Branching $\beta$1–6N-Acetylglucosaminetranferases and Polylactosamine Expression in Mouse F9 Teratocarcinoma Cells and Differentiated Counterparts*

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$\beta$-All-trans-retinoic acid (RA)-induced endodermal differentiation of mouse F9 teratocarcinoma cells is accompanied by changes in glycoprotein glycosylation, including expression of SSEA-1 (i.e. polylactosamine) and leukophytohemagglutinin-reactive oligosaccharides (i.e. -GlcNAc$\beta$1–6Man$\alpha$1–6-branched N-linked). We have used the F9 teratocarcinoma cells as a model to study developmental regulation of glycosyltransferase activities which are responsible for the biosynthesis of $\beta$1–6GlcNAc-branched N- and O-linked oligosaccharides and polylactosamine. Growth of F9 cells in the presence of $10^{-8}$ RA for 4 days increased core 2 GlcNAc transferase and GlcNAc transferase V activities by 13- and 6-fold, respectively, whereas the activities of GlcNAc transferase I, $\beta$1–3GlcNAc transferase (I), $\beta$1–4Gal transferase, and $\beta$1–3Gal transferase increased 2-4-fold. Induction of glycosyltransferase activities by RA was dose-dependent and showed a biphasic response with approximately half of the increase observed 3 days after RA treatment and the remainder occurred by day 4. PYS-2, a parietal endoderm cell line, showed levels of glycosyltransferase activities similar to those of RA-treated F9 cells. Glycosyltransferase activities in the RA-resistant F9 cell line (RA-3-10) were low and showed only a small induction by RA. These observations suggest that differentiation of F9 cells is closely associated with induction of multiple glycosyltransferase activities, with most pronounced increases in GlcNAc transferase V and 2', 5'-tetradenylate (core 2) GlcNAc transferase. The increase in GlcNAc transferase V was also reflected by the 4-6-fold increase in the binding of 125I-leukophytohemagglutinin to several cellular glycoproteins, which occurred after 3 days of RA treatment.

The endo-$\beta$-galactosidase-sensitive polylactosamine content of membrane glycoproteins and, in particular, the LAMP-1 glycoprotein was markedly increased after RA treatment of F9 cells. Consistent with these observations, fucosylated polylactosamine (i.e. dimeric Le*) was also increased in RA-treated cells. Analysis of the aryl oligosaccharides produced by F9 cells cultured in the presence of aryl $\alpha$-D-GalNAc showed that RA treatment enhanced the synthesis of disialyl core 2 O-linked oligosaccharides and increased the polylactosamine content of the aryl oligosaccharides by >20-fold. The results suggest that differentiation of F9 cells into endoderm is closely associated with increased GlcNAc transferase V and core 2 GlcNAc transferase activities, enzymes which control the level of $\beta$1–6GlcNAc-branched N- and O-linked oligosaccharides, the preferred substrates for polylactosamine addition.

The mouse teratocarcinoma cell line F9 exhibits characteristics of embryonic cells from inner cell mass of the blastula stage, and following RA treatment, the cells differentiate into primitive endoderm-like cells (1–3). F9 differentiation occurring over 4–5 days in the presence of RA is associated with changes in gene expression typified by sequential expression of homeotic (i.e. Hox) genes, and the expression of collagen type IV and laminin (4–6). In addition, cell surface carbohydrates change following RA-induced differentiation as determined using anti-carbohydrate antibodies (7, 8). In particular, loss of SSEA-1 (i.e. Le*, Gal$\beta$1–4(Fuc$\alpha$1–3)GlcNAc$\beta$) (9) and acquisition of SLex* (10) and Gal$\alpha$1–3Gal (11) occurs in RA-induced F9 cells. These antigenic carbohydrate sequences are commonly found at the nonreducing termini of polylactosamine (i.e. repeating Gal$\beta$1–4GlcNAc$\beta$1–3 which also increases following differentiation of embryonal carcinoma cells (7, 12). Polylactosamine or i antigen expressed on differentiated EC cells was found almost exclusively on glycoproteins (8), presumed to be associated with N- and/or O-linked oligosaccharides. Expression of SSEA-1 at the 8–16-cell stage has been implicated in compaction of the mouse embryo (13, 14), suggesting that developmentally regulated changes in oligosaccharide biosynthesis and processing may be required for embryo development.

Following RA-induced differentiation of F9 cells, the LAMP-1 glycoprotein, a major cellular carrier of N-linked 1

Acknowledgments

The abbreviations used are: RA, $\beta$-all-trans-retinoic acid; L-PHA, leukophytohemagglutinin; pNP, para-nitrophenyl; MESS, 4-morpholineethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PBS, phosphate-buffered saline; Me$_3$SO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; SA, sialic acid; BtZcAMP, N',O'-dibutyryl cAMP.

