Molecular Cloning of a Fourth Member of a Human α(1,3)Fucosyltransferase Gene Family

MULTIPLE HOMOLOGOUS SEQUENCES THAT DETERMINE EXPRESSION OF THE LEWIS x, SIALYL LEWIS x, AND DIFUCOSYL SIALYL LEWIS x EPITOPES*

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We and others have previously described the isolation of three human α(1,3)fucosyltransferase genes which form the basis of a nascent glycosyltransferase gene family. We now report the molecular cloning and expression of a fourth homologous human α(1,3)fucosyltransferase gene. When transfected into mammalian cells, this fucosyltransferase gene is capable of directing expression of the Lewis x (Galβ1-4[Fucα1-3] GlcNAc), sialyl Lewis x (NeuNαcβ2-3Galβ1-4[Fucα1-3] GlcNAcβ1-3Galβ1-4[Fucα1-3]GlcNAc) epitopes. The enzyme shares 85% amino acid sequence identity with Fuc-TIII and 89% identity with Fuc-Tc but differs substantially in its acceptor substrate requirements. Polymerase chain reaction analyses demonstrate that the gene is syntenic to Fuc-TIII and Fuc-TV on chromosome 19. Southern blot analyses of human genomic DNA demonstrate that these four α(1,3)fucosyltransferase genes account for all DNA sequences that hybridize with the Fuc-TIII catalytic domain. Using similar methods, a catalytic domain probe from Fuc-TV identifies a new class of DNA fragments which do not cross-hybridize with the chromosome 19 fucosyltransferase probes. These results extend the molecular definition of a family of human α(1,3)fucosyltransferase genes and provide tools for examining fucosyltransferase gene expression.

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molecular cloning of a fourth human fucosyltransferase gene. 24576

A myeloid-expressed enzyme (Fuc-TIV) (23, 26, 45, 46), and a third α(1,3)fucosyltransferase gene whose cognate enzyme (Fuc-TV) exhibits properties distinct from those of Fuc-TII and Fuc-TIV (47). We now report the cloning and expression of a fourth distinct human α(1,3)fucosyltransferase gene, whose predicted amino acid sequence is very similar to Fuc-TII and Fuc-TV and substantially different from Fuc-TIV. Disparate acceptor substrate requirements characterize the enzymes encoded by these four genes. These homologous DNA sequences define a glycosyltransferase gene family, serve as templates for structure/function analyses, and represent tools to study α(1,3)fucosyltransferase expression in human cells.

Experimental Procedures

Nomenclature — The gene described here represents the fourth distinct human α(1,3)fucosyltransferase DNA sequence and will be called Fuc-TVI. We previously designated the human Lewis blood group α(1,3)/α(1,4)fucosyltransferase Fuc-TII and a myeloid-expressed α(1,3)fucosyltransferase Fuc-TIV (47). The Fuc-TVI gene encodes an α(1,3)fucosyltransferase with properties similar, although not identical, to those ascribed to an α(1,3)fucosyltransferase found in human plasma (42, 47).

Human Genomic Library and Screening — The preparation and screening of a human genomic DNA library has been described previously (48). The library was screened with a radiolabeled 1.7-kb XbaI fragment isolated from the insert in pcDNA1-Fuc-TIV (44, 47).

DNA Sequence Analysis — Southern blot analyses were used to identify phage clones with strong homology to the human Fuc-TVI probe. A 1.3-kb fragment from a strongly hybridizing phage with a disparate Fuc-TII restriction pattern was subcloned into the plasmid pT7Z18R (Pharmacia LKB Biotechnology Inc.). The DNA sequence of this fragment was determined by the dyeoxy chain termination method (48) using T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.). Oligonucleotides were synthesized according to flanking plasmid sequences and subsequently according to insert sequence. The DNA sequence of this fragment was nearly identical to the 3' end of the Fuc-TVI coding region but diverged immediately distal to the stop codon of the Fuc-TVI coding segment (see “Results”). To isolate a single restriction fragment encompassing a possible novel coding region, a 7.1-kb EcoRI fragment that cross-hybridized with the Fuc-TVI probe was isolated from this phage DNA. This fragment was ligated into the EcoRI site of pcDNA1, to yield the vector pcDNA1-Fuc-TVI. The DNA sequence of the insert in this vector was determined as described above.

To construct an expression vector consisting largely of the coding sequence of this gene, PCR (49) was used to amplify this segment from pcDNA1-Fuc-TVI. Fifty-nanogram aliquots of plasmid template were used in reactions with the GeneAmp kit (Perkin-Elmer Cetus). Twenty cycles of amplification were performed, consisting of 1.5 min at 94 °C, followed by annealing and extension for 3.5 min at 72 °C. Primers immediately flanking the opening reading frame (Fig. 1) were chosen to specifically amplify the Fuc-TVI coding sequence (upper strand primer: gcggatcttctcttcctccacctccattccagcagctctgta, nucleotides −22 through −3; lower strand primer: gcgatcctggctgaaagcttgcctgcagcctttagg, corresponding to reverse complement of nucleotides 1154–1183; endogenous HindIII site underlined). The PCR product was digested with HindIII, and the 1.2-kb fragment was cloned into the HindIII site of pcDNA1. A representative phage containing the insert in the sense orientation with respect to the plasmid's cytomegalovirus promoter was designated pcDNA1-Fuc-TVI. The insert in plasmid pcDNA1-Fuc-TVI was sequenced in its entirety to exclude possible PCR errors. Sequence analyses were performed using the sequence analysis software package of the University of Wisconsin Genetics Computer Group (50) (version 5.5). The sequence was analyzed using the IBM Pasteur Sequence Analysis Software package (Kodak/IIBI).

