A Cloned Human DNA Restriction Fragment Determines Expression of a GDP-L-fucose;β-D-Galactoside 2-α-L-fucosyltransferase in Transfected Cells

EVIDENCE FOR ISOLATION AND TRANSFER OF THE HUMAN H BLOOD GROUP LOCUS

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We have described previously a gene transfer system for the isolation of human DNA sequences that determine expression of α-(1,2)-fucosyltransferase (α-(1,2)-fucosyltransferase) (Ernst, L. K., Rajan, V. P., Larsen, R. D., Ruff, M. M., and Lowe, J. B. (1989) J. Biol. Chem. 264, 3436-3447). With this system, we found that de novo expression of the fucosyltransferase in mouse recipient cells was associated with the transfer and stable genomic integration of characteristic human DNA restriction fragments. We report here the results of experiments designed to determine the genetic origin of the fucosyltransferase determined by these sequences. First, we characterize the fucosyltransferases found in these mouse transfectants and in the human cell line used as a DNA donor. We compare their properties to those displayed by the human H and Secretor blood group fucosyltransferases. We find that the enzymes in the transfected cells have properties similar or identical to those of the human H α-(1,2)-fucosyltransferase. However, their properties differ significantly from the properties of the human Secretor α-(1,2)-fucosyltransferase and are also distinct from the properties of a murine fucosyltransferase.

To confirm further that these transfected human sequences determine the H phenotype of the transfectants, we cloned the two human EcoRI restriction fragments common to each H-expressing secondary transfectant. The larger of these two fragments directs de novo expression of an α-(1,2)-fucosyltransferase when transfected into COS-1 cells. The pH activity profile of this α-(1,2)-fucosyltransferase and its apparent Michaelis constants for substrate and acceptor mirror those we determined for the human H α-(1,2)-fucosyltransferase. We conclude that genetic information sufficient to determine expression of this α-(1,2)-fucosyltransferase resides within the 3.4-kilobase pair human EcoRI restriction fragment and that this most likely represents the human H blood group locus.

The human blood group H determinant serves as a common precursor for the action of specific glycosyltransferases that construct the A and B blood group antigens. The H structure is a terminal α-L-fucosyl-(1,2)-β-D-galactosyl moiety whose synthesis is catalyzed by α-(1,2)-fucosyltransferases. These enzymes have been identified in a number of tissues and fluids in many different mammalian species (1-4). They act upon Type II precursors with nonreducing N-acetyllactosamine termini (Gal-β(1,4)-GlcNAc) or upon Type 1 precursors with nonreducing termini (Gal-β(1,3)-GlcNAc), to catalyze the addition of L-fucose in α linkage to the C-2 position of their terminal galactose residues (2, 5).

Genetic and biochemical observations are consistent with the idea that the human H blood group locus encodes an α-(1,2)-fucosyltransferase (1). This enzyme may be readily detected in hematopoietic tissues and plasma in all individuals excepting those of the rare Bombay and para-Bombay phenotypes (1). The Fucα1→2Gal linkage it constructs is also synthesized in hematopoietic tissues. By contrast, in secretory fluids and tissues, expression of Fucα1→2Gal linkages and a cognate α-(1,2)-fucosyltransferase is determined by the Secretor locus (1). Oriol et al. (6) have reconciled these observations with a model in which the H and Se loci independently determine the expression of distinct α-(1,2)-fucosyltransferases whose expression is tissue specific. Nonsecretors are presumed to be homozygous for a null allele at the Se locus; para-Bombay and Bombay individuals are homozygous for a null allele at the H locus.

Surface-expressed glycoconjugates containing terminal Fucα1→2Gal linkages show striking temporal and spatial changes in their expression during human and murine embryogenesis (7-9). Moreover, these linkages may be aberrantly expressed in association with oncogenic transformation (10-12). These observations indicate that the expression of these structures is precisely orchestrated and suggest that these surface molecules play important roles during the developmental process. The expression of these cell surface oligosaccharide structures is to a large extent dependent upon the α-(1,2)-fucosyltransferase(s) responsible for their synthesis (13, 14). An understanding of the molecular basis for the regulation of expression of H structures will require the use of cloned segments of the gene(s) controlling expression of these enzymes.

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§§ The abbreviations used are: α-(1,2)-fucosyltransferase, GDP-fucose;β-D-galactoside 2-α-L-fucosyltransferase; Se, Secretor; [14C]fucosylphenyl-β-D-galactoside, phenyl-2-O-[14C]fucopyranosyl)-β-D-galactopyranoside; PBS, phosphate-buffered saline; kb, kilobase pairs. Unless otherwise noted, GDP-fucose specifies GDP-β-L-fucose.
We have described previously a DNA-mediated transfection system designed to isolate these genes (15). Gene transfer methods were used to generate murine cell lines that express cell surface H structures and a cognate α-(1,2)-fucosyltransferase. This phenotype was then correlated with the structure of the human DNA sequences integrated within the genomes of the mouse transfectants. Our observations indicated that a limited set of transfected human DNA fragments determines expression of the H phenotype in these cell lines. However, our results suggested, but could not distinguish between, several possible functions for these transfected human sequences. For instance, our observations were consistent either with the notion that these human sequences represent a structural gene encoding a human fucosyltransferase or that they function to activate an endogenous mouse α-(1,2)-fucosyltransferase gene (15). We report here the results of studies that address these issues.

