*	*Glycopeptide fraction*
F9	D-[6-^{3}H]Gal	12.3 (51)	4.0 (17)	7.7 (32)
	D-[6-^{3}H]GlcN	1.4 (28)	0.9 (18)	2.7 (54)
RA/F9	D-[6-^{3}H]Gal	3.0 (44)	2.1 (31)	1.7 (25)
	D-[6-^{3}H]GlcN	0.7 (23)	1.1 (35)	1.3 (42)

[a] Percent of total counts incorporated into glycolipids and glycopeptide fractions in parentheses.

in the Experimental section. The distribution of radioactivity incorporated into glycolipid and glycopeptide fractions is shown in Table I. Since the differentiated cells grew more slowly during the labeling period and fewer cells were available for extraction, less radioactivity was incorporated into glycoconjugates of RA/F9 cells than into the

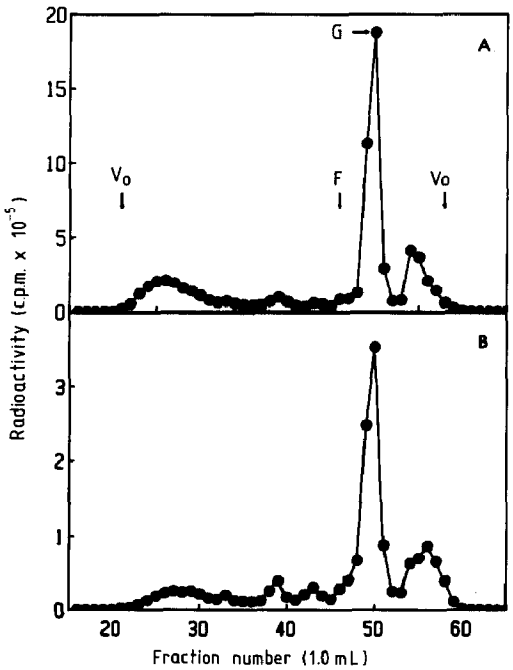

Fig. 1. Gel filtration of metabolically labeled, glycolipid-derived oligosaccharides from F9 and RA/F9 cells. Neutral glycolipid-derived oligosaccharides from F9 cells (A) or RA/F9 cells (B), cultured in the presence of D-[6-^{3}H]galactose were applied to a column of Bio-Gel P-6 equilibrated and eluted with pyridine–acetic acid buffer. Radioactivity was measured by counting samples of each fraction (1.0 mL). The column void volume (V_o) and the total volume (V_t) were determined by the elution of Dextran and D-glucose, respectively. The elution volumes of authentic Forssman oligosaccharide (F) and globoside oligosaccharide (G) are indicated.

glycoconjugates of F9 cells. Comparison of the relative amounts of specific glycolipid-derived oligosaccharides was, therefore, made on the basis of percentages of oligosaccharide within a common fraction.

Isolation of neutral glycolipid-derived oligosaccharides. — The neutral glycolipid fractions from D-[^{3}H]galactose- and D-[^{3}H]glucosamine-labeled F9 and RA/F9 cells were subjected to ozonolysis and alkali fragmentation. The yields of free [^{3}H]oligosaccharides from the neutral [^{3}H]glycolipid fractions were 60–80%. The free [^{3}H]oligosaccharides derived from cells labeled with D-[6-^{3}H]galactose were subjected to gel filtration on a column of Bio-Gel P-6 (Fig. 1). Only minor differences were observed in the profile of radioactivity obtained with [^{3}H]oligosaccharides from F9 cells (Fig. 1A) when compared to the profile from RA/F9 cells (Fig. 1B). The major oligosaccharide component (tubes 48–52) was eluted with a distribution coefficient of a tetrasaccharide and was identified as the globoside oligosaccharide. A slight decrease in the higher-molecular-weight oligosaccharides (fractions 23–35) was observed in the sample from the RA/F9 cells. The results of the analysis of the [^{3}H]oligosaccharides derived from cells labeled with D-[6-^{3}H]glucosamine were identical (data not shown).