Transfection of COS-1 and CHO-T Cells — COS-1 cells, cultured as described (23, 51), were transfected with plasmid DNAs (pcDNA1-Fuc-TIV, this work; pcDNA1-Fuc-TII, Refs. 44 and 47; pcDNA1-Fuc-TV, Ref. 45; pcDNA1-Fuc-TVI, Ref. 47; or pcDNA1, Invitrogen) using a DEAE-dextran procedure (44, 52). A Chinese hamster ovary cell line (53) that stably expresses the polyoma large T antigen (CHO-T cells)2 and that replicates polyoma origin-containing plasmids (54, 55) was transfected with plasmid DNAs using a liposome-based reagent (N-[1-(2,3-dioleoyloxypropyl)-N,N,N-trimethylammoniummethylsulfate, DOTAP, Boehringer Mannheim) and a modification of the manufacturer's protocol.

Antibodies — Anti-Lewis a antibody (anti-SSEA-1, IgM, Ref. 3) was provided by Dr. David Smith (Wistar Institute, Philadelphia). Anti-H and anti-Lewis a monoclonal antibodies (IgM) were purchased from Chemicon Ltd. (Edmonton, Alberta, Canada). A monoclonal anti-VIM-2 antibody (IgM) was purchased from An-der-Grub (Kamberg, Austria). Anti-sialyl Lewis x monoclonal antibody CSLEX1 (IgM) and anti-sialyl Lewis x monoclonal antibody CSLEX1 (IgG) were purchased from the UCLA Tissue Typing Laboratory (Los Angeles, CA). The IgM monoclonal antibody FH6 was provided by Dr. Reiji Kannagi (Aichi Cancer Research Institute, Nagoya, Japan). FH6 recognizes difucosyl sialyl Lewis x (16, 17, 24, 56). Fluorescein-conjugated goat anti-mouse IgM and IgG antibodies were purchased from Sigma.

Flow Cytometry Analysis — COS-1 or CHO-T cells transfected with fusocyltransferase expression vectors, or with a control vector, were harvested (57) 72 h after transfection and stained with monoclonal antibodies diluted in staining media (23, 44, 45, 47). Anti-Le- a and anti-Lewis x antibodies were used at 1:5000 (23, 44, 45, 47). Anti-sialyl Lewis x was used at 1:500 (10.8 μg/ml). Anti-sialyl Lewis x was used at a dilution of 1:500 (10.8 μg/ml). Anti-VIM-2 was used at 1:50. Anti-difucosyl sialyl Lewis x (FH6) was used at 1:50. Cells were then stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgM and anti-IgG and analyzed on a Becton-Dickinson FACScan (23, 44, 45, 47). Approximately 27–32% of the population of COS-1 and CHO-T cells transfected with each fusocyltransferase vector express the Lewis x determinant. Over 95% of control transfectants (pcDNA1-transfected COS-1 and CHO-T cells) stained with the anti-Lewis x antibody exhibit a fluorescein intensity below the gated settings.

Fucosyltransferase Assays — Cell extracts containing 1% Triton X-100 were prepared from transfected COS-1 cells (44, 47). Fusocyltransferase assays with neutral acceptor substrates were performed in a volume of 20 μl and contained 25 mM sodium cacodylate (pH 6.2), 5 mM ATP, 10 mM L-fucose, 20 mM MnCl2, 5 μM GDP-[3H]fucose, and 10 μg of cell extract protein. Acceptor substrates were added to a final concentration of 20 mM (N-acetyllactosamine, Ga1β1→4GlcNAc; lactose, Galβ1→4Glc; and lacto-N-biose I, Galβ1→3GlcNAc) or 5 mM (2’-fucosyllactose, Fucα1→2Galβ1→4Glc). Control assays with no added acceptor were performed using the same conditions. Reactions were incubated at 37 °C for periods of time to yield linear rates (<20% of GDP-fucose consumed during the course of the reaction; typically 1 h). Assays were terminated by addition of 20 μl of ethanol, followed by addition of 560 μl of distilled water. The terminated assays were centrifuged at 12,000 × g for 5 min, and the supernatants were collected. The flow rate of 1 ml/min. The trisaccharide product of these reactions, an aliquot of each terminated reaction supernatant was subjected to scintillation counting. Another aliquot was applied to a column containing Dowex X200-400, formate form (44, 47). To quantify incorporation of radioactive fucose into product, the flow-through fraction, and 2 ml of a subsequent water elution, were collected, pooled, and counted.

Amine adsorption HPLC (44, 58) was used to confirm the structure of the product formed with N-acetyllactosamine. The neutral product in the Dowex eluate was lyophilized, resuspended in 130 μl of 70% acetonitrile/water (70:30), and compounds were purchased from the UCLA Tissue Typing Laboratory (Los Angeles, CA). The IgM monoclonal antibody FH6 was provided by Dr. Reiji Kannagi (Aichi Cancer Research Institute, Nagoya, Japan). FH6 recognizes difucosyl sialyl Lewis x (16, 17, 24, 56). Fluorescein-conjugated goat anti-mouse IgM and IgG antibodies were purchased from Sigma.
was prepared using a previously described procedure (64). To control primer specificity for Fuc-TVI. 

Negative controls for these experiments consisted of genomic DNA from non-cleaving Fuc-TI11 or Fuc-TV at the site shown in Fig. 1) to verify the identity of amplified fragments. PCR products and restriction digests were subjected to restriction endonuclease digestion with NcoI (which digests 75% of 5′-actinonucleotide, 25% 2′ m triethyleneamine acetate (pH 5.5)) for subsequent product analysis by HPLC as described below. To verify that the Dowex chromatography procedure effectively fractionated radioactive product from radioactive GDP-fucose substrate, a single aliquot of the terminally diluted assay was lyophilized to dryness, re-suspended in 130 μl of 75% acetone, 25% 2′ m triethyleneamine acetate (pH 5.5), and was also subjected to analysis by HPLC, as described below.

Lyoophilized re-suspended samples derived from un-fractionated assays or from Dowex column-fractionated assays were subjected to ion-suppressed amine-adsorption HPLC using a Varian AX-5-column equilibrated in 75% acetone, 25% 2′ m triethyleneamine acetate (pH 5.5). Products were analyzed by electrospray ionization mass spectroscopy. Mass products were identified by comparison to elution times shown by radiolabeled standards as described below.