We characterized the α-(1,2)-fucosyltransferase in the H-expressing mouse transfectants, as well as the α-(1,2)-fucosyltransferase activity in extracts prepared from the human A431 cell line that served as the DNA donor for these transfections. We find the properties of these enzymes to be virtually identical to each other and to the properties of the blood group H α-(1,2)-fucosyltransferase in human serum. Furthermore, the characteristics of these enzymes are found to be significantly different from the human Se α-(1,2)-fucosyltransferase and from those of an α-(1,2)-fucosyltransferase expressed by the mouse strain from which the recipient L cells were derived. Moreover, we show that a cloned human DNA segment identified in each H-expressing transfectant contains sufficient information to direct the synthesis of an α-(1,2)-fucosyltransferase when transfected into COS-1 cells. We find that the properties of this enzyme reflect those we determined for the human H α-(1,2)-fucosyltransferase. Considered together, these results strongly suggest that transfected human DNA sequences encode the α-(1,2)-fucosyltransferase found in the H-expressing transfected mouse cells and imply that these cloned sequences represent part or all of the human H α-(1,2)-fucosyltransferase gene.

EXPERIMENTAL PROCEDURES

RESULTS

We have described previously the use of human gene transfer and immunologic selection procedures to generate murine cell lines that express cell surface H structures and a cognate α-(1,2)-fucosyltransferase (15). Our intent was to establish a system for isolating human DNA sequences that determine the expression of α-(1,2)-fucosyltransferases. The results of these experiments were consistent with, but could not confirm, the notion that transfected human sequences encode the α-(1,2)-fucosyltransferase found in these cells. In preparation for molecular cloning experiments designed to isolate those sequences, we first sought to define the nature of the α-(1,2)-fucosyltransferase activity determined by the transfected human DNA sequences. We anticipated that analysis of the properties of these enzymes would provide a clue to their genetic origin (i.e. encoded by the transfected human sequences or by an “activated” endogenous mouse fucosyltransferase gene).

A431 Cells Express an α-(1,2)-Fucosyltransferase Analogous to the Human Blood Group H α-(1,2)-Fucosyltransferase—We first characterized the α-(1,2)-fucosyltransferase expressed by the human A431 donor cell line, anticipating that if the gene encoding this enzyme was expressed after gene transfer, its particular enzymatic properties would be displayed in the transfected murine cells and would, therefore, confirm its human origin. Phenyl-β-D-galactoside was used as the acceptor for these assays. This compound functions as a specific acceptor for α-(1,2)-fucosyltransferases; fucosyltransferases that generate α-(1,3), α-(1,4), or α-(1,6) linkages are unable to use phenyl-β-D-galactoside as an acceptor substrate (16). Furthermore, this acceptor can discriminate between the human H and Se α-(1,2)-fucosyltransferases (17, 18), is commercially available, and allows simple and rapid determination of α-(1,2)-fucosyltransferase activity (see “Experimental Procedures”). Crude cell extracts were used for the characterization of the properties of the α-(1,2)-fucosyltransferase in A431 cells. Analyses performed with crude extracts or body fluids are subject to artifact as a result of substrate and/or product hydrolysis during assay. We, therefore, carefully tested for these hydrolytic activities when assaying each enzyme source described in this paper; when found, these hydrolytic activities were either eliminated by fractionation of the enzyme source or were neutralized by inclusion in assays of substances that inhibit these activities but that do not affect α-(1,2)-fucosyltransferase activity (see “Experimental Procedures”).

As reported previously, an α-(1,2)-fucosyltransferase activity is present in Triton-solubilized extracts of human A431 cells (15). Enzyme activity was detectable in the absence of added manganese or other divalent cations, as reported for the human H and Se α-(1,2)-fucosyltransferases (16, 19). Activity was enhanced approximately 4-fold by the use of Triton X-100 in cell extract preparation (data not shown), suggesting that much of this enzyme exists in a membrane-bound form in these cells. Substrate activity and pH activity determinations were done with A431 cell extracts in order to further characterize the α-(1,2)-fucosyltransferase activity. These extracts were assayed in parallel with an ammonium sulfate fraction of human serum enriched for blood group H α-(1,2)-fucosyltransferase activity (Ref. 20 and “Experimental Procedures”) and with a fraction of human milk enriched for the α-(1,2)-fucosyltransferase activity thought to be the product of the Se locus (Ref. 17 and “Experimental Procedures”).

The pH activity profile determined for the α-(1,2)-fucosyltransferase activity in A431 extracts (Fig. 1C) is similar to that determined for the human serum blood group H α-(1,2)-fucosyltransferase (Fig. 1A). These profiles differ only slightly from the profile exhibited by the Se α-(1,2)-fucosyltransferase (Fig. 1B).

Assays were also performed to determine apparent Michaelis constants for the α-(1,2)-fucosyltransferase donor substrate GDP-fucose. The α-(1,2)-fucosyltransferase activity in A431 extracts exhibits an apparent Km for GDP-fucose of 10.9 μM (Fig. 2C). This is similar to the apparent Km determined for GDP-fucose in assays containing serum-derived H α-(1,2)-fucosyltransferase (16.2 μM, Fig. 2A), but significantly different from the apparent Km exhibited by the milk enzyme (122.7 μM, Fig. 2B).

