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Molecular cloning, chromosomal assignment and tissue-specific expression of a murine α(1,2)fucosyltransferase expressed in thymic and epididymal epithelial cells

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Terminal Fucα(1-2)Galβ epitopes have been proposed to play significant roles in cell–cell interactions in development, cell adhesion, and malignant transformation. To begin to investigate the regulation and function of α(1-2)fucosylated epitopes in an animal model, we have isolated and characterized a mouse genomic DNA segment encoding a protein orthologous to the human *H* blood group locus α(1,2)fucosyltransferase (FUT1). This segment maintains an open reading frame encoding 376 amino acids sharing 75 % sequence identity with the enzyme encoded by human *FUT1*, and 55 % sequence identity with the enzyme encoded by the human *Secretor* blood group locus (*FUT2*). Expression of the open reading frame in COS-7 cells yields an α(1,2)fucosyltransferase activity with a K_m of 7.6 mM for phenyl-β-D-galactoside. Southern blotting and interspecific backcross analyses indicate that this murine locus represents a single copy sequence mapping to a novel locus 2.1 centimorgans from the *Klk1* locus, in a region of homology between mouse chromosome 7 and the human *FUT1* locus on the long arm of chromosome 19. Mouse *FUT1* yields a 2.8 kb mRNA transcript identifiable in many organs, including thymus, lung, stomach, pancreas, small intestine, colon, uterus and epididymis. Hybridization analyses *in situ* localize expression of FUT1 transcripts to thymic medullary and epididymal epithelial cells, implying that this gene determines the expression of cell surface Fucα(1-2)Galβ epitopes in these tissues.

INTRODUCTION

Fucosylated glycoconjugates have been implicated in multiple cell–cell interactions, including inflammation and cell trafficking, differentiation and development, and malignancy [1–3]. Fucose, in α(1-2) linkage to terminal β-galactosides, is a necessary component of the human ABO, H, Lewis b (Le^b) and Lewis y (Le^y) blood group antigens synthesized by the sequential action of glycosyltransferases [4]. In humans, the α(1,2)fucosyltransferase (α(1,2)FT) activities (GDP-L-fucose:β-D-galactosyl-R 2-α-L-fucosyltransferase, EC 2.4.1.69) that catalyse the synthesis of the Fucα(1,2)Galβ epitope, or H blood group antigen, are encoded by two closely linked α(1,2)FT genes corresponding to the *H* blood group locus (*FUT1*) and the *Secretor* (*Se*) blood group locus (*FUT2*) [5–8]. Both α(1,2)FTs are expressed in gastric mucosa and salivary glands, whereas the *H* locus also directs expression of the H antigen in the erythroid lineage, in epithelial cells of the epidermis, and in vascular endothelial cells (reviewed in [9]). By contrast, the *Se* locus is restricted in its expression to endoderm-derived epithelial of the stomach, intestine, and salivary glands [9,10].

In addition to an essential role in A and B blood group antigen expression, multiple other functions have been proposed for α(1,2)fucosylated epitopes. These include roles in intestinal maturation and host–microbe interactions [11,12], vaginal epithelial–microbe interactions [13], and mouse blastocyst–uterine epithelial adhesion [14]. Much of the evidence supporting these proposed roles is indirect, and is dependent, in part, on the patterns of H epitope expression developed through histochemical staining with lectins or antibodies that recognize the H epitope, together with limited evidence for inhibitory effects of exogenous H-containing oligosaccharides on adhesive processes in H-expressing tissues.

To begin to define the regulation and function of Fucα(1-2)Galβ epitopes in a genetically tractable mammalian model organism, we have isolated and characterized a murine genetic locus orthologous [15] to the human *H* α(1,2)FT locus. This murine locus encodes an α(1,2)FT with catalytic properties similar to the human FUT1, and maps to mouse chromosome 7 in a region of homology with the long arm of human chromosome 19 where the human *H* locus resides. Transcripts derived from murine *FUT1* are unexpectedly widespread in the mouse, accumulating in the thymus and reproductive tissues, in the lung and spleen, and in the gastrointestinal tract. Hybridization *in situ* localizes FUT1 transcripts to epithelial cells within the thymic medulla, and within the epididymis. Expression of this locus in these tissues and cell types implies a regulatory role for this α(1,2)FT gene in expression of terminal Fucα(1-2)Galβ epitopes by these tissues, and provides a framework for transgenic and gene-ablation strategies capable of defining the function of these epitopes in these locations.