The structure predicted for the fucosylated product generated with α(3)GalNAc-2→3Gal-α(1→4)GlcNAc, 9 min; Galβ1→4[Fucα1→3]GlcNAc, 27 min; NeuNAc2→3Galβ1→4[Fucα1→3]GlcNAc, 52 min; GDP-[14C]fucose, 51 min. Parallel HPLC separations made with the Dowex column-fractionated assays, and the unfracti- onated assays indicated that the Dowex column procedure removes more than 95% of the radiolabeled GDP-fucose in the assay, yet does not retain detectable amounts of the sialylated product.

The product structure predicted for this fucosylated product generates with α(3)GalNAc-2→3Gal-α(1→4)GlcNAc was confirmed by neuraminidase digestion followed by HPLC analysis. The HPLC-purified fucosylated tetrasaccharide was digested with Clostridium perfringens neuraminidase (Sigma Type X; 4 milliunits/10,000 cpm of tetrasaccharide product) for 8 h at 37°C in a buffer consisting of 20 mM sodium cacodylate (pH 6.2) and 10 mM 1-fucose. The digest was then subjected to fractionation by amine adsorption HPLC. The radiolabeled product of neuraminidase digestion co-eluted with a 3-fucosyl-N-acetyllactosamine standard (47).

PCR Analysis of Human Chromosomal Somatic Cell Hybrids—Genomic DNA was prepared from two panels of hamster-human somatic cell hybrids (47) that were obtained from Dr. S. L. Murphy (New Haven, Conn.). Murine microcell hybrids containing human chromosome 11 (61, 62) and 7 (63) have been described previously (47). Hybrid cell genomic DNAs were analyzed using the PCR primers detailed above for the cloning of pcDNA1-Fuc-TV (Fig. 1). Amplification using these two primers yields a 1.2-kb fragment from the Fuc-TV gene. Thirty cycles of amplification were performed, consisting of 1.5 min at 94°C, followed by annealing and extension for 3.5 min at 72°C. Multiple negative controls with no added genomic DNA template were run in parallel with test samples to exclude the presence of contaminating DNA sequences. Other negative controls included a single cell hybrid containing human genomic DNAs, which were used to rule out the possibility of non-specific amplification of homologous sequences across species. Positive controls for these experiments consisted of genomic DNA from the Lewis-positive individual who served as the source of the genomic DNA library and other human genomic DNAs. Cloned segments of Fuc-TII, Fuc-TIV, Fuc-TV, and Fuc-TI were used to confirm primer specificity for Fuc-TV. A portion of each PCR reaction was subjected to restriction endonuclease digestion with NcoI (which does not cleave Fuc-TIII or Fuc-TV at the site shown in Fig. 1) to verify the identity of amplified fragments. PCR products and restriction digests were analyzed on 1.2% agarose gels.

Southern Blot Analyses of Human Genomic DNA—Genomic DNA was prepared using a previously described procedure (64). To control for DNA sequence polymorphisms previously found in human α(3)fucosyltransferase genes, genomic DNA was prepared from the same individual whose DNA was used to construct the original genomic library (45, 47). Genomic DNA was digested with restriction endonucleases, fractionated through 0.8% agarose gels, and subjected to Southern transfer using previously described methods (45, 64). For comparability in subsequent experiments with several probes, multiple blots were prepared from identical sets of restriction digests generated by digestion of a single gel. Hybridization and wash conditions are as described previously (45, 64) and as detailed in the legend to Fig. 4. The hybridized and washed blots were subjected to autoradiography for 48–120 h, and the resulting autoradiograms were then scanned using an OmniMedia 650 scanner (XR5, Torrence, CA). The images were grouped and overall contrast was adjusted using Adobe Photoshop (version 2.0), followed by labeling with Adobe Illustrator 3.2 (Mountain View, CA).

Southern blots were probed with “gene-specific” probes or with probes that recognize conserved portions of the genes that correspond to their putative catalytic domains (“catalytic domain” probes). Gene-specific probes were derived from regions 3′ to the highly homologous coding portions of each gene (Figs. 2, A and B). Each gene-specific probe was chosen to have less than 70% homology to the other fucosyltransferase sequences or to other sequences in the GenBank data base, and human repetitive sequences were avoided (44, 65). Catalytic domain probes, derived from either Fuc-TI11 or from Fuc-TV, were chosen from regions of highest DNA sequence conservation among the four human fucosyltransferase genes (Figs. 1 and 2; see also Refs. 45 and 47). The Fuc-TV gene-specific probe was isolated by XbaI digestion of a clone encompassing 1.3 kb of Fuc-TV 3′ untranslated region (immediately downstream of the insert in pcDNA1-Fuc-TV, Ref. 47). The remaining probes were generated using PCR amplification of cloned templates as described above and the primers listed in Table I. The positions and sizes of each probe are illustrated in Fig. 2B.

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RESULTS

Molecular Cloning of a Human Genomic DNA Segment Homologous to, but Distinct from, the Human Fuc-TIII, -IV, and -V Genes—Four or more distinct α(1,3)fucosyltransferase activities have been described previously in biochemical analyses of human tissues and cells (35–42). Candidate genes encoding fucosyltransferases similar to three of these activities have been reported (26, 44–47). Two of these enzymes, Fuc-TIII and Fuc-TV, maintain extraordinarily high levels of protein and nucleic acid similarity, as depicted schematically in Fig. 2A but display marked differences in acceptor substrate specificities (47). Another gene termed Fuc-TV (45, 46) or ELFT (26) is less similar in its sequence, and its corresponding enzyme also exhibits disparate substrate requirements (45). The Fuc-TIII and Fuc-TV genes were isolated from a human genomic DNA phage library screened at low stringency with the cDNA encoding Fuc-TIII (45, 47). Eighteen phages isolated from this screening were characterized. Nine of these phages exhibited relatively weak hybridization to the Fuc-TIII probe and proved to contain the Fuc-TV gene. Nine other phages exhibited strong hybridization signals. Seven of these phages proved to contain the Fuc-TV gene, whereas one other was found to contain sequences co-linear with the Fuc-TIII cDNA.