Apparent Michaelis constants were also determined for the artificial acceptor phenyl-β-D-galactoside. The enzyme in A431 extracts displays an apparent Km of 1.9 mM (Fig. 3C), whereas an apparent Km of 3.1 mM was obtained with fractionated human serum (Fig. 3A). By contrast, the milk α-(1,2)-fucosyltransferase exhibits a considerably higher apparent Km for phenyl-β-galactoside (15.1 mM, Fig. 3B).
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FIG. 1. Effect of pH on α-(1,2)-fucosyltransferase activities. Enzyme activity was assayed using 25 mM phenyl-β-D-galactoside and 3 μM GDP-[14C]fucose, as described under "Experimental Procedures." A, sodium acetate; B, sodium phosphate; C, Tris-HCl. Activity was determined for the α-(1,2)-fucosyltransferases in fractionated human serum (A), fractionated human milk (B), or in extracts prepared from A431 cells (C), mH1-12 cells (D), s2-2 cells (E), and in COS-1 cells transfected with pH3.4 (F).

together, these results suggest that human A431 cells express an enzyme similar or identical to the human blood group H α-(1,2)-fucosyltransferase, but distinct from the Se enzyme found in human milk.

Properties of the α-(1,2)-Fucosyltransferase in H-expressing Mouse Transfectants—We also defined the pH and substrate activity characteristics of the α-(1,2)-fucosyltransferase in H-expressing mouse transfectants (15) to determine if these properties were similar to or distinct from those of the enzyme expressed in the A431 donor cell line. We found that the pH activity profiles exhibited by the α-(1,2)-fucosyltransferase in extracts prepared from the primary transfectant mH1-12, and from a representative secondary transfectant s2-2, were indistinguishable from each other (Fig. 1, D and E). These profiles were similar to those exhibited by serum H α-(1,2)-fucosyltransferase (Fig. 1, A) and by the A431 α-(1,2)-fucosyltransferase (Fig. 1, C).

The substrate concentration activity properties exhibited by the transfectant α-(1,2)-fucosyltransferases were consistent with the notion that these enzymes are derived from transfected human sequences. Apparent Michaelis constants were determined for the substrate GDP-fucose, using extracts prepared from transfectants mH1-12 and s2-2. The apparent

FIG. 2. Apparent Michaelis constants for GDP-[14C]fucose, determined for human α-(1,2)-fucosyltransferases. Apparent $K_m$ values were determined (see "Experimental Procedures") in the presence of 25 mM phenyl-β-D-galactoside acceptor for all but the milk-derived enzyme. This was assayed with 75 mM phenyl-β-D-galactoside, since the apparent $K_m$ exhibited by this enzyme for this acceptor is 15.1 mM (see Fig. 3). A, serum, $K_m = 16.2 \mu M$; B, milk, $K_m = 122.7 \mu M$; C, A431 cells, $K_m = 10.9 \mu M$.

$K_m$ values exhibited by the α-(1,2)-fucosyltransferases in these extracts were essentially identical (mH1-12, 12.8 μM; s2-2, 12.4 μM, Fig. 4, A and B). These are similar to the apparent $K_m$ values exhibited by the A431 α-(1,2)-fucosyltransferase activity (10.9 μM, Fig. 2C) and by serum H α-(1,2)-fucosyltransferase (16.2 μM, Fig. 2A), but significantly different from
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FIG. 4. Apparent Michaelis constants for GDP-[14C]fucose, determined for α-(1,2)-fucosyltransferases in transfected cells and in mouse intestine extracts. Apparent \( K_m \) values were determined (see "Experimental Procedures") in the presence of 25 m\(\text{M} \) phenyl-\(\beta\)-D-galactoside acceptor. A, mH1-12 cells, \( K_m = 12.8 \mu\text{M} \); B, s2-2 cells, \( K_m = 12.4 \mu\text{M} \); C, mouse intestinal mucosa, \( K_m = 68.7 \mu\text{M} \); D, COS-1 cells transfected with pH3.4, \( K_m = 17.5 \mu\text{M} \).

Similar results were obtained when apparent \( K_m \) values were determined for the acceptor phenyl-\(\beta\)-D-galactoside. The apparent \( K_m \) determined for this substrate, using the α-(1,2)-fucosyltransferase activity in mH1-12 extracts, was 2.4 m\(\text{M} \) (Fig. 5A). The α-(1,2)-fucosyltransferase activity in s2-2 cells exhibited a similar apparent \( K_m \) for this acceptor (3.1 m\(\text{M} \), Fig. 5B). Moreover, each of the other cloned secondary transfectants exhibited apparent \( K_m \) values for this acceptor that ranged between 2.0 and 3.2 m\(\text{M} \) (Table I). These values are very similar to the apparent \( K_m \) values obtained for phenyl-\(\beta\)-D-galactoside when A431 extracts (1.9 m\(\text{M} \)) or fractionated human serum (3.1 m\(\text{M} \)) were assayed, but markedly different from the value obtained with the Se α-(1,2)-fucosyltransferase (15.1 m\(\text{M} \)) (Table I).