MATERIALS AND METHODS

Library screening

Approx. 1.0×10^6 recombinant λ phage from a murine 3T3 cell genomic library (Stratagene, La Jolla, CA, U.S.A.) were screened

Abbreviations used: Le^b, Lewis b blood group antigen; Le^y, Lewis y blood group antigen; α(1,2)FT, α(1,2)fucosyltransferase; *FUT1*, α(1,2)fucosyltransferase *H* gene; *FUT2*, α(1,2)fucosyltransferase *Secretor* gene; GM1, monosialyl ganglioside 1.

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The nucleotide sequence of murine *FUT1* appears in the EMBL/GenBank Data Bank under accession number U90553.

by plaque hybridization with nitrocellulose filters (Schleicher and Schuell, Keene, NH, U.S.A.) [16]. Filters were prehybridized at 42 °C for 2 h in 50 % formamide, 5 × SSC (SSC = 0.15 M NaCl/0.015 M sodium citrate), 10 × Denhardt's solution and 0.1 % SDS, then sequentially hybridized for 16 h at 32 °C in prehybridization solution containing 10 % (w/v) dextran sulphate, 100 μg/ml denatured salmon sperm DNA, and [α-^{32}P]dCTP-labelled 1.2-kb *Hin*fI fragment of the human *FUT1* gene [17,18]. Filters were rinsed three times for 20 min each at room temperature in 2 × SSC and then once for 30 min at 55 °C in 2 × SSC containing 0.5 % (w/v) SDS, and were subjected to autoradiography. Fifteen independent hybridization-positive plaques were identified after two additional cycles of plaque hybridization. Phage DNAs were prepared from liquid lysates and were characterized as described below.

Classification of clones and sequence analysis

Southern-blot analysis was used to classify phage clones according to restriction pattern and strength of homology to the human 1.2-kb *Hin*fI probe (results not shown). Two *Eco*RI fragments (3.4 kb and 1.6 kb) and an overlapping 2.3 kb *Pst*I fragment from the hybridizing phage designated E45 were subcloned into the plasmid pTZ19R (Pharmacia Biotech, Piscataway, NJ, U.S.A.). Both strands of each fragment were sequenced using the dideoxy chain termination method using T7 DNA polymerase Sequenase (U.S. Biochemical, Cleveland, OH, U.S.A.), yielding a total of 6.9 kb of genomic sequence. DNA sequence alignments were performed with the sequence analysis software MacVector (Oxford Molecular Group, Oxford, U.K.). The MacVector scoring matrix for alignment was a DNA identity matrix containing a default deletion penalty of −12 and default gap penalty of −4 per base. The Kyte–Doolittle scale with a window of 7 residues was used for hydrophilicity analysis.

Hybridization probe generation by the PCR

The PCR was used to amplify various segments from α(1,2)FT clones as probes. In general, 50 ng aliquots of plasmid template were used in reactions with the GeneAmp kit (Perkin–Elmer, Foster City, CA, U.S.A.) or Expand PCR System (Boehringer Mannheim, Indianapolis, IN, U.S.A.). Twenty cycles were performed, consisting of denaturation at 94 °C, followed by annealing at 64 °C–72 °C and extension for 1–3 min at 72 °C, depending on the length of the probe. Reactions were checked by agarose-gel electrophoresis; PCR-generated probes were gel-purified before use.