The remaining phage exhibited a unique ParI restriction pattern, suggesting that it represents a sequence distinct from the other α(1,3)fucosyltransferase genes. A 7.1-kb hybridization-positive EcoRI fragment from this phage was cloned and sequenced.

DNA sequence analysis of this genomic fragment identifies a single long open reading frame (Fig. 1, bp 1–1080) predicted to yield a protein with striking amino acid sequence similarity to Fuc-TIII (Fig. 1). This reading frame begins at a methionine codon found within a sequence context corresponding to Kozak’s consensus rules for translation initiation sites (66). A single strongly hydrophobic peptide segment corresponding

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3 B. W. Weston, R. Morales, and J. B. Lowe, unpublished data.

TABLE I

<table>
<thead>
<tr>
<th>Probe</th>
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<tr>
<td>Fuc-TII1 catalytic domain</td>
<td>U: CCGACACGAAAGCAGAGCATACTACAGAAGGTCTCTGGCC L: GGCAGATGAGGTTCTCCGAGCCGCGGAC</td>
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<tr>
<td>Fuc-TII1 gene-specific</td>
<td>U: CCACCCGGGAGTGATGGTGCAAGAGCTGTGTCAAGACAGG T: CAGAAAAGTGAAATGGAGAAACAGTTGAGT</td>
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<tr>
<td>Fuc-TIV catalytic domain</td>
<td>U: GGTGCCCGAAATTTGGGCTCCTGACAC L: CAGAAAAACGTGAATCGGGAAACAGTTG</td>
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<tr>
<td>Fuc-TIV gene specific</td>
<td>U: AGCTGTTGTCAGGGTGGTGAAGGGGCGCT L: CAGAAAAACGTGAATCGGGAAACAGTTG</td>
</tr>
<tr>
<td>Fuc-TV gene specific</td>
<td>U: AAAGGGGAGTGTCCTTCTTCTGAGTGCCAA L: CCTGTTCCTCCACACAGGACCCGGCCAAA</td>
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* U, upper; L, lower.

FIG. 1. Comparison of DNA and derived protein sequences of Fuc-TVI and Fuc-TIII. Residue 1 of the nucleotide sequence of the Fuc-TVI genomic DNA segment is assigned to the A residue of the putative initiation codon. The derived protein sequence of Fuc-TVI, in single letter code, is displayed above the Fuc-TVI DNA sequence. Dashed lines below the numbered Fuc-TVI DNA sequence denote DNA sequence identity between Fuc-TII1 and Fuc-TVI. Nucleotide sequence differences are indicated within the dashed line representing the Fuc-TII1 sequence. Blank areas correspond to regions of Fuc-TVI that have no counterpart in the Fuc-TII1 sequence or vice versa. The derived amino acid sequences for Fuc-TII1 and Fuc-TVI are identical except where indicated by the inclusion of differing amino acids (in single letter code) under the corresponding codons in Fuc-TII1. The positions of the two PCR primers used to construct pcDNA1-Fuc-TVI and to analyze chromosomal hybrid DNAs are underlined. The location of the NcoI site in Fuc-TVI used to confirm the identity of the PCR product is italicized and labeled. Circled asparagine residues in the Fuc-TVI protein sequence represent potential asparagine-linked glycosylation sites. The DNA sequence in the 5′-untranslated region highlighted by stippled overlining corresponds to a sequence motif consistent with a splice acceptor consensus sequence. Similar analyses for Fuc-TIV and Fuc-TV have been published previously (45, 47).

The open reading frame predicts a 358-amino acid protein that is identical to Fuc-TII1 at 306 of 361 corresponding amino acid positions (Fig. 1) and identical to Fuc-TV at 334 of 374 amino acids (data not shown). Because of this strong sequence similarity to previously described human α(1,3)fucosyltransferases, we term the derived protein sequence Fuc-TVI. Marked sequence discontinuities occur between these derived protein sequences in a region corresponding to base pairs 121-449 of Fuc-TVI (Fig. 1). This region of Fuc-TVI does not contain the 33-bp nucleotide sequence insertion found in Fuc-
TV relative to Fuc-TIII (47). A total of 97 other amino acid sequence differences between Fuc-TIII, Fuc-TV, and Fuc-TVII are observed in this region; by contrast, only 14 amino acid sequence differences are noted between the COOH-terminal halves of these three proteins (Fig. 2A). Four asparagine residues that represent potential N-glycosylation sites are found within the Fuc-TVII predicted protein (Fig. 1). Two of these, located at amino acid residues 153 and 184, correspond to N-linked glycosylation sites present at precisely corresponding positions in Fuc-TIII and Fuc-TV (Fig. 2A). The other two, located at positions 46 and 91, are not found in corresponding locations in Fuc-TIII, but are present in Fuc-TV (Fig. 2A).

The Fuc-TVII DNA sequence diverges from the Fuc-TIII sequence at a position approximately 18 base pairs 5' to the initiator codons of the two sequences (data not shown). A sequence corresponding to the consensus for splice acceptor sites (68, 69) is found in this region of the Fuc-TVII sequence, beginning at a position 96 base pairs 5' to the initiator codon (Fig. 1). The Fuc-TIII and Fuc-TVII DNA sequences also diverge at a position 57 base pairs 3' to their respective termination codons.

In summary, the cross-hybridizing open reading frame has the potential to encode a type II transmembrane protein with marked amino acid sequence similarity to two other cloned human fucosyltransferases. This relationship is most salient in a region corresponding to the catalytic domain of these latter two enzymes and is analogous to the structural similarities previously described for Fuc-TIII and Fuc-TV (Fig. 2A and Ref. 47). Given the sequence discontinuities identified in the DNA sequence flanking the open reading frame, these comparisons suggest that this cross-hybridizing sequence represents the coding region of an α(1,3)fucosyltransferase gene.