A Murine Tissue α-(1,2)-Fucosyltransferase Diffs from the α-(1,2)-Fucosyltransferase in H-expressing Mouse Transfectants—Our previous experiments indicated that H-expressing mouse transfectants can be isolated from mouse cells transfected with human DNA, but not from mouse cells transfected with mouse DNA (15). A straightforward interpretation of these results is that transfected human sequences determine the phenotype of H-expressing transfectants. The enzymatic data presented above are also consistent with the notion that these transfected human sequences encode the enzyme found in the H-expressing mouse transfectants and that this represents the blood group H α-(1,2)-fucosyltransferase. Nonetheless, these observations do not exclude the possibility that the transfected human DNA sequences have activated an endogenous murine α-(1,2)-fucosyltransferase gene not normally expressed by L cells. This event might have been selected for in the primary transfectant, and in turn each H-expressing secondary transfectant may have been selected because it had

TABLE I

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Apparent ( K_m ) for phenyl-(\beta)-D-galactoside</th>
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<tbody>
<tr>
<td>Serum (fractionated)</td>
<td>3.1</td>
</tr>
<tr>
<td>Milk (fractionated)</td>
<td>15.1</td>
</tr>
<tr>
<td>A431 cells</td>
<td>1.9</td>
</tr>
<tr>
<td>Mouse intestinal mucosa (fractionated)</td>
<td>7.7</td>
</tr>
<tr>
<td>mH1-12 cells</td>
<td>2.4</td>
</tr>
<tr>
<td>s1 cells</td>
<td>2.0</td>
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<tr>
<td>s2-2 cells</td>
<td>3.1</td>
</tr>
<tr>
<td>s3 cells</td>
<td>2.1</td>
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<tr>
<td>s4 cells</td>
<td>3.2</td>
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<tr>
<td>s5 cells</td>
<td>3.1</td>
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<tr>
<td>COS-1 cells transfected with pH3.4</td>
<td>4.4</td>
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received the human sequence capable of "activating" an endogenous mouse α-(1,2)-fucosyltransferase gene. A consequence of these events would be that the activated endogenous mouse α-(1,2)-fucosyltransferase gene should encode an enzyme exhibiting substrate activity characteristics that parallel those we determined for the α-(1,2)-fucosyltransferase in the H-expressing transfectants. To address this possibility, we isolated tissues and serum from the C3H mouse strain from which the original L cell line was derived (21) and assayed these for α-(1,2)-fucosyltransferase activity that might represent the product of an activated murine α-(1,2)-fucosyltransferase gene.

Serum, bone marrow, spleen, submaxillary glands, small intestinal mucosa, and lung tissue were solubilized with Triton X-100 and assayed for α-(1,2)-fucosyltransferase (see "Experimental Procedures"). Significant quantities of enzyme activity were detectable in extracts prepared from intestinal epithelium, but only trace amounts of activity were identified in spleen extracts, and no activity was found in the other tissues assayed (data not shown). Moreover, no activity was found in serum, even after prolonged incubation. The apparent lack of α-(1,2)-fucosyltransferase activity in the enzyme-negative tissues and serum was not due to consumption of product or substrate (see "Experimental Procedures," data not shown). Furthermore, mixing experiments with mH1-12 α-(1,2)-fucosyltransferase preparations indicated that "inhibitors" were not responsible for the apparent lack of activity (data not shown). The absence of α-(1,2)-fucosyltransferase activity in the serum of these mice is consistent with the possibility that the genome of this strain, and thus the L cell genome, does not include a gene analogous to the human H α-(1,2)-fucosyltransferase gene.

Analysis of the substrate activity properties of the α-(1,2)-fucosyltransferase found in the small intestinal mucosa indicated that this enzyme is different from the α-(1,2)-fucosyltransferase expressed in the transfected cell lines. Extracts prepared from C3H intestinal mucosa were first fractionated by ion exchange chromatography to eliminate acceptor-independent GDP-fucose hydrolysis activity (see "Experimental Procedures"). The α-(1,2)-fucosyltransferase activity in this fractionated tissue extract was then assayed to determine apparent Michaelis constants for GDP-fucose and phenyl-P-D-galactoside. The enzyme in these fractionated extracts exhibited an apparent Michaelis constant of 68.7 μM (Fig. 4C). This is markedly different from the K_m exhibited by the α-(1,2)-fucosyltransferase expressed in the transfected cell lines. Extracts prepared from C3H intestinal mucosa were first fractionated by ion exchange chromatography to eliminate acceptor-independent GDP-fucose hydrolysis activity (see "Experimental Procedures"). The α-(1,2)-fucosyltransferase activity in this fractionated tissue extract was then assayed to determine apparent Michaelis constants for GDP-fucose and phenyl-β-D-galactoside. The enzyme in these fractionated extracts exhibited a K_m for GDP-fucose of 68.7 μM (Fig. 4C). This is markedly different from the K_m exhibited by the α-(1,2)-fucosyltransferase expressed in the transfected cell lines (12.8 μM; Fig. 2A) or in the human A431 donor cells (10.9 μM; Fig. 2C).