Decreased levels of Forssman-derived oligosaccharide in RA/F9 cells. — In order to detect the relative amounts of Forssman glycolipid-derived oligosaccharide, the fractions eluted from the P-6 columns in the area of pentasaccharides and tetrasaccharides

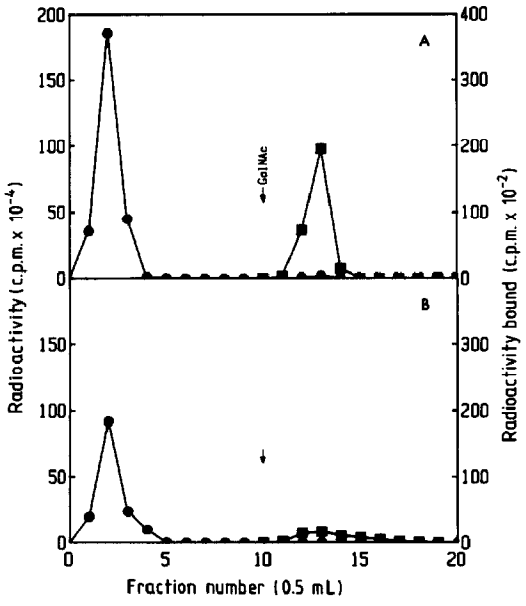

Fig. 2. Affinity purification of Forssman-derived oligosaccharides from F9 and RA/F9 cells. The mixture of tetra- and penta-saccharides (fractions 43–52, Fig. 1) from F9 cells (A) or RA/F9 cells (B), cultured in the presence of D-[6-^{3}H]galactose were applied to a column (0.5 x 6 cm) of *Helix pomatia* lectin-Affigel (9 mg of lectin/mL of gel), equilibrated in PBS at room temperature and allowed to stand for 30 min before elution at a flow rate of 10 mL/h. Radioactivity was measured on 0.5-mL fractions (●). Radioactivity bound by the lectin column and specifically eluted with D-GalNAc (0.1 mg/mL) is shown in an expanded scale (■).

(fractions 43–52) were pooled and subjected to affinity chromatography on a column of immobilized *Helix pomatia* lectin, which specifically binds oligosaccharides having nonreducing 2-acetamido-2-deoxy-α-D-galactopyranosyl end-groups[37]. For the D-[^{3}H]galactose-labeled oligosaccharides, 1.5% of the F9 and 0.4% of the RA/F9 material bound to immobilized HPA. Similarly, for the D-[^{3}H]glucosamine-labeled oligosaccharides, 2.5% of the F9 and 0.8% of the RA/F9 material bound to HPA. Thus, there was a 3–4-fold decrease in the amount of Forssman glycolipid in the RA/F9 cells relative to the F9 cells. In general, these small amounts of radiolabel bound by immobilized HPA indicated that the affinity-purified [^{3}H]oligosaccharides from D-[^{3}H]galactose-labeled cells represent minor components of the tetrasaccharide and pentasaccharide fraction (Fig. 2). The metabolically radiolabeled, affinity-purified oligosaccharide was a pentasaccharide based on its behavior on gel filtration and cochromatography with authentic Forssman-derived oligosaccharide on paper chromatography.

Composition of the Helix pomatia*-bound oligosaccharide.* — The radioactively labeled monosaccharides, incorporated into the affinity-purified pentasaccharides from F9 and RA/F9 cells, were identified by cochromatography with authentic standards. *N*-Acetylgalactosamine was identified as the only amino sugar in the oligosaccharides isolated from D-[^{3}H]glucosamine-labeled cells. The affinity-purified pentasaccharides from the D-[^{3}H]galactose-labeled cells contained both glucose and galactose. The ratio of galactose to glucose in the pentasaccharide from D-[^{3}H]galactose-labeled F9 cells and RA/F9 cells was ~ 6:1 and 7:1, respectively, based on the relative amounts of radioactivity in each peak (data not shown). The actual molar ratio of galactose to glucose in the Forssman glycolipid is 2:1. This difference is presumably due to differences in the specific radioactivities of the intracellular pools of UDP-D-glucose and UDP-D-galactose.

To determine more precisely the relationship between radioactivity incorporated as glucose and galactose into glycolipids of F9 and RA/F9 cells, two disaccharides, one isolated from the glycolipid-derived oligosaccharides of D-[^{3}H]galactose-labeled F9 and one from RA/F9 cells (Figs. 1A and 1B, fractions 53–60), were analyzed. Both disaccharides were identified as lactose by their cochromatography with standards on paper chromatography, their retardation on a column of RCA-1, and their complete hydrolysis to D-galactose and D-glucose with *E. coli* β-D-galactosidase (data not shown). Paper chromatography of the component [^{3}H]monosaccharides from these disaccharides, which have a molar ratio of galactose to glucose of 1:1, indicated a [^{3}H]galactose-to-[^{3}H]glucose ratio of 2.7:1 for the F9 structure and 3.6:1 for the RA/F9 structure (data not shown). These ratios were essentially one half those found for the affinity-purified pentasaccharides from F9 and RA/F9 cells. These observations permitted the calculation of the molar ratio galactose to glucose in the pentasaccharides as 2:1. This is the value expected for the oligosaccharide derived from the Forssman glycolipid.