The Genomic DNA Restriction Fragment Determines Expression of a Related but Distinct α(1,3)Fucosyltransferase—To determine if this open reading frame encodes a functional α(1,3)fucosyltransferase, the putative coding region segment was cloned into a mammalian expression vector and tested in transfection experiments. The expression plasmid pcDNA1-Fuc-TVII (see “Experimental Procedures”) was first transfected into the COS-1 cell line, a mammalian host that does not normally express α(1,3)fucosyltransferase activity (44). Extracts prepared from transfected cells were tested for α(1,3)- and α(1,4)fucosyltransferase activity in assays containing low molecular weight oligosaccharides previously shown to function in vitro as acceptor substrates (44-47). Extracts prepared from pcDNA1-Fuc-TVII-transfected COS-1 cells were found to contain an α(1,3)fucosyltransferase activity that efficiently used N-acetyllactosamine to generate 3-fucosyl-N-acetyllactosamine (specific activity of 880 pmol/mg/h). Under the same assay conditions, the α(1,3)fucosyltransferase activity in this same extract preparation did not use two other neutral type II acceptor molecules, 2'-fucosyllactose and lactose (specific activity less than 0.5 pmol/mg/h). In addition, no detectable α(1,4)fucosyltransferase activity was found in multiple assays with the type I substrate lacto-N-biose I (specific activity less than 0.5 pmol/mg/h). As noted previously (44, 45, 47), extracts prepared from pcDNA1-transfected COS cells contain no detectable fucosyltransferase activity with any of the four acceptors tested.

A second set of experiments were completed to determine the relative efficiency with which the enzyme could use the sialylated acceptor α(2,3)sialyl-L-N-acetyllactosamine. Extracts from pcDNA1-Fuc-TVII-transfected COS-1 cells used α(2,3)sialyl-N-acetyllactosamine with a specific activity of 1048 pmol/mg/h. The fucosyltransferase activity in these same extracts consistently used the non-sialylated acceptor N-acetyllactosamine with slightly less efficiency (932 pmol/mg/h). Control extracts prepared from pcDNA1-transfected COS-1 cells transferred no detectable fucose to α(2,3)sialyl-N-acetyllactosamine. These results indicate that the genomic DNA fragment in pcDNA1-Fuc-TVII encodes an α(1,3)fucosyltransferase.

The acceptor substrate preferences exhibited by pcDNA1-Fuc-TVII-transfected COS-1 cells contrast with those described previously for the other cloned human α(1,3)fucosyltransferases. Fuc-TIII can use all of the five acceptors tested with high efficiency, although highest relative activity is found with the type I acceptor lacto-N-biose I (44, 47). Fuc-TVII utilizes N-acetyllactosamine, but not the low molecular weight acceptor substrates α(2,3)sialyl-N-acetyllactosamine, 2'-fucosyllactose, or lacto-N-biose I (45, 46). Both Fuc-TV and Fuc-TVII fucosylate N-acetyllactosamine and α(2,3)sialyl-N-acetyllactosamine with similarly high efficien-
cies. Fuc-TV, however, uses 2'-fucosylactose at approximately 42% of the efficiency with which it uses N-acetyllactosamine (Ref. 47). Fuc-TV also shows low but reproducible activity with lactose and lacto-N-biose I (approximately 10% of the activity demonstrated with N-acetyllactosamine, Ref. 47). By contrast, Fuc-TV shows no detectable activity with any of these other neutral type II or type I acceptors. Thus, these results indicate that Fuc-TV maintains catalytic properties that are similar to, but nonetheless distinct from, those exhibited by the other cloned α(1,3)fucosyltransferases.

To examine acceptor substrate specificities in vivo, COS-1 cells transfected with human fucosyltransferase vectors were analyzed by flow cytometry. COS-1 cells maintain substrate levels of GDP-fucose and glycosylated acceptors and can construct surface-localized α(1,3)- and α(1,4)-fucosylated oligosaccharides when transfected with fucosyltransferase expression vectors (23, 44, 45, 47). As shown in Fig. 3A, COS-1 cells transfected with pcDNA1-Fuc-TVI exhibit surface expression of the Lewis x and sialyl Lewis x determinants but do not express the Lewis a or sialyl Lewis a antigens. By contrast, COS-1 cells transfected with pcDNA1-Fuc-TIII stain with antibodies specific for the Lewis x, sialyl Lewis x, Lewis a, and sialyl Lewis a determinants (Fig. 3A and Ref. 44). The results obtained with pcDNA1-Fuc-TV also differ from those obtained when COS-1 cells are transfected with pcDNA1-Fuc-TIV, which constructs surface-localized Lewis x determinants when expressed in COS-1 cells, but does not determine expression of the sialyl Lewis x antigen (Fig. 3A and Ref. 45). Comparison of the flow cytometric profiles for pcDNA1-Fuc-TV and pcDNA1-Fuc-TIV in COS-1 cells shows that these two cloned fucosyltransferases are functionally more similar to each other than to either Fuc-TIII or Fuc-TV; both direct expression of Lewis x and sialyl Lewis x molecules, but not Lewis a or sialyl Lewis a determinants (Fig. 3A).

To further examine the cloned α(1,3)fucosyltransferases for their abilities to fucosylate terminal and internal N-acetyllactosamine residues, CHO cells containing the polyoma large T-antigen (CHO-T cells, see “Experimental Procedures” and Refs. 54 and 55) were transiently transfected with each expression plasmid. Unlike COS-1 cells, CHO cells are known to contain glycosylated type II precursors for the VIM-2 determinant (45, 53) but do not construct type I precursors (23, 30, 45). CHO cells also maintain type II precursors for the Lewis x and sialyl Lewis x epitopes. As shown in Fig. 3B, all four cloned fucosyltransferase sequences are capable of determining surface-localized Lewis x in CHO cells. Fuc-TIII, -IV, and -V, but not Fuc-TV, are able to construct the VIM-2 structure. In addition, plasmids pcDNA1-Fuc-TIII, -Fuc-TV, and -Fuc-TVI each direct expression of sialyl Lewis x and difucosylated sialyl Lewis x determinants, whereas pcDNA1-Fuc-TIV does not.