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oligosaccharides (15–18), shows greatly increased binding to leukophytohemagglutinin (L-PHA) (19). This lectin binds specifically to the more branched tri- and tetrantennary complex-type N-linked oligosaccharides (20). L-PHA reactivity with LAMP-1 also increases in oncogene-transformed rat2 fibroblasts and in metastatic sublines of the SP1 murine mammary carcinoma 1 cells as a result of a 3–10-fold increase in UDP-GlcNAcα6Manβ6-N-acetylgalactosaminyltransferase V (i.e. GlcNAc transferase V) activity (21, 22). The polylactosamine content in N-linked oligosaccharides of MDAY-D2 lymphoma cells and SP1 cell lines correlated with changes in GlcNAc transferase V activity, whereas UDP-GlcNAcGalβ4GlcNAc-Rβ3-N-acetylgalactosaminyltransferase (GlcNAc to Gal) (i.e. GlcNAc transferase (ii)) activity remained unchanged (22). This suggests that GlcNAc transferase V catalyzes a rate-limiting reaction in expression of polylactosamine in N-linked oligosaccharides and is consistent with earlier substrate specificity studies on partially purified GlcNAc transferase (i) which showed the enzyme preferentially substituted acceptors with the Galβ1–4GlcNAcβ1–6Manα-antennas (23).

Polyolactosamine is also found in O-linked oligosaccharides (24), and its expression in SP1 tumor cells appeared to be dependent upon UDP-GlcNAcGalβ3Gallα-Rβ6-N-acetylgalactosaminyltransferase (GlcNAc to GalNAc) (i.e. core 2 GlcNAc transferase) activity which substitutes Galβ1–3GalNAc producing Galβ1–3(GlcNAcβ1–6)GalNAc (22). Substitution of the GlcNAc by UDP-Galβ3Gallα-Rβ3-galactosyltransferase (i.e. β3–4Gal transferase) provides a lactosamine substrate which can be extended by GlcNAc transferase (i) into polylactosamine. Core 2 GlcNAc transferase activities in human B lymphocytes have recently been shown to regulate polylactosamine levels in the O-linked oligosaccharides of CD43 (i.e. leukosialin) (25).

These observations suggest that during embryogenesis, expression of polylactosamine, as well as the extended chain antigens (i.e. pol-Leα and pol-Leβ) based on this linear sequence, may be regulated by the β1–6 branching GlcNAc transferases. To address this question, we have compared glycosyltransferase activities and polylactosamine levels in N- and O-linked oligosaccharides of F9 cells and RA-treated F9 cells. The results show that differentiation of F9 cells into primitive or parietal endoderm is associated with distinct patterns of glycosyltransferase activity. Furthermore, enhanced GlcNAc transferase V and core 2 GlcNAc transferase activities in differentiated F9 cells were associated with expression of endo-β-galactosidase-sensitive polylactosamine sequences in both N- and O-linked oligosaccharides.

**MATERIALS AND METHODS**

**Chemicals and Glycosyltransferase Substrates**

UDP-6-[3H]N-acetylgalactosamine (26.8 Ci/mmol, Du Pont-New England Nuclear) and UDP-6-[3H]galactose (20 Ci/mmol, Amer- sham, United Kingdom) were diluted with the respective unlabeled sugar nucleotides purchased from Sigma. GalNAcα2-3NP and Galβ1–3Gallα–2NP were purchased from Toronto Research Chemicals (Toronto). GlcNAcβ3–3Galβ–OCH3 was purchased from Sigma. GlcNAcβ1–2Manα1–6Manβ3–O(CH2)6COOH; Manα1–3(Manα1–6)Manβ1–O(CH2)6COOH; Galβ1–4GlcNAcβ1–2Manα1–6Manβ3–O(CH2)6COOH were kindly provided by Dr. O. Hindsaul, University of Alberta. RA was a gift from BASF Aktiengesellschaft (Ludwigshafen, Germany). L-PHA was purchased from Vector Labor- atories (Burlingame, CA). SH2 monodonal antibody was kindly provided by Drs. A. K. Singhal and S. Hakomori, Biomembrane Institute, Seattle (26).
plates were incubated with a 1:1,000 dilution in PBST + 1% BSA of alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) at room temperature for 2 h. Plates were washed with PBST and water prior to a 1-h color development using a p-nitrophenyl phosphate substrate solution (Bio-Rad). Absorbance at 410 nm was determined using a Bio-Tek EL-312 enzyme immunoassay plate reader. All assays were performed in duplicate at six to eight lysate protein concentrations. Activities (i.e. A200 units/mg/h) in different cell lysates were compared in experiments preformed on the same day.