COS-7 cell expression

An expression vector containing the open reading frame within phage E45 (pcDNAI-mα(1,2)FT) was constructed by cloning a 1.7 kb *Sac*I and *Eco*RV fragment from phage E45 between the *Sac*I and *Eco*RV sites of the mammalian expression vector pcDNAI (Invitrogen, San Diego, CA, U.S.A.). COS-7 cells were transfected with pcDNAI-mα(1,2)FT, or with control plasmids, using a DEAE–dextran procedure described previously [17,19]. Control plasmids included pcDNAI and pcDNAI containing a 3.2 kb *Xho*I fragment encoding the human H α(1,2)FT [termed pcDNAI-hα(1,2)FT] [5].

Antibodies

Mouse IgM anti-H, anti-A and anti-B monoclonal antibodies were purchased from Chembiomed Ltd. (Edmonton, Alberta, Canada). 1-12F (anti-sialyl Lewis x) mouse IgM monoclonal antibody was provided by Dr. Reiji Kannagi (Aichi Cancer Center, Nagoya, Japan). Fluorescein-conjugated goat anti-murine IgM was purchased from Sigma (St. Louis, MO, U.S.A.).

Flow-cytometry analysis

COS-7 cells, harvested 72 h after transfection, were stained with monoclonal antibodies diluted in staining media, using methods described previously [19,20]. Anti-A, -B and -H antibodies were used at 10 μg/ml. Anti-sialyl Lewis x antibody 1-12F was used at a dilution of 1:500 (12 μg/ml.). Cells were then stained with fluorescein-isothiocyanate-conjugated goat anti-murine IgM (40 μg/ml) and analysed on a Becton-Dickinson FACScan as described previously [19].

Enzymic Assays

Blood group A and B glycosyltransferases were assayed using extracts prepared from COS-7 cells transfected with pcDNAI-mα(1,2)FT, with pcDNAI-hα(1,2)FT or with plasmid DNA. Cell extracts were assayed as previously published for α(1,3)*N*-acetylgalactosaminyltransferase (blood group A) in a reaction mixture containing 0.05 M cacodylate buffer, pH 6.8, 25 mM $MnCl_2$, 0.15 M NaCl, 2 mM 2′-fucosyl-lactose and 0.5 mM [^{3}H]UDP-GalNAc (0.01 mCi) [21]. Blood group B α(1,3)galactosyltransferase assays contained 0.05 M imidazole buffer, pH 6.5, 25 mM $MnCl_2$, 0.15 M NaCl, 2 mM 2′-fucosyl-lactose and 0.5 mM [^{3}H]UDP-Gal (0.01 mCi) [22].

In experiments analysing α(1,2)FT activity, cell extracts containing 1 % (v/v) Triton X-100 and 10 % (v/v) glycerol were prepared from transfected COS-7 cells and activity was assayed under the following conditions [17,19]. Assay A contained 3 μM (30000 c.p.m.) GDP-[^{14}C]fucose, 1–50 mM phenyl-β-D-galactoside, 5 mM ATP, 10 mM L-fucose in 20 mM sodium phosphate buffer, pH 6.1, in a total volume of 20 μl. Control assays, with no added acceptor, were run in parallel under the same conditions. The amounts of added enzyme were adjusted to ensure that reactions were linear throughout the incubation period (2 h at 37 °C). Under these condition, less than 25 % of the substrate was converted during the incubation period. The reactions were stopped by the addition of ethanol (20 μl), followed by dilution with 1 ml of water. The solutions were then centrifuged at 15000 ***g*** for 5 min to remove precipitated protein and the supernatants were collected for further processing. Fucosylated product was isolated by reverse-phase chromatography on Sep-Pack C_{18}cartridges (Waters Corp., Milford, MA, U.S.A.) [17]. After washing with 6 ml of water, the product was eluted with 50 % acetonitrile and quantified by liquid scintillation spectroscopy.