Thus, the pattern of sialylated lactosaminoglycan fucosylation shared by Fuc-TIII, -V, and -VI differs from that demonstrated by the less homologous Fuc-TV, which is unable to fucosylate the terminal N-acetyllactosamine on sialylated precursors regardless of the presence or absence of fucose on the internal N-acetyllactosamine residue. These results suggest a correlation between shared structural determinants in Fuc-TIII, -V, and -VI and their abilities to express sialyl Lewis x and difucosyl sialyl Lewis x moieties. Likewise, these results suggest a correlation between enzyme-specific peptide motifs and the enzymes' disparate abilities to determine expression of the VIM-2 epitope. Such differences in the ability to construct the VIM-2 determinant likely depend, at least in part, upon variance in the rates with which each enzyme can fucosylate internal and terminal N-acetyllactosamine residues found in α(2,3)-sialylated lactosaminoglycans and upon modification of such rates by prior fucosylation at alternate positions (differences in order of fucosylation, see “Discussion”).

The Fuc-TVI Gene Is Syntenic to the Homologous Fuc-TIII and Fuc-TV Genes on Chromosome 19—Biochemical and genetic analyses indicate that human α(1,3)fucosyltransferase activities can be assigned to loci on chromosomes 19 (43) and 11 (35, 41). We reported previously the molecular localization of Fuc-TIII and Fuc-TV to chromosome 19 and Fuc-TIV to chromosome 11 (47). To determine the chromosomal location of Fuc-TVI, PCR was used to analyze a series of somatic cell hybrid DNAs informative for each human chromosome (see “Experimental Procedures” and Ref. 47). Diagnostic fragment sizes for Fuc-TVI (1.2 kb) and confirming NcoI restriction digests (450- and 750-bp fragments, data not shown) were obtained only when using genomic DNA template from hybrids containing human chromosome 19 (cell lines 683, 750, 756, 860, 867, 1006, and 1099; Ref. 47). All other human chromosomes were excluded, since these fragments were not
detected with hybrid DNAs containing, in aggregate, every other human chromosome. These data assign the Fuc-TV1 locus to chromosome 19.

Two Distinct Classes of Human Genomic DNA Fragments Are Defined by Homology with the Catalytic Domains of Fuc-TIII and Fuc-TV1—Several human α(1,3)fucosyltransferase activities have been described (10, 35–43), suggesting the existence of an equivalent number of α(1,3)fucosyltransferase genes. We used Southern blot analyses and probes derived from each of the four cloned human α(1,3)fucosyltransferase genes to determine if these sequences fully represent all structurally similar human α(1,3)fucosyltransferase genes or if others remain to be isolated.

One pair of probes (catalytic domain probes, see "Experimental Procedures") was used to sample the genome for additional DNA sequences that cross-hybridize to the most highly conserved segments of the four α(1,3)fucosyltransferase genes. Another set of probes (gene-specific probes, see "Experimental Procedures") was used to assign specific genes to bands detected with the catalytic domain probes. Restriction enzymes used to digest the genomic DNA were chosen so that, for each known gene, a single restriction fragment would be identified by its gene-specific probe, allowing comparison with the catalytic domain probe analyses. Any band(s) detected with a catalytic domain probe, but not detected with any of the gene-specific probes, would represent new cross-hybridizing sequence(s) and would thus be candidate(s) for novel fucosyltransferase gene segment(s).

The DNA sequence of the Fuc-TIII catalytic domain probe is 96 and 95% identical, respectively, to corresponding positions in the Fuc-TV and Fuc-TV1 genes and is 62% identical to the corresponding segment in the Fuc-TV1 gene. This probe identifies several bands when used under conditions of decreased hybridization stringency (Fig. 4A). Regardless of the restriction enzyme used, however, each of the bands detected with the Fuc-TIII catalytic domain probe used at low stringency can be assigned to one of the four cloned α(1,3)fucosyltransferase genes, by comparison to the blots probed at high stringency with the four gene-specific probes (Fig. 4B).

The DNA sequence of the Fuc-TV1 catalytic domain probe is 73, 72, and 70% identical, respectively, to corresponding positions in the Fuc-TIII, -V, and -VI genes. Like the Fuc-TIII catalytic domain probe, this probe also identifies several bands when used under conditions of decreased hybridization stringency (compare Fig. 4, C and A). Most, but not all, of these fragments may be assigned to one of the four cloned α(1,3)fucosyltransferase genes. Specifically, this probe identifies several cross-hybridizing restriction fragments (Fig. 4C, asterisks) that do not correspond to bands detected with the gene specific probes (Fig. 4B). These fragments exhibit cross-hybridization intensities similar to those observed when the Fuc-TV1 gene is detected at low stringency with the Fuc-TIII probe (Fig. 4A) but are not detected with the Fuc-TIII catalytic domain probe using the same hybridization conditions (Fig. 4C). These results indicate that the Fuc-TV1 catalytic domain probe is detecting novel cross-hybridizing DNA sequences and suggest the possibility that these sequences may correspond to one or more additional, yet uncloned, fucosyltransferase genes, whose sequences may be more similar to Fuc-TV1 than to the chromosome 19-localized genes Fuc-TIII, -V, and -VI.