Structural analysis of the affinity purified, Helix pomatia*-bound pentasaccharide.* — The position and anomeric configuration of the amino sugars in the pentasaccharide from D-[^{3}H]glucosamine-labeled cells was determined by exoglycosidase digestion. Treatment of the pentasaccharide from the F9 cells with *N*-acetyl-α-D-galactosamini-

TABLE II

Methylation analysis of products from sequential exoglycosidase digestion of the D-[6-^{3}H]Gal-labeled pentasaccharide from F9 cells

	Methylated digestion products migrating with methylated standards (c.p.m.)[a]		
Methylated sugar	*Tetrasaccharide*	*Trisaccharide*	*Disaccharide*
2,3,4,6-Tetra-*O*-methylgalactose		187 (0.9)	284 (1.1)
2,3,6-Tri-*O*-methylgalactose	177 (0.9)	244 (1.1)	
2,4,6-Tri-*O*-methylgalactose	244 (1.1)		
1,2,3,5,6-Penta-*O*-methylglucitol	57 (1.0)	65 (1.0)	64 (1.0)

[a] In parentheses, mols of methylated sugar/mol of oligosaccharide based on the relative amounts of radioactivity in galactose and glucose from glycolipid-derived lactose in these cells, and on the assumption of one mol of glucose/mol of oligosaccharide.

dase resulted in its conversion to equal amounts of radiolabeled tetrasaccharide and free D-[^{3}H]GalNAc. Digestion of the tetrasaccharide with *N*-acetyl-β-D-hexosaminidase resulted in the complete release of D-[^{3}H]GalNAc from the tetrasaccharide. Exoglycosidase digestion of the corresponding RA/F9 pentasaccharide yielded identical results. These data indicated that the affinity-purified pentasaccharides have two D-GalNAc units, one (1→3)-linked terminal α-D-GalNAc group, and one penultimate β-D-GalNAc residue. The remaining three residues of the affinity-purified pentasaccharides are galactose and glucose in a molar ratio of 2:1.

The substitution pattern of the neutral sugars in the pentasaccharide was obtained by methylation analysis. The intact, affinity-purified pentasaccharides from D-[^{3}H]galactose-labeled F9 and RA/F9 cells were reduced with sodium borohydride, permethylated, and hydrolyzed. The resulting, partially methylated [^{3}H]monosaccharides were identified by cochromatography with standards. As summarized in Table II, the intact pentasaccharide consisted of 3-O- and a 4-O-substituted galactose units, and a reducing terminal glucose unit, which is consistent with the structure of the Forssman pentasaccharide.

The linkages of the neutral monosaccharides in the reduced D-[^{3}H]galactose-labeled pentasaccharide from F9 cells was carried out by methylation analyses of the products of sequential exoglycosidase digestions. The pentasaccharide was quantitatively converted into a tetrasaccharide by incubation with *N*-acetyl-α-D-galactosaminidase, and methylation analysis of this tetrasaccharide gave results identical to those of the reduced pentasaccharide. Methylation analysis of the trisaccharide generated by treatment of the tetrasaccharide with *N*-acetyl-β-D-hexosaminidase indicated that this enzyme treatment converted a 3-O-substituted galactosyl residue into a terminal galactosyl group and left the 4-O-substituted galactosyl and glucitol residues intact (Table II). Incubation of the trisaccharide with α-D-galactosidase converted it into a monosaccharide and a disaccharide. Analysis of the partially methylated monosaccharides derived from the permethylated reduced disaccharide indicated a terminal galactosyl

group and a reduced glucitol residue in a 1:1 ratio (Table II). Although the reduced disaccharide was resistant to digestion with *E. coli* β-D-galactosidase, the disaccharide generated from a nonreduced sample of the pentasaccharide by an identical sequence of enzyme digestions was hydrolyzed to D-glucose and D-galactose by β-D-galactosidase (data not shown). Thus, the results of the structural analyses of the affinity-purified pentasaccharide, isolated from D-[^{3}H]galactose- and D-[^{3}H]glucosamine-labeled F9 and RA/F9 cells, are consistent with the pentasaccharide being the Forssman-derived oligosaccharide (**1**).