Measurement of Endo-β-Galactosidase-sensitive Polylactosamine

Endo-β-galactosidase-sensitive polylactosamine was quantitated as described previously (22). Briefly, microsomal membranes in 1 mM HEPES, pH 7.0, were heated to 65°C for 15 min to inactivate β-galactosidases. The samples were diluted with 0.15 M NaCl, 10 mM HEPES, pH 7.0, 0.5% Triton X-100 to 3 mg/ml, and terminal GlcNAc residues, particularly O-linked GlcNAc residues (29), were blocked with cold galactose by bovine β-Gal transferase. The reaction buffer contained 100 mM galactose, 100 mM HEPES, pH 7.3, 0.15 M NaCl, 50 mM MnCl2, 1.2 mM AMP, 1 mM UDP-Gal, 0.5% Triton X-100, 900 milliunits/ml bovine Gal transferase (Sigma) and 1.0 mg/ml microsomal membrane protein. The reaction was incubated for 2 h at 37°C, and then the samples were exhaustively dialyzed against 0.15 M NaCl, 50 mM MnCl2, and 25 mM sodium acetate, pH 5, to remove the cold UDP-Gal. Half of each sample was treated with 5 milliunits of endo-β-galactosidase (Miles) per 100 μg of protein for 16 h at 50°C. Samples (50 μl), both endo-β-galactosidase-treated and untreated, were diluted 1:1 with the reaction buffer, this time with 2 μCi of UDP-[3H]Gal ([H]Gal) instead of cold UDP-Gal. After 2 h the reaction was terminated by the addition of ice-cold 10% trichloroacetic acid, and the pellet was washed three times with 10% trichloroacetic acid and counted in a liquid scintillation counter.

To release N-linked oligosaccharides, samples were labeled with [3H]Gal as described above and then heated to 65°C for 15 min, made 0.2 M Tris, pH 7.4, and left at 4°C overnight, and incubated with and without 40 units of glycopeptidase F from Flavobacterium meningosepticum (Boehringer) for 16 h at 37°C. The samples were then precipitated and washed three times with 10% trichloroacetic acid and counted in a liquid scintillation counter.

Binding of 125I-L-PHA to Cellular Glycoproteins

Cells were solubilized by suspending 2.5 × 10^7 cells/ml in 0.5% Nonidet P-40 in 10 mM Tris-HCl, pH 7.4, and incubating them for 20 min on ice. The cell suspension was centrifuged at 12,000 × g, and the supernatant fraction (cell extract) was collected. The cellular proteins were subjected to polyacrylamide gel electrophoresis (PAGE), in the presence of sodium dodecyl sulfate (SDS), and then the proteins were transferred electrophoretically to a nitrocellulose membrane. Following an overnight blocking of nonspecific binding sites with a solution containing 0.05% Polysorbate 20 and 0.15 M NaCl in PBS, 0.1% BSA. Following three 5-min washes in PBS, 0.1% BSA, lectin blots were incubated for 1 h with horseradish peroxidase-conjugated anti-mouse antibody diluted 1:1,000 in TBS, 0.1% BSA. Blots were washed four times (5 min each) followed by one wash in TBS, 0.1% Tween 20, and one wash in TBS. The blots were developed using the ECL chemiluminescence Western blotting kit as per manufacturer’s instructions (Amersham).

Western Blotting

Proteins separated by 12% SDS-PAGE under reducing conditions were transferred electrophoretically onto nitrocellulose sheets in buffer consisting of 25 mM Tris, 0.2 M glycine which contained 20% methanol. Blots were blocked for 1 h at 37°C in 50 mM Tris-HCl, pH 8.0, 0.15 M NaCl (TBS), 5% BSA, then incubated with a 1:50 dilution of culture supernatant containing SH2 monoclonal antibody in PBS, 0.1% BSA. Following three 5-min washes in PBS, 0.1% BSA, lectin blots were incubated for 1 h with horseradish peroxidase-conjugated anti-mouse antibody diluted 1:1,000 in TBS, 0.1% BSA. Blots were washed four times (5 min each) followed by one wash in TBS, 0.1% Tween 20, and one wash in TBS. The blots were developed using the ECL chemiluminescence Western blotting kit as per manufacturer’s instructions (Amersham).
and approximately 30 pmol/mg/h using radiolabeled UDP-GlcNAc.