DISCUSSION

Subsets of α(1,3)- and α(1,4)-fucosylated cell surface oligosaccharides figure importantly in the inflammatory process in their roles as ligands for the selectin family of cell adhesion proteins (23–32). These molecules, and related ones, may also maintain other important functions, including ones critical to morphogenic processes during early mammalian embryogenesis (1–7). Such other postulated functions remain to be definitively demonstrated, however. Expression of these molecules is controlled in large measure by tissue-specific and developmentally regulated expression of α(1,3)fucosyltransferases (10, 33, 34). The number of these human enzymes and corresponding genes has not been defined. Similarly, the mechanisms that control their expression, and the functions of the oligosaccharides whose biosynthesis they determine are not completely understood. We have sought to study these issues by isolating and characterizing human α(1,3)fucosyltransferase genes. Previous reports have described three members of a structurally related α(1,3)fucosyltransferase gene family (26, 44–47). In this report, we describe the isolation and sequence of a fourth member of this family, its expression in mammalian cells, and an analysis of its functional and structural relationship to known and unknown sequences in the human genome.

This gene, termed Fuc-TV1, is most similar in its structure to the Fuc-TV gene. Both genes maintain intronless coding segments, whose nucleic acid sequences share 91% identity. Their respective protein products are also very similar, sharing 99% primary sequence identity. These enzymes are nearly identical in their COOH-terminal regions, and most sequence differences lie in a region between the enzymes' transmembrane segments and the NH2-terminal ends of their catalytic domains. This is a relationship we previously noted when comparing the sequences of Fuc-TIII and Fuc-TV (47), whose sequences also differ most in an analogously-positioned "hypervariable" region. The repetition of this relationship in a group of three distinct α(1,3)fucosyltransferases reinforces the concept (47) that residues within this hypervariable region play important roles in determining the efficiency with which particular α(1,3)fucosyltransferases use different acceptor substrates and, by inference, participate in binding interactions between the enzymes and acceptor molecules during fucosylation reactions.

The genes encoding Fuc-TV and Fuc-TV1 are localized to human chromosome 19, along with the Fuc-TIII gene. This latter gene also maintains an intronless coding region with a high degree of sequence identity to the coding portions of Fuc-TV and Fuc-TV1 (although these latter two sequences are more similar to each other than they are to Fuc-TIII). By contrast, the other known human α(1,3)fucosyltransferase gene (Fuc-TV1) is located on human chromosome 11 and is substantially less similar to the three chromosome 19-localized α(1,3)fucosyltransferase genes. These observations are consistent with a hypothesis that a relatively distant gene duplication event generated two distinct α(1,3)fucosyltransferase genes on two different chromosomes and that their sequences have diverged substantially in the interim. It seems probable that more recent duplicative events have yielded the family of structurally similar chromosome 19-localized α(1,3)fucosyltransferase genes, whose sequences have not yet diverged substantially. This notion is also consistent with analyses of pedigrees with individuals deficient in α(1,3)fucosyltransferase activities, wherein Lewis blood group-negative phenotype is genetically linked to plasma α(1,3)fucosyltransferase deficiency (38). It is also consistent with preliminary Southern blot analyses of the chromosome
19-localized α(1,3)fucosyltransferase genes, suggesting that these genes maintain close physical linkage. In this context, it will be interesting to define the genomic organization of these latter three genes to more precisely determine their physical relationships and to aid in the definition of the molecular basis for null alleles at these loci.

Important issues that remain to be addressed include defining the total number of human α(1,3)fucosyltransferase genes and assigning existing (and potential) α(1,3)fucosyltransferase genes to the described α(1,3)fucosyltransferase activities that have been described. Biochemical (37, 39, 40, 42, 70-74) and genetic (38) analyses suggest that at least five different α(1,3)fucosyltransferase activities may be found in human tissues or cells. It is difficult to directly compare many of these results, however, because of differences in assay conditions used or substrates tested, and because the activities assayed have variously been derived from tissue or cell homogenates or enzymes variably purified from tissues and fluids. Nonetheless, tentative molecular correlates for some of these enzyme activities may be proposed, based on acceptor substrate specificities of α(1,3)fucosyltransferase activities generated with cloned α(1,3)fucosyltransferase genes.

The Fuc-TIII gene has been proposed to correspond to the human Lewis blood group locus, based on its chromosomal assignment, and on the ability of its corresponding enzyme to exhibit both α(1,3)fucosyltransferase and α(1,4)fucosyltransferase activities (44, 71). Definitive confirmation of this assignment will require a demonstration of tight linkage between a molecular defect in this gene and null alleles at the Lewis blood group locus. This is especially true in light of recent evidence indicating that α(1,4)fucosyltransferase activity may be detected in some rare Lewis blood group-negative individuals (72).

The Fuc-TIV gene is known to correspond to an α(1,3)fucosyltransferase activity found in myeloid-lineage cells, based on acceptor substrate specificity and Northern blot analyses (26, 39, 45, 46). This gene is also transcribed in a wide variety of other tissues, however, as is the Fuc-TIII gene, and transcripts from both genes are detectable in some tissues. These latter observations imply that caution must be exercised when interpreting the results of α(1,3)fucosyltransferase assays on tissue extracts because of the very real possibility that the “activity” detected in a tissue may be a composite of multiple distinct α(1,3)fucosyltransferases.

Fig. 4. Southern blot analyses of human genomic DNA using α(1,3)fucosyltransferase catalytic domain and gene-specific probes. Human genomic DNA was digested with the restriction enzymes BamHI/EcoRI, EcoRV/EcoRI, or EarI, electrophoresed on agarose gels, and subjected to Southern blot analysis. The results shown are derived from a master gel and blot containing reiterated sets of the three digests (10 μg of digested DNA/lane). The master blot was subdivided into strips after transfer, and each component strip containing three digests was separately hybridized with gene-specific or catalytic domain probes. Hybridizations were performed in a described previously solution for at least 18 h. For hybridizations with catalytic domain probes, the temperature was maintained at 35 °C. Blots hybridized with gene-specific probes were maintained at 42 °C. Following hybridization, the blots were rinsed three times with room temperature 2× SSC, washed using the final conditions noted below, and subjected to autoradiography (see "Experimental Procedures"). Roman numeral labels denote genomic fragments corresponding to specific cloned α(1,3)fucosyltransferases. A, Fuc-TIII catalytic domain probe. The final wash was with 2× SSC, 1% sodium dodecyl sulfate, at 60 °C for 30 min. B, gene-specific probes. Fuc-TIII, final wash was 0.1× SSC, 0.1% sodium dodecyl sulfate, 65 °C for 45 min. Fuc-TIV, final wash was 0.5× SSC, 0.2% sodium dodecyl sulfate, 65 °C for 30 min. Fuc-TVI, final wash was 0.1× SSC, 0.1% sodium dodecyl sulfate, 65 °C for 45 min. C, Fuc-TIV catalytic domain probe. The first three separated lanes derive from a blot which was washed with 2× SSC, 1% sodium dodecyl sulfate, at 63 °C for 90 min. The block of juxtaposed lanes at the far right in C was washed identically except that the temperature was maintained at 60 °C. Asterisks mark fragments that do not correspond to previously cloned fucosyltransferase genes.