Likewise, the C3H intestinal α-(1,2)-fucosyltransferase displays a characteristic K_m for the acceptor phenyl-β-D-galactoside (7.7 mM; Fig. 5C and Table I). By contrast, each of the enzymes in human serum, in A431 cells, or in cloned H-expressing mouse transfec-


DISCUSSION

Many mammalian proteins are subject to extensive post-translational modification by enzymatic glycosylation. This is a dynamic yet highly controlled process regulated primarily through expression of the glycosyltransferases responsible for oligosaccharide biosynthesis (13, 14). There is a substantial amount of information available concerning the catalytic properties of these enzymes, yet there is a paucity of knowledge about their primary structures and the molecular basis for their regulation. Cloned glycosyltransferase gene segments and corresponding cDNAs represent tools to address these subjects. However, despite the fact that more than 30 distinct mammalian glycosyltransferases have been described (14),
cloned cDNAs have been reported for only two of these enzymes (24-28). This is primarily because it is difficult or even impossible to purify these proteins in quantities sufficient for standard molecular cloning strategies requiring amino acid sequence information or anti-glycosyltransferase antibodies. It, therefore, seems logical to develop cloning strategies that instead use existing information about the substrate and acceptor properties of these enzymes and that take advantage of the multitude of antibody and lectin reagents specific for the oligosaccharide products of these enzymes. We report here the successful application of this type of strategy to isolate DNA sequences that determine expression of a human α-(1,2)-fucosyltransferase.

Our previous results implied that specific transfected human DNA sequences determine expression of an α-(1,2)-fucosyltransferase in H-expressing mouse transfected cell lines (15). However, those results could not allow us to distinguish between a number of possible functions for the transfected sequences. Others have demonstrated that the human H and Se α-(1,2)-fucosyltransferases exhibit distinct kinetic properties (17-19). We, therefore, anticipated that characterization of the α-(1,2)-fucosyltransferase found in the H-expressing mouse transfected cell lines would allow us to determine if transfected human sequences are responsible for expression of one or the other of these human enzymes. In the studies described here, we find that the α-(1,2)-fucosyltransferases in human serum, in A431 cells, in H-expressing mouse L cell transfected, and in COS-1 cells in which expression of this enzyme is determined by the transfected, cloned 3.4-kb human gene segment, each exhibit an apparent $K_m$ for phenyl-β-D-galactoside of between 1.9 and 4.4 mM. These values are similar to those reported by others for this acceptor when assaying partially purified human serum H α-(1,2)-fucosyltransferase (apparent $K_m$ values of 4.6 and 6.4 mM at pH 7.5, with 1.35 mM GDP-fucose; Ref. 17) or the H α-(1,2)-fucosyltransferase in whole serum (apparent $K_m$ of 1.4 mM at pH 7.2, with 12 mM GDP-fucose; Ref. 18). By contrast, our results indicate that the Se α-(1,2)-fucosyltransferase found in human breast milk exhibits a significantly higher apparent $K_m$ for this acceptor (15.1 mM). Other researchers have also found that the Se α-(1,2)-fucosyltransferase exhibits a higher apparent $K_m$ for phenyl-β-D-galactoside (46 mM, Ref. 17; 10 mM, Ref. 18).

Extensive published kinetic data for GDP-fucose do not exist for the human H and Se enzymes since the nonradioactive form of this compound has not been commercially available. Our own analyses indicate that the α-(1,2)-fucosyltransferases in human serum and in A431 cells exhibit apparent $K_m$ values for GDP-fucose of 16.2 and 10.9 μM, respectively. These results are in reasonable agreement with those reported by Le Pendu et al. (18) who found that human serum H α-(1,2)-fucosyltransferase displays an apparent $K_m$ for GDP-fucose of 8 μM, using assay conditions somewhat different from ours (pH 7.2, with 9.4 mM lacto-N-biose I as the acceptor; Ref. 18). We found that the x-(1,2)-fucosyltransferase in H-expressing mouse L cell transfected, and in COS-1 cells in which expression of this enzyme is determined by the transfected, cloned 3.4-kb human gene segment, each exhibited similar apparent $K_m$ values for GDP-fucose (12.8, 12.4, and 17.5 μM, respectively). By contrast, we found that the human Se enzyme displays a significantly higher apparent $K_m$ for this substrate (122.7 μM).

To address the possibility that the α-(1,2)-fucosyltransferase in the transfected cells may represent the product of an activated mouse gene, we also surveyed mouse tissues for this activity and examined the properties of the only mouse tissue α-(1,2)-fucosyltransferase we could identify and characterize. These analyses indicated that this enzyme has characteristics distinct from those displayed by the α-(1,2)-fucosyltransferase found in H-expressing mouse transfected. These data suggest that the α-(1,2)-fucosyltransferase in H-expressing mouse transfected is not derived from an activated endogenous mouse fucosyltransferase gene.

In aggregate, our results are most consistent with the notion that the transfected human sequences determine expression of an α-(1,2)-fucosyltransferase similar or identical to the human H α-(1,2)-fucosyltransferase found in human serum, but distinct from the Se enzyme. However, the precise nature of the molecule(s) encoded by this segment remains to be defined. Southern blot analyses indicate that the 3.4-kb EcoRI fragment does not hybridize to mouse genomic DNA se-
quences. This implies that this segment is primarily or exclusively comprised of human DNA sequences and virtually eliminates the possibility that this segment represents a mouse α-(1,2)-fucosyltransferase gene activated in cis by insertion of human regulatory sequences. But we have not yet completely excluded the possibility that the 3.4-kb EcoRI fragment encodes a trans-acting molecule (29–31) capable of activating endogenous fucosyltransferase genes in both mouse and COS-1 cell (African Green monkey) genomes. It also remains possible that a protein encoded by the 3.4-kb segment operates in a manner analogous to α-lactalbumin and “modulates” the substrate or acceptor specificity of an endogenous glycosyltransferase in these cells, causing them to exhibit α-(1,2)-fucosyltransferase activity (reviewed in Ref. 32). Other possibilities might also exist, yet we are most satisfied with the notion that these sequences encode an α-(1,2)-fucosyltransferase. This is an especially compelling hypothesis, since the enzymatic properties determined by these sequences in transfected cells mirror those of the human blood group H α-(1,2)-fucosyltransferase, yet are distinct from those displayed by a murine α-(1,2)-fucosyltransferase. Final resolution of these issues will ultimately require structural analysis of these sequences, isolation and study of their cognate transcript(s) from human cells, and functional analysis of polypeptide(s) encoded by the transcript(s).