Increased expression of LAMP-1- and L-PHA-reactive oligosaccharides in F9 cells was detected previously at $10^{-8}$ M and optimal at $10^{-5}$ M RA (19). GlcNAc transferase V activity showed a similar dose-dependent increase, with maximal activity at $10^{-5}$ M RA (i.e. a 5.5-fold increase) (Fig. 3). The O-linked glycosyltransferases core 2 GlcNAc transferase and β1-3Gal transferase also showed a dose-dependent increase in activity with a 12- and 3-fold increase at $10^{-6}$ M RA, respectively (Fig. 3B). The RA dose response for core 2 GlcNAc transferase (Fig. 3C) was similar to that of GlcNAc transferase V, whereas β1-3Gal transferase required higher concentrations of RA > $10^{-6}$ M (Fig. 3D) similar to that reported for induction of the LAMP-2 in F9 cells (19).

Time courses for RA-stimulated induction of GlcNAc transferase V, core 2 GlcNAc transferase, β1-3Gal transferase, and β1-4Gal transferase activities showed biphasic responses with an increase occurring over days 1–3 followed by a further more precipitous increase on 4th day after RA treatment (Fig. 4). A similar time course was observed for induction of L-PHA-reactive glycoconjugates (i.e. LAMP-1, LAMP-2, and laminin), with a small increase on day 3 and the major change 4 days after RA treatment (Fig. 5). Under the same experimental conditions, induction of laminin B1, LAMP-1, and LAMP-2 also began 2–3 days after RA treatment (19). In human and mouse embryonal carcinoma cells, RA treatment results in the sequential and ordered induction of Hox 2 genes begins within 3 h and extending over 5 days (5, 6). The biphasic induction of glycosyltransferases may also be caused by sequential regulatory events in this complex process.

The data in Fig. 6 summarize the fold increase in GlcNAc transferase V, core 2 GlcNAc transferase, β1-4Gal transferase, and β1-3Gal transferase as well as GlcNAc transferase I and GlcNAc transferase (i) activities after 4 days in the presence of $10^{-6}$ M RA. GlcNAc transferase V and core 2 GlcNAc transferase showed the largest induction, 6- and 12-fold, respectively, whereas increases in the other glycosyltransferases were 2-3.6-fold. GlcNAc transferase V assay using UDP-[^3H]GlcNAc showed only a 3-fold increase compared with 6-fold with the ELISA. The difference may be because of the concentration of UDP-GlcNAc used in the two assays. The ELISA contained 4 mM UDP-GlcNAc. The radioactive assay was performed at 1 mM, approximately the $K_m$ for UDP-GlcNAc (34), and may therefore have underestimated the difference between enzyme activity in untreated and RA-treated cells.

To examine further the relationship between F9 cell differentiation and glycosyltransferase induction, cells were treated with agents previously shown to have distinct effects on F9 differentiation pathways. RA induces F9 cells to differentiate into primitive endoderm, Bt2cAMP plus RA induces parietal endoderm, and Bt2cAMP alone does not induce differentiation (35). In contrast, butyrate has been shown to inhibit RA-dependent differentiation of F9 cells (36). Consistent with this relationship, induction of GlcNAc transferase V and core 2 GlcNAc transferase activities was RA > RA + butyrate > Bt2cAMP (Fig. 7A). RA + Bt2cAMP stimulated GlcNAc transferase V activity, but less than RA alone. Butyrate alone also showed intermediate levels of GlcNAc transferase V and core 2 GlcNAc transferase stimulation, which suggests that it may affect F9 cell differentiation in a manner independent of RA. The patterns of induction of GlcNAc transferase V and core 2 GlcNAc transferase activities in F9 cells treated with combinations of RA, butyrate, Bt2cAMP were similar for these two enzymes and differed from that of the Gal transferases. β1-4Gal transferase and β1-3Gal transferase activities showed a 2–3-fold induction with all treatments except RA + Bt2cAMP, in which induction was 3–4-fold (Fig. 7). L-PHA reactivity of glycoproteins from F9 cells treated with these agents correlated with GlcNAc transferase V activity in cells treated with RA or with RA + Bt2cAMP as shown in Fig. 5. However, there was no increase in L-PHA binding in cells treated with butyrate alone.