A. B. W. Weston, unpublished data.

Assignment of the Fuc-TV and Fuc-TVI gene products to specific previously described α(1,3)fucosyltransferase activities remains problematic, however. One prominent candidate is α(1,3)fucosyltransferase activity found in the plasma of all except rare individuals (39, 40). Preliminary analyses of the acceptor substrate specificity of this activity indicate that it does not correspond either to the Lewis blood group enzyme (Fuc-TIII) nor to the myeloid type enzyme (Fuc-TIV) (38–40). While a minor fraction of α(1,3)fucosyltransferase activity in human plasma appears to be derived from myeloid lineage cells (approximately 10%, Ref. 39), the cellular origin(s) of the remainder of the circulating α(1,3)fucosyltransferase activity are not yet well defined (39). Nonetheless, analysis of pedigrees with individuals deficient in this activity are consistent with the hypothesis that it corresponds to a single gene product (38, 40, 41). This activity has recently been purified to homogeneity (42), and its acceptor substrate specificity has been examined in detail. The plasma enzyme efficiently uses N-acetyllactosamine and α(2,3)sialylactosamine (apparent $K_m$ values of less than 1 nM, Ref. 42) but does not use lactose or 2'-fucosyllactose with significant efficiency (apparent $K_m$ values in excess of 60 and 26 mM, respectively, Ref. 42). This enzyme exhibits no detectable α(1,4)fucosyltransferase activity when assayed with lacto-N-biose I (42). As we have noted previously, these properties are similar, but not identical, to those maintained by Fuc-TV (47); this enzyme operates efficiently with both N-acetyllactosamine and α(2,3)sialylactosamine, but it can also utilize 2'-fucosyllactose to some extent. Perhaps most importantly, Fuc-TV can also use lacto-N-biose I at a low but significant rate (47).

By contrast, the properties of the purified plasma enzyme are in many ways more similar to those we describe here for Fuc-TVI. Specifically, Fuc-TV uses both N-acetyllactosamine and α(2,3)sialylactosamine with high efficiency but does not operate on 2'-fucosyllactose nor on lacto-N-biose I. Since nonidentical methods (44, 45, 47) and acceptors (39, 40, 42, 47) have been used to analyze the purified and nonpurified plasma enzyme and the recombinant Fuc-TV, direct comparison is difficult, and we therefore cannot yet be sure that Fuc-TVI in fact corresponds to the plasma enzyme.

It should also be noted that the activity of Fuc-TVI also resembles that of an α(1,3)fucosyltransferase in lung carcinoma cells (74, reviewed in Ref. 10). This latter enzyme maintains an acceptor substrate specificity that closely resembles that of the plasma enzyme, yet exhibits physical and affinity chromatographic properties distinct from the plasma α(1,3)fucosyltransferase (10). It is interesting to note that the lung enzyme does not form the VIM-2 determinant when tested in vitro with glycolipid acceptor substrates (74), presumably because this enzyme prefers to perform its initial fucosylation reaction using the terminal N-acetyllactosamine moiety on α(2,3)-sialylated polyacetylatedglycans. Thus, terminal fucosylation to form the sialyl Lewis x determinant occurs first and is followed by fucosylation of the internal N-acetyllactosamine to form difucosyl sialyl Lewis x. The reverse reaction, with the potential to form a singly internally fucosylated product corresponding to the VIM-2 moiety, does not occur to any significant degree. This result is analogous to the results with Fuc-TVI reported here, which also does not construct the VIM-2 molecule when expressed in transfected cells that maintain glycoprotein-based (53) VIM-2 precursors. In particular, Fuc-TVI yields high amounts of surface-localized mono- and di-fucosylated sialyl Lewis x determinants but little if any VIM-2 epitopes. These data suggest that Fuc-TVI maintains an ordered fucosylation preference analogous to that proposed for the lung α(1,3)fucosyltransferase. In any event, further detailed biochemical and molecular genetic studies will be required to confirm this prediction and to determine if the Fuc-TVI gene corresponds to the plasma and/or lung α(1,3)fucosyltransferase activities or to an enzyme expressed elsewhere.

It also remains possible that the plasma and lung α(1,3)fucosyltransferase activities, and those in other tissues, are encoded by other as yet uncharacterized human α(1,3)fucosyltransferase genes. Such genes may include the sequences identified by the Southern blot analyses reported here that were detected on blots probed at low hybridization stringency with the Fuc-TIV probe. Alternatively, α(1,3)fucosyltransferase genes may exist whose primary structures are distinct from the α(1,3)fucosyltransferases defined to date and which will require expression cloning approaches, or protein purification methods, for their isolation. Once isolated, this group of molecular reagents should prove useful as tools to define the tissue-specific expression patterns of α(1,3)fucosyltransferase genes, the mechanisms that regulate them, and ultimately, the functions of their oligosaccharide products. They should also prove useful in experiments designed to address the α(1,3)fucosyltransferase-based determinant that allow these enzymes to quantitatively distinguish among a variety of oligosaccharide acceptor substrates.

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Molecular Cloning of a Fourth Human Fucosyltransferase Gene