There is no reliable published information concerning the molecular weight of the human H α-(1,2)-fucosyltransferase, since this enzyme has not been purified. However, a purified porcine α-(1,2)-fucosyltransferase has been shown to have a subunit molecular mass of approximately 55,000–60,000 Da (2). Since it appears that glycosyltransferases may themselves be moderately glycosylated (see Ref. 24, for example), it seems likely that the primary translation product of this porcine gene might be somewhat smaller, perhaps as small as 40,000–45,000 Da. In light of the similarities that have been observed between the porcine and human genomes (Ref. 33, for example), a comparable size might be inferred for the human H α-(1,2)-fucosyltransferase. Since the minimal amount of genetic coding information required for a protein this size is roughly 1 kb, and since most mammalian genes are interspersed with multiple (and frequently large) intervening sequences (34), we were somewhat surprised to find that enzyme activity could be determined by a segment of genomic DNA as small as 3.4 kb. Explanations for this observation include the possibility that this α-(1,2)-fucosyltransferase gene is simply small or that it perhaps encodes a protein much smaller than the porcine enzyme. Alternatively, it may be one of the rare human genes that lacks introns (35). However, in consideration of the fact that these sequences were isolated by selecting only for α-(1,2)-fucosyltransferase function, it also seems possible that they represent a truncated form of a larger (intact) α-(1,2)-fucosyltransferase gene. As has been observed in similar systems (36), such ζ rearrangement might have occurred during the transfection process, resulting in deletion of noncoding (or even coding) segments not essential for catalytic activity. It is also possible that deletion of nonessential segments could occur as a result of the cloning procedures we used. It should also be noted, however, that the pWE15 vector used in these experiments is not specifically equipped to direct transcription of DNA sequences cloned within its EcoRI sites (22). Thus, it appears that control sequences necessary for transcription of the gene(s) within the borders of the 3.4-kb EcoRI fragment have remained intact during the cloning procedures. These issues should be resolved upon analysis of the structure of these sequences and of the transcript(s) derived from this segment.

Finally, it should be noted that cloned DNA sequences like the one we describe here represent useful reagents for studying oligosaccharide function. For example, such sequences will enable the construction of mammalian cell lines that differ in specific glycosylation capabilities, but that are otherwise iso- genetic, offering a straightforward opportunity to examine biological functions imparted to glycoproteins or cells by specific oligosaccharide linkages. Moreover, with the advent of technologies that provide for tissue-specific transcriptional control of heterologous sequences in transgenic animals (reviewed in Ref. 53), and that allow gene disruption by homologous recombination in murine embryonic stem cells (reviewed in Ref. 54), cloned glycosyltransferase genes and cDNAs may be used to investigate the functions of specific oligosaccharide structures during development and differentiation.

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Supplementary Material To:

by
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EXPERIMENTAL PROCEDURES

Materials

β-Fucose was purchased from Fluka (Buchs, Switzerland). α-Phosphorylphosphohydrolate was obtained from Aldrich. Glucuronol-3-phosphate, α-L-α-Fucose-1-phosphate (dicyclohexylammonium salt), phenyl-β-D-galactose, and bovine serum α-1,4-galactosidase were from Sigma. GDP-[3H]-fucose (44.8 Ci/mmol) and [1-14C]fucose (58.7 mCi/mmol) were from Amersham. Female C3H mice were obtained from the Charles River Co. (Wilmington, MA).

Methods

Paper Chromatography

Descending paper chromatography was performed using Wharman No. 40 in ethyl acetate/pbpyridine/4% water (10:4:3; solvent A) (16) or using Wharman No. 3MM in 95% ethanol/1 M ammonium acetate/7.5% (v/v) Solulight (37). The labelled compounds were located by autoradiography of dried chromatograms. Alternatively, the dried chromatograms were cut into 1 cm strips and the radioactive-labelled compounds were eluted with water. An aliquot of each strip was mixed with starch solution and radioactivity was determined in a scintillation counter.