PYS-2 and PSA-5E are murine embryonal lines with parietal and visceral phenotypes, respectively (37), and both show L-PHA reactivity comparable to RA-treated F9 cells (19). Consistent with this phenotype, both PYS-2 and PSA-5E cells showed elevated levels of GlcNAc transferase V activity similar to that of RA-induced F9 cells (Table I). PYS-2 cells also had core 2 GlcNAc transferase activity comparable to that of RA-treated F9 cells, but the visceral endoderm line PSA-5E showed a distinctive phenotype with very low levels of core 2 GlcNAc transferase activity, below that of untreated F9 cells. Differentiation into parietal endoderm, represented by both the PYS-2 cell line and RA + Bt2cAMP-treated F9 cells, was distinguished by elevated β1-4Gal transferase and -2 GlcNAc transferase activity, below that of untreated F9 cells.
Fig. 3. Dose-response curve for RA-induced increase in GlcNAc transferase V (panels A and B), core 2 GlcNAc transferase (panel C), and β1-3Gal transferase (panel D) activities in F9 teratocarcinoma cells. F9 cells were treated with various concentrations of RA (●) or 0.01% Me2SO (△) for 4 days. GlcNAc transferase V activity was measured at seven lysate protein concentrations using the ELISA. Panel A shows each GlcNAc transferase V activity curve as a function of log[protein], and in panel B the data are expressed as specific activity versus RA concentration. ●, ▽, △, □, ■, and ▲ in panel A are in order of decreasing RA concentration.

Fig. 4. Time course for RA-dependent induction of GlcNAc transferase V (panel A), β1-4Gal transferase (panel B), core 2 GlcNAc transferase (panel C), and β1-3Gal transferase (panel D) in F9 cells. F9 cells were treated with 10^{-8} M RA (●) or 0.01% Me2SO (△). The cells were harvested, and glycosyltransferases activities were measured as described under "Materials and Methods."

β1-3Gal transferase activities compared with primitive endoderm (RA-treated F9) (Table I and Fig. 7). The results suggest that embryonal cell differentiation is closely associated with changes in glycosyltransferase activity, which are characteristic of the differentiated cell lineage.

Glycosyltransferase activities were measured in RA-3-10, a RA-resistant F9 cell line which does not differentiate into parietal endoderm in the presence of RA (38). Partial induction of GlcNAc transferase V activity (i.e. 2-fold) was observed following treatment of RA-3-10 cells for 4 days with 10^{-5} M
β1-6GlcNAc Transferases and Polylactosamine in F9 Cells

**Fig. 5. Identification of L-PHA-reactive oligosaccharides in F9 cells treated with 0.01% Me2SO (control) (first lane), 1 mM Bt2cAMP (second lane), 10 μM RA (third lane), RA plus Bt2cAMP (fourth lane), 2 mM butyrate (fifth lane), and RA plus butyrate (sixth lane).** Cells were cultured for 4 days with the different agents, harvested, and subjected to SDS-PAGE. The electrophoretic migration of each sample is indicated by the numbers above each lane of the autoradiogram. The numbers on the left indicate the migration of the prestained molecular weight standards, 125I-labeled L-PHA followed by autoradiography. The numbers on the left indicate the migration of the prestained molecular weight standards, α2-macroglobulin (M, 180,000), β-galactosidase (M, 116,000), and fructose-6-phosphate kinase (M, 84,000). The electrophoretic migration of the glycoprotein bands was estimated by densitometric scanning and related to the control, which was assigned a value of 1. Bottom panel, cells were grown for the indicated numbers of time points, and the ratio of the two values is presented.

**RA (Table I).** However, basal levels of core 2 GlcNAc transferase and Gal transferase in the mutant cells were lower than untreated F9 cells, and RA induced only a small increase. β1-4Gal transferase activity in RA-3-10 cells was similar to that of F9 cells and was unaffected by RA treatment (Table I). Both differentiation and induction of glycosyltransferases are deficient in this RA-resistant cell line, supporting an association between these phenotypes.

**Endo-β-Galactosidase-sensitive Polylactosamine—LAMP** glycoproteins in F9 cells become L-PHA reactive and show greater size heterogeneity on SDS-PAGE after 4 days in RA, consistent with increased β1-6GlcNAc branching of N-linked oligosaccharides (19) (Fig. 5). Polylactosamine may also contribute to the size of L-PHA reactivity of the glycoconjugates in RA-treated cells. To examine this possibility, polylactosamine content in F9 cell lysates was measured using α method based upon labeling the GlcNAc termini produced by the action of endo-β-galactosidase with bovine milk Gal transferase/UDP-[3H]Gal (22). E. freundii endo-β-galactosidase cleaves polylactosamine sequences including those substituted with SA or α1-2/3/4 fucose, but not those substituted at the 6 position of Gal (39). Detergent cell lysates were pretreated with unlabeled UDP-Gal and Gal transferase to saturate the O-linked and other terminal GlcNAc residues (29). After exhaustive dialysis to remove UDP-Gal, the samples were digested with endo-β-galactosidase and then treated with UDP-[3H]Gal and Gal transferase. Protein was trichloroacetic acid-precipitated and the radioactivity measured in a β-counter. To distinguish N- and O-linked polylactosamine, the portion of radioactivity that became soluble in ice-cold 10% trichloroacetic acid after glycopeptidase F treatment was determined. F9 cell lysates showed negligible levels of endo-β-galactosidase-sensitive polylactosamine in N- and O-linked oligosaccharides (Table II). In contrast RA-treated F9 cells had 68.3 pmol/mg compared with 139.7 pmol/mg in the MDAY-D2 tumor cell. MDAY-D2 cells served as a positive control which was previously determined to have 207 pmol of polylactosamine/mg of microsomal membrane protein (i.e. this value is taken from Table IV of Ref. 22 and corrected for the 10% efficiency of the Gal transferase reaction for comparison with the present data in Table I). Polylactosamine in RA-treated F9 and MDAY-D2 cells was approximately equally distributed between N- and O-linked oligosaccharides.