α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-D-Glc

1

Determination of the activity of UPD-D-GalNAc:GbOse$_4$Cer (1→3)-N-acetyl-α-D-galactosaminyltransferase in homogenates of F9 and differentiated F9 cells. — The decrease in the amount of Forssman pentasaccharide relative to the total neutral glycolipid-derived oligosaccharides (>70%) is consistent with immunological data indicating the loss of the Forssman antigen in established, differentiated F9 cells[9,19]. The decreased expression of Forssman glycolipid in differentiated cells could be due to several factors, including a decreased amount of globoside, the precursor to the Forssman glycolipid, or a decreased level of the glycosyltransferase catalyzing the conversion of globoside to Forssman glycolipid. In both differentiated and nondifferentiated cells, the oligosaccharide derived from globoside was the major glycolipid-

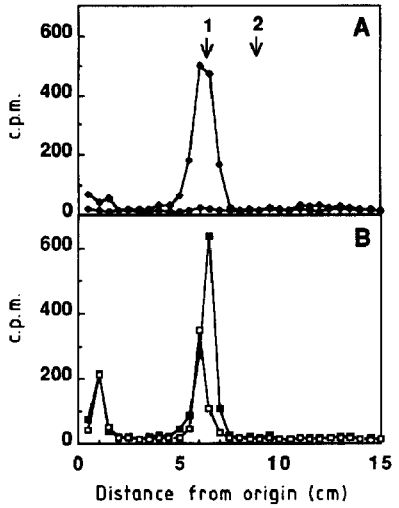

Fig. 3. Identification of the *N*-acetyl-α-D-galactosaminyltransferase reaction products by comigration with authentic standards on t.l.c.: (A) The F9 extract (3 mg of protein) was incubated with the globoside acceptor (50 μg) (◆) or without acceptor ( ) for 4 h at 37°. The organic extract of the assay mixture was analyzed by t.l.c. as described in the Experimental section. (B) Identical analyses of a RA/F9 extract (■) and an equal mixture of F9 and RA/F9 extracts (□) incubated with globoside as the acceptor. The positions of migration for authentic glycolipid standards are as follows. (1) Forssman glycolipid and (2) globoside.

TABLE III

UPD-D-GalNAc:GbOse$_4$Cer (1→3)-*N*-acetyl-α-D-galactosaminyltransferase activity in mixed extracts of F9 and RA/F9 cells

Enzyme source	*Product formed mg^{-1} h^{-1} (pmol)*[a]	
	Experiment No. 1	*Experiment No. 2*
F9 Extract	1.02	0.95
RA/F9 Extract	0.36	0.28
F9 Extract + RA/F9 Extract	0.79[b]	[c]

[a] The specific activities of the *N*-acetyl-α-D-galactosaminyltransferase were determined from the amount of radiolabeled Forssman glycolipid synthesized as shown in Fig. 3. The expected specific activity in the mixture in pmol mg^{-1} h^{-1} was calculated from the separately determined activities in each extract. [b] Calc. 0.69. [c] Not determined.

derived oligosaccharide, comprising 30–45% of the total D-[^{3}H]galactose incorporated into neutral glycolipid from these cells, and the ratio of incorporated D-[^{3}H]galactose in globoside relative to Forssman glycolipid was 59:1 in F9 cells and 160:1 in the differentiated cells. Thus, the depressed level of Forssman glycolipid associated with cellular differentiation is not due to decreased levels of its precursor.