Preparation of Radioactively Labeled Standards

[14C]Fucose-1-phosphate was prepared by enzymatic cleavage of GDP-[14C]-fucose (1 nmol) with trisaccharide phosphodiesterase (EC 3.1.4.1, 1 μl of 0.005 units, Bucherich-Mannheim) in 20 μl of 100 mM Tris-HCl pH 8 at 37°C for 3 h. The reaction was then fractionated by descending paper chromatography on Wharman No. 3MM using Solvent B for 20 h (37) in parallel with GDP-[14C]-fucose and [14C]fucose 1-phosphate. [14C]Fucose-1-phosphate (38 μg) was then eluted from the chromatogram with water and concentrated under vacuum. [14C]Fucosylphenyl-β-D-galactoside was generated from GDP-[14C]-fucose (1.4 μM) and phenyl-β-D-galactoside (2.5 mM) by the action of α-L-fucosyltransferase activity in human serum, using the reaction conditions described below for assay of α-L-fucosyltransferase. The products of this reaction were fractionated by descending paper chromatography on Wharman No. 40 for 6 h in Solvent B (A: 10:4:3, B: 0:100:40) and were then eluted from the paper with water and concentrated under vacuum. Alternatively, [14C]Fucosylphenyl-β-D-galactoside was isolated from fucosyltransferase assay mixtures using the Sep-Pak procedure described below.

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by


Cell Lines and Cell Culture

COS-1 cells were obtained from the American Type Culture Collection and were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cultures were maintained in 5% CO2 at 37°C.

Preparation of Cell Extracts

Cells were washed with PBS, removed from culture dishes with a rubber policeman, and pelleted by centrifugation. Cell pellets were resuspended in 2 volumes of cold 1% Triton X-100 (Serva Feinbio, Heidelberg, Germany) and sonicated for 15 seconds using a Branson sonicator equipped with a probe (20,000 watts). Inactive supernatants were centrifuged for 10 minutes at 10,000 x g and the pellets were resuspended in 2 volumes of cold 1% Triton X-100 and sonicated as described above. Centrifuged at 15,000 x g for 5 minutes and the supernatants were collected. Mouse intestinal mucosal extracts were prepared by digesting the small intestine into a fine polypropylene rod, shaking the mucosal cells in buffered saline, and collecting the cells by centrifugation at 1500 x g. Extracts were then prepared as described above.

Partial Purification of Membrane-Associated (α-L-Fucosyltransferase)

Preliminary experiments indicated that extracts prepared from mouse intestinal mucosa contained large amounts of an activity that hydrolysed GDP-fucose in an acceptor-independent manner (see below and Table 1). Since subcellular hydrolysates were isolated with a lower activity than the total mucosal extract, further attempts were made to isolate the enzyme from the total mucosal extract, using the procedure described below.

Assay of Naltosyl Fucosyltransferase

Serum was prepared from a rabbit which had been injected with a drawn blood obtained from a non-Fucosyltransferase individual. The blood was drawn into a glass tube at 37°C for 1 h, and was immediately fractionated by absorption with activated charcoal exactly as described (26). The 20-40% ammonium sulfate fraction was dialyzed against 5 changes of 4 liters of water (8 h each) at 4°C. Assay of (α-L-Fucosyltransferase) was done immediately. Alternatively, the fractionated serum was aliquoted and stored at -20°C until use.
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Fucosyltransferase assays were performed by a modification of the procedure reported by Chester et al. (16). The standard assay contained GDP-[14C]fucose (5 μM), β-phenyl-d-glucoside (25 mM), ATP (5 mM) and the enzyme solution (1-10 μl) in 20 mM sodium cacodylate, pH 7.0. The reaction mixture was incubated for 1 h at 37°C. After the addition of 25 mM sodium cacodylate, pH 6.0, and 500 mM NaCl in 20 mM cacodylate, pH 6.0. Fractions (1 ml) were collected and assayed for (α-1→2)fucosyltransferase activity with 25 mM sodium acetate, sodium phosphate, or Tri-HCl, using concentrated solutions of these buffers previously adjusted to various pH values. The final pH value of each reaction was determined after 1 h incubation at room temperature. In assaying for GDP-fucose, GDP-[14C]fucose was diluted with unlabeled GDP-fucose to a final specific activity of 26.3 μCi/μmol. The GDP-fucose was then counted in a Beckman scintillation counter (model 78.2508). The retention of the purified acceptor in the Sep-Pak cartridge was monitored by aspiration with three 2 ml portions of water. The enzyme was then washed with 1 liter of methanol and then washed with 1 liter of ethanol.

A separation based upon hydrophilic interaction chromatography (42) was developed for rapid purification of the fucosyltransferase. The enzyme preparation was applied to a C18 column that had been equilibrated with 100 mM triethylammonium acetate, pH 7.0. The enzyme was then washed with 1 liter of acetonitrile in 100 mM triethylammonium acetate, pH 7.0, and then eluted with 1 liter of 0.5 M triethylammonium acetate, pH 7.0. The enzyme was then subjected to reaperization in dry pyridine and evaporation to dryness in vacuo. The enzyme preparation was then dried under nitrogen and dissolved in PBS (pH 7.4) to give a concentration of 10 units/ml. The enzyme was then dialyzed against PBS (pH 7.4) at 4°C.

A second step of purification was carried out using a Sep-Pak C18 column (Waters-Millipore) that had been equilibrated with 100 mM triethylammonium acetate, pH 7.0. The enzyme was then applied to the column, which had been equilibrated with PBS (pH 7.4), and was eluted with a linear gradient of 100 mM triethylammonium acetate, pH 7.0, to 100% acetonitrile in PBS (pH 7.4) over a period of 60 min. The enzyme was eluted at 240 nm. The enzyme was then dialyzed against water and the dialyzed enzyme was then applied to a hydrophobic cartridge (AX 300, 4.6 mm x 22 cm, Phenomenex, Torrance, CA). An aliquot (10 μl) of the enzyme solution was applied to the column in 100 mM triethylammonium acetate, pH 7.0, and was eluted at a linear gradient from 100 mM triethylammonium acetate, pH 7.0, to 100% acetonitrile in 100 mM triethylammonium acetate, pH 7.0, over a period of 60 min. The enzyme was eluted at 240 nm.