To examine further the RA-dependent changes in GlcNAc branching and polylactosamine of O-linked oligosaccharides, F9 cells were incubated with GalNAc-pNP which partitions into cell membranes, enters the Golgi, and serves as an acceptor for O-glycosylation (40). Previous studies using MDAY-D2 tumor cells have shown that aryl oligosaccharides are synthesized and expelled into the culture medium with a
Fig. 7. Induction of several glycosyltransferases in F9 cells induced to differentiate along distinct pathways. Panel A, GlcNAc transferase V; panel B, 3-4Gal transferase; panel C, core 2 GlcNAc transferase; and panel D, 3-3Gal transferase in F9 cells treated with (from bottom to top) 2 mM butyrate (first row), 1 mM Bt2cAMP (dbcAMP; second row), RA (third row), RA plus butyrate (fourth row), RA plus Bt2cAMP (fifth row). Basal levels of transferase activity in vehicle-treated F9 cells were: panel A, 0.021 A" units/pg/h; panel B, 9.8 nmol/mg/h; panel C, 0.41 nmol/mg/h; panel D, 1.50 nmol/mg/h.

Table I

Glycosyltransferase activities in F9 cells, RA-resistant F9 cells, their RA-treated counterparts, and parietal and visceral endodermal cell lines

Glycosyltransferase activities were measured as described under "Materials and Methods." RA-treated cells were cultured in the presence of M RA for 4 days, and 0.01% Me2SO was added to controls cultures. The type of endodermal differentiation is specified in parentheses. T, transferase.

<table>
<thead>
<tr>
<th>Cells</th>
<th>GlcNAc-TV</th>
<th>Core 2 GlcNAc-T</th>
<th>/5Gal-T</th>
<th>/4Gal-T</th>
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<tr>
<td></td>
<td>A&quot; units/pg/h</td>
<td>nmol/mg/h</td>
<td></td>
<td></td>
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<tr>
<td>F9 (wild type)</td>
<td>0.02</td>
<td>0.39 ± 0.09</td>
<td>1.30 ± 0.16</td>
<td>10.8 ± 2.5</td>
</tr>
<tr>
<td>F9 + RA (primitive)</td>
<td>0.11</td>
<td>5.2 ± 1.26</td>
<td>4.65 ± 0.94</td>
<td>24.7 ± 5.5</td>
</tr>
<tr>
<td>PYS-2 (parietal)</td>
<td>0.13</td>
<td>3.23</td>
<td>9.44</td>
<td>50.8</td>
</tr>
<tr>
<td>PSA-5E (visceral)</td>
<td>0.12</td>
<td>&lt;0.1</td>
<td>5.05</td>
<td>63.8</td>
</tr>
<tr>
<td>RA-3-10</td>
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<td>0.29</td>
<td>0.30</td>
<td>11.9</td>
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<tr>
<td>RA-3-10 + RA</td>
<td>0.05</td>
<td>0.56</td>
<td>0.70</td>
<td>12.7</td>
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</tbody>
</table>

Table II

Endo β-galactosidase-sensitive polylactosamine in N- and O-linked oligosaccharides of F9 cells following RA treatment