Therefore, we tested the possibility that the decreased expression of Forssman glycolipid in differentiated F9 cells is due to decreased activity of the enzyme UDP-D-GalNAc:GbOse$_4$Cer (1→3)-*N*-acetyl-α-D-galactosaminyltransferase, which converts globoside into Forssman glycolipid. This enzyme has been assayed in extracts from a number of cells and tissues[47–51]. Extracts from both F9 cells and F9 cells induced to differentiate by treatment with retinoic acid for three days were incubated with globoside and UDP-D-[^{14}C]GalNAc, and the radiolabeled glycolipid products were isolated and analyzed by t.l.c. The product of the enzyme reaction from both cell types comigrated with authentic Forssman glycolipid (Fig. 3). In a first experiment (1), the specific activity of the transferase was calculated to be 1.02 pmol mg^{-1} h^{-1} for F9 cells and 0.36 pmol mg^{-1} h^{-1} for differentiated cells (Table III). To verify these results, this experiment was repeated and the results of the second experiment (2) are shown also in Table III. To detect possible soluble inhibitors or activators in the extracts, which could indirectly account for the differences in activity, equal amounts of the extracts from the two cell types were mixed and assayed for Forssman glycolipid synthesis. The amount of radiolabeled Forssman glycolipid synthesized by the mixed cell types was very close to that predicted, which indicates that the activity differences are not likely to be due to soluble inhibitors or activators (Fig. 3 and Table III). These results indicated an approximately 70% decrease in the activity of the enzyme UDP-D-GalNAc:GbOse$_4$Cer (1→3)-*N*-acetyl-α-D-galactosaminyltransferase upon differentiation of F9 cells in the presence of retinoic acid for three days.

DISCUSSION

The Forssman antigen, which has been detected immunologically on F9 embryonal carcinoma cells[9,18–20], is considered a differentiation antigen in mice since the antigen is not detected on the endoderm of simple embryoid bodies or on differentiated teratocarcinoma cells after long term culture[4,6,9,20]. Our results demonstrated that decreased Forssman glycolipid expression in F9 cells can be detected within three days following retinoic acid treatment and that the reduction is due to decreased levels of activity of Forssman synthase. Because long-term cultures of retinoic acid-differentiated F9 cells are reported to not express Forssman antigen[9,20], the residual Forssman synthesis observed after three days of retinoic acid treatment in our study is presumably due to residual activity of the Forssman synthase in these cells at that time.

The mechanism by which retinoic acid exerts its effect on target cells is the subject of much current interest. These is evidence that the hormonal and inducing effects of retinoic acid may be mediated through a cellular retinoic acid-binding protein[52–54]. Retinoic acid promotes the differentiation of a wide variety of cultured animal cells[11,55–57] and has specific effects on the activities of enzymes and other gene products[58–60]. The precise mechanism of action of retinoic acid, however, is not known and the precise role of the retinoic acid-binding proteins in mediating action of the drug is being intensely studied[61,62]. In several systems, it has been shown that retinoic acid-induced differentiation of a number of cell lines, in addition to F9 cells, results in altered glycoconjugate biosynthesis[63–67]. Based on the results of our findings, it would be interesting to observe whether retinoic acid-induced differentiation of other cell lines expressing Forssman antigen also results in alterations in Forssman synthase activity.

Immunological methods employing specific antioligosaccharide antibodies have been useful in detecting developmental changes in carbohydrate antigens on embryos or embryonal carcinoma cells[4–9,20,22]. Chemical characterization of potential carbohydrate antigens, however, is often difficult owing to low levels of materials available, difficulties in verifying biosynthesis rather than acquisition by the cells in question, and the difficulty in fractionating and purifying small quantities of oligosaccharides from complex mixtures. We have demonstrated that it is possible to accomplish the chemical characterization of a minor glycolipid antigen and to follow alterations in its biosynthesis during cell differentiation by the analysis of metabolically radiolabeled, glycolipid-derived oligosaccharides purified by lectin-affinity chromatography. For example, the Forssman oligosaccharide characterized in our study contained less than 2% of the D-[^{3}H]galactose and D-[^{3}H]glucosamine incorporated into the total neutral glycolipid-derived oligosaccharides. The application of this approach using additional immobilized lectins to the characterization of all the neutral and acidic glycolipid-derived oligosaccharides of F9 cells and their retinoic acid differentiated counterparts is in progress.

Although our study has demonstrated that Forssman synthase activity declines upon retinoic acid-induced differentiation of F9 cells, it should be noted that we have previously reported that retinoic acid-induced differentiation of these cells results in an

elevation in the activity and transcript levels of another glycosyltransferase, UPD-D-Gal:β-D-galactoside (1→3)-α-D-galactosyltransferase[21,68]. Considering the observations that many other glycosyltransferase activities in F9 cells are not altered upon differentiation[21], and that the levels, for example, of many neutral glycosphingolipids such as globoside are not altered, retinoic acid differentiation is specific and does not result in general changes in the activities of all glycosyltransferases[69]. Studies in progress are addressing the factors determining decreased Forssman synthase in differentiated cells and the biological consequences of the loss of Forssman glycolipid expression.

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