The Sep-Pak method does not separate GDP-[14C]fucose from [14C]fucose-l-phosphate and thus cannot direct nucleotide peroxisome activity that may consume GDP-fucose while slowing the rate of GDP-fucose synthesis. This method is therefore limited in its ability to separate GDP-fucose from other nucleotides. The Sep-Pak method is therefore limited in its ability to separate GDP-fucose from other nucleotides. The Sep-Pak method is therefore limited in its ability to separate GDP-fucose from other nucleotides. The Sep-Pak method is therefore limited in its ability to separate GDP-fucose from other nucleotides.
Cloned Human DNA Sequences Determine Expression of a Fucosyltransferase

**Figure 7. Fractionation of fucosyltransferase assay mixtures and products using Sep-Pak cartridges.** See *Experimental Procedures* for details of the separation.

| Enzyme source | % initial cpm found as [14C]fucose | [14C]fucose-1-Po4 | [14C]fucose
<table>
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<tr>
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<tbody>
<tr>
<td>Fractionated human serum</td>
<td>53.10</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A431 cells</td>
<td>0.95</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NIH-3T3 cells</td>
<td>1.48</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>s2-2 cells</td>
<td>0.96</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fractionated mouse intestine</td>
<td>20.94</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fractionated mouse intestine + 10 mM L-fucose</td>
<td>0.16</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>COS-1 cells transfected with pJ834</td>
<td>0.26</td>
<td>ND</td>
<td>ND</td>
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</table>

**Table II. GDP-fucose hydrolysis activity in crude or fractionated preparations containing [14C]-fucose.** Enzyme preparations were incubated in the standard (α1,2)fucosyltransferase assay mixture, but in the absence of phenyl-β-D-galactoside acceptor. After the standard incubation time (4 h for serum and 3 h for other enzymes), the amount of fucose and fucose-1-phosphate formed from GDP-[14C]fucose were determined by paper chromatography on Whatman No. 3MM in Solvent B, as described in the text. Approximately 1% of the radioactivity in the GDP-[14C]fucose is obtained from the manufacturer's reagent [14C]fucose. The values presented in this table have been corrected for this. ND, not detectable.

| Enzyme source | % initial cpm found as [14C]fucose | [14C]fucose-1-Po4 | [14C]fucose
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<tr>
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<tr>
<td>Fractionated human serum</td>
<td>99.1</td>
<td>ND</td>
<td>0.9</td>
</tr>
<tr>
<td>A431 cells</td>
<td>98.4</td>
<td>ND</td>
<td>1.6</td>
</tr>
<tr>
<td>NIH-3T3 cells</td>
<td>99.5</td>
<td>ND</td>
<td>0.7</td>
</tr>
<tr>
<td>s2-2 cells</td>
<td>99.5</td>
<td>ND</td>
<td>0.5</td>
</tr>
<tr>
<td>Mouse intestine</td>
<td>10.0</td>
<td>11.8</td>
<td>11.4</td>
</tr>
<tr>
<td>Fractionated mouse intestine</td>
<td>97.0</td>
<td>ND</td>
<td>3.0</td>
</tr>
<tr>
<td>Fractionated milk (Secretor)</td>
<td>98.6</td>
<td>ND</td>
<td>1.4</td>
</tr>
<tr>
<td>COS-1 cells transfected with pH3.4</td>
<td>99.2</td>
<td>ND</td>
<td>0.8</td>
</tr>
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**Isolation of Human DNA Restriction Fragments from Transfectant Clone s2-2**

High molecular weight genomic DNA was isolated from the H-expressing secondary transfecent s2-2 (15), digested with EcoRI, and fractionated through a 1% agarose gel buffered in Tris-borate EDTA (45). The region of the gel containing the 2.7 kb and the 3.4 kb human EcoRI fragments was excised from 3 mm slices and the DNA in these was isolated by electroelution. Aliquots of the non-fractionated DNA were analysed by Southern blotting (46) with a radiolabelled Alu probe (BLURR ref. 47), using hybridisation and wash conditions described previously (15). Fractions containing either the 2.7 kb or the 3.4 kb fragment were excised separately to prepare plaque libraries in lambda g1 (48). These libraries were screened (49) with a radiolabelled M13 BLURR probe. Positive plaques isolated from a tertiary screen were used to prepare phage DNA (51), and phages containing either the 2.7 kb EcoRI fragment or the 3.4 kb EcoRI fragments were identified by Southern blotting. The 3.4 kb or the 2.7 kb insert was released from the phage arms by EcoRI digestion, purified by agarose gel electrophoresis and elution (43), and individually sequenced between the EcoRI sites in pWE15 (22).

**COS-1 Cell Transfactions**

Plasmid DNAs were transfected into COS-1 cells by the DEAE-dextran procedure (27). Seventy-two hours after transfection, cells were harvested, extracts were prepared as described above, and extracts were subjected to assays for (α1,2)fucosyltransferase activity, for GDP-fucose hydrolytic activity, and for α-fucosidase activity.