F9 cells were cultured for 4 days in either the presence or absence of 10^{-8} M RA. Cells were harvested and polylactosamine measured as described under "Materials and Methods." The results are corrected for the efficiency of the Gal transferase reaction (i.e., 10%) and expressed per mg of cellular protein.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Total</th>
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<th>Glycopeptidase F-insensitive pmol/mg</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F9</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>F9 + RA</td>
<td>68.3</td>
<td>36.8</td>
<td>31.5</td>
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<tr>
<td>MDAY-D2</td>
<td>139.7</td>
<td>67.0</td>
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</tbody>
</table>

half-time of transit to the cell surface of 13.6 min, consistent with their movement by bulk flow (30). Production of disialyl aryl oligosaccharides, both core 1 and core 2 structures, increased in a linear manner over a 6-h period. The disialyl aryl oligosaccharides were analyzed since this fraction can be readily purified by DEAE-HPLC, and further separated by Sep-Pak C8 and HPLC (30). The disialyl aryl oligosaccharides produced by F9 cells with and without RA treatment were separated by DEAE-HPLC and quantitated by absorbance at 303 nm as described previously (30) (Fig. 8). RA-treated F9 cells showed approximately twice as much disialyl aryl oligosaccharide per cell as mock-treated cells (Table III). This may be because of a general increase in cellular glycosylating activity or a more specific increase in sialylation (10, 41). When aliquots of the disialyl fraction were treated with mild acid and separated by Ultrahydrogel HPLC, the ratio of core 1 to core 2 oligosaccharides was 1:2 for RA-treated cells and 2:1 for untreated F9 cells, consistent with the observed increase in core 2 GlcNAc transferase activity following RA treatment. Endo-β-galactosidase-sensitive polylactosamine in the disialyl aryl oligosaccharide fraction was readily detected in RA-treated F9 cells, whereas only background levels were observed in vehicle-treated F9 cells (Table III). Polylactosamine sequences labeled by endo-β-galactosidase digestion and Gal transferase/UDP-[3H]Gal were separated on DEAE-HPLC and found to be 55% neutral and 45% monosialylated. The reduction in charge of the latter fraction from 2 to 1 following endo-β-galactosidase treatment is consistent with the core 2 structure (i.e., Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAca), where polylactosamine is located only on the (β1-6)-linked lactosamine arm. The proportion of aryl oligosaccharide containing polylactosamine produced by RA-treated F9 cells was less than 1%, and therefore further
charides desalted on a Bio-Gel P2 column. A monosialyl standard at positions marked 0.01% days with data are corrected for the efficiency of the Gal transferase reaction endo-β-galactosidase or incubation buffer, followed by was desalted on P2 Bio-Gel column. Aliquots were treated with either concentrated by lyophilization, desalted on a P2 Bio-Gel column, and separated on a DEAE-HPLC column (Fig. 8). The disialyl fraction (i.e. SAa2-3Galβ1-3(SAa2-3Galβ1-4GlcNAcβ1-6)GalNAc-pNP) eluted at positions marked 1 and 2, respectively.

### TABLE III

<table>
<thead>
<tr>
<th>Cells</th>
<th>Total</th>
<th>Disialyl core 1</th>
<th>Disialyl core 2</th>
<th>Endo-β-galactosidase-sensitive polylactosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/h/10^6 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F9</td>
<td>48.2</td>
<td>14.6</td>
<td>7.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>F9 + RA</td>
<td>109.3</td>
<td>26.6</td>
<td>53.8</td>
<td>0.88</td>
</tr>
</tbody>
</table>

structural analysis will require a larger preparation of aryl oligosaccharides.

The N-linked oligosaccharides of the LAMP-1 glycoprotein bear a large proportion of polylactosamine found in lymphoid tumor cells (16, 18). F9 cells, both control or RA-treated, were metabolically labeled with [3H]glucosamine to compare endo-β-galactosidase-sensitive polylactosamine content in a specific glycoprotein. LAMP-1 in RA-treated cells showed greater size heterogeneity and [3H]glucosamine labeling, and this was reduced by endo-β-galactosidase to levels found in LAMP-1 of untreated F9 cells (Fig. 9). Polylactosamine is the backbone for polyfucosylated Lewis x and y antigens; and as expected, RA-treated F9 cells also showed increased endo-β-galactosidase-sensitive dimeric Le^a, as determined by Western blotting with the monoclonal antibody SH2 (Fig. 10).

### DISCUSSION

RA-induced differentiation of F9 embryonal carcinoma cells results in the expression of L-PHA-reactive oligosaccharides which increases the apparent molecular weight of the LAMP-1 glycoprotein (19). Cossu et al. (41) compared glycopeptides from F9 cells and a primitive endodermal subline and found that the latter had a larger fraction of glycopeptides which were excluded from a concanavalin A-Sepharose affinity column, presumably because of more GlcNAc branching at the trimannosyl core. These observations suggest that differentiation into primitive endoderm cells is associated with increased β1-6GlcNAc branching of N-linked oligosaccharides caused by up-regulation of GlcNAc transferase V activity, similar to that observed following malignant transformation in human and rodent cells (21, 22). We have examined this possibility in the present study by comparing glycosyltransferase activities in F9, differentiated F9 cells, and two endodermal cell lines. Earlier studies on the murine tumor cell lines MDAY-D2 and SP1 suggested that polylactosamine content in N- and O-linked oligosaccharides is dependent upon a critical level of GlcNAc transferase V and core 2 GlcNAc transferase activities. Therefore, we have also measured polylactosamine expression in F9 and RA-differentiated F9 cells.

Differential of F9 cells into primitive endoderm (i.e. RA-treated) and into parietal endoderm (RA + Bt2cAMP) resulted in increased GlcNAc transferase V and core 2 GlcNAc transferase (i.e. approximately 6- and 13-fold, respectively) compared with 2-3-fold induction of GlcNAc transferase I, β1-
3Gal transferase, β-1,4Gal transferase, and GlcNAc transferase (i). GlcNAc transferase I, β-1,3Gal transferase are widely expressed and involved in the biosynthesis of core N- and O-linked oligosaccharides, respectively. The differentiation-associated increase in their activities suggests a general increase in cellular glycosylation capacity. This may account in part for the observed increase in disialyl aryl oligosaccharides made by RA-treated F9 cells compared with untreated F9 cells. However, elevated glycosyltransferase activity is not caused by a general increase in catabolism, as [1H]leucine incorporation into proteins is similar in untreated and RA-treated F9 cells (42).

β-1,4Gal transferase and GlcNAc transferase (i) are required for polylactosamine biosynthesis, and although the change in their activities paralleled that of the ubiquitous GlcNAc transferase I and β-1,3Gal transferase enzymes, the larger increase in GlcNAc transferase V and core 2 GlcNAc transferase may be responsible for the observed up-regulation of polylactosamine content of O- and N-linked oligosaccharides. The increase in GlcNAc transferase V when compared with the increase in other enzymes examined was only 2-fold greater. However, as little as a 2-3-fold increase in GlcNAc transferase V activity in the SP1 tumor cells and T24H ras-transfected rat2 fibroblasts is associated with a dramatic increase in L-PHA reactivity of membrane glycoproteins including LAMP-1 (21, 31) and greatly increased polylactosamine, whereas β-1,4Gal transferase and GlcNAc transferase (i) were not altered (22). Conversely, a 3-4-fold reduction in GlcNAc transferase V activity in KBL-1 cells, a somatic mutant of the MDAY-D2 lymphoma, results in almost complete loss of polylactosamine in N-linked, whereas O-linked polylactosamine remained unchanged (22).

Endo-β-galactosidase-sensitive polylactosamine in membrane glycoconjugates and LAMP-1 increased dramatically following RA-dependent differentiation of F9 cells, an observation consistent with those made earlier on whole mouse embryos using antibodies reactive with "i" antigen (43). Endo-β-galactosidase is inhibited by six substitutions to Gal, such as in the glycoprotein fraction of developing mouse embryos by mouse embryos (43) and embryonal carcinoma cells (8). Glycosyltransferase activity in the parietal endoderm cell line PYS-2 was similar to that of RA-treated F9 cells, suggesting that changes are not strictly RA-dependent but are associated with cell differentiation. The visceral endoderm cell line PAS-5E showed a pattern of glycosyltransferase activity similar to that of RA + BtzcAMP-treated F9 cells, with the exception that core 2 GlcNAc transferase was very low in PAS-5E. This may reflect a further phase of differentiation occurring after that of 4-day RA + BtzcAMP-treated F9 cells. Based on expression of laminin, collagen, and Hox genes in RA-treated F9 cells, differentiation appears to require ordered and interdependent changes in gene expression (4-6) and phenotypic changes, particularly in glycosyltransferase activities which extend up to and beyond 4 days (42, 47).

In conclusion, the results of this study demonstrate that differentiation of F9 cells into distinct types of endodermal cells is accompanied by marked and characteristic changes in specific glycosyltransferases and in polylactosaminoglycans. Developmental regulation of polylactosamine and associated Lewis antigens may have functional consequences for cellular interactions during embryogenesis (13, 14). Transgenic experiments will be required to test this hypothesis, as has recently been done by Variki et al. (48) to explore the role of 9-O-acetylmuramidic acid in murine embryogenesis. The mechanism(s) by which RA alone or in combination with BtzcAMP increases the activity of several glycosyltransferases is not known. It is likely that RA regulates the transcription of their genes via nuclear retinoic acid receptors (49-51). The elucidation of such a mechanism must await the cloning of these enzymes and the isolation and analysis of their promoters.

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31-66GlcNAc Transferases and Polylactosamine in F9 Cells

1251