Assay B, using free oligosaccharide acceptors, contained 3 μM (30000 c.p.m.) GDP-[^{14}C]fucose, 0.2–50 mM free oligosaccharide acceptor, 5 mM ATP, 10 mM L-fucose, 25 mM $MnCl_2$ and 25 mM sodium cacodylate, pH 6.1, in a total volume of 20 μl. Incubations were carried out at 37 °C for 1 h and were terminated by addition of ethanol (20 μl), followed by the addition of 560 μl of distilled water. After centrifugation, an aliquot (50 μl) of the supernatants was subjected to scintillation spectroscopy for determination of total radioactivity. Another aliquot (200 μl) was applied to a column containing Dowex 1X2-400, formate form [5,19]. The flow-through fraction and a subsequent water elution (2 ml) were collected and pooled and the amount of fucosylated product was estimated by scintillation spectroscopy. Control assays without acceptors were performed to correct for the presence of endogenous acceptor and the hydrolysis of GDP-[^{14}C]fucose. One unit of α(1,2)FT activity was defined as 1 pmol of product formed per hour.

Apparent K_m values for α(1,2)FT activity were calculated from Lineweaver–Burk plots of initial velocity for various oligosaccharide acceptors within the concentration range of 1.0–50.0 mM phenyl-β-D-galactoside, 0.5–50.0 mM *N*-acetyl-lactosamine, 0.2–50.0 mM lactose, or 0.2–50.0 mM of lacto-*N*-biose I. Apparent K_m values for GDP-fucose were calculated within the concentration range of 5–500 μM with a saturating concentration of phenyl-β-D-galactoside (25 mM).

Fucosyltransferase assays using lipid acceptors [monosialyl ganglioside 1 (GM1) and lactosylceramide] were carried out according to previously published procedures [23] in a 200 μl volume assay containing 0.3 mM acceptor, 0.1 mM GDP-[^{14}C]fucose, 1.0 mg/ml detergent (CF-54), 0.2 M cacodylate buffer, pH 6.5, 10 mM $MnCl_2$ and transfected COS-7 cell extracts (5 μg of protein). Blank incubations, omitting acceptors, were regularly run in parallel. After incubation at 37 °C for 1 h, the reaction mixtures were applied to Whatman 3MM chromatography paper and constituents were separated by descending chromatography in 1 % (w/v) tetraborate [23]. The radioactivity co-migrating with lipid standards was measured by liquid scintillation spectroscopy. The apparent K_m value for GDP-fucose was calculated within the concentration range of 5–500 μM with a saturating concentration of GM1 (300 μM). Characterization of the product obtained with the GM1 acceptor substrate was achieved by fractionation of the radiolabelled products by HPTLC in chloroform/methanol/0.02 % $CaCl_2$ in water (50:42:11, by vol.), followed by fluorography, according to Ghidoni et al. [24]. The product of the reactions containing GM1 acceptor substrate co-migrated with an authentic [^{3}H]fucosyl-GM1a standard and migrated faster than an authentic [^{3}H]GM1a standard, both generously provided by Dr. Marco Trinchera (Universita di Pavia, Pavia, Italy) (results not shown).

Southern blotting

Murine genomic DNA was prepared from C57BL/6 mouse liver, subjected to restriction digestion, fractionated by electrophoresis on 1.2 % (w/v) agarose gels and transferred to Hybond-N membranes (Amersham Life Sciences, Arlington Heights, IL, U.S.A.) as described previously [20,25]. Blots were probed with either the 1.2 kb *Hin*fI fragment of the human FUT1 [5,6], or a murine FUT1 gene-specific probe (probe A) generated by PCR amplification of cloned template DNA using the following primers: forward primer TTCCTGTCCTGAGCAGTCCTCC-TCACTCTCTGGGA; reverse primer GAATTCCCTACGAA-TCTGTTCCCGAAGATGATG (the locations are indicated on Figure 1B). This 352 bp mouse FUT1-specific probe was chosen in order to maintain less than 60 % similarity with other fucosyltransferase sequences or other sequences in the EMBL/GenBank Data Bank. Genomic DNA Southern-blot hybridizations were carried out in the presence of 50 % (w/v) formamide, 5 × SSC containing 0.2 % (w/v) SDS at 32 °C for 48 h. The blots were then washed three times in 2 × SSC containing 0.2 % (w/v) SDS at room temperature for 30 min each wash and subjected to autoradiography.

Chromosomal mapping of the mouse *FUT1* locus

The mouse chromosomal location of the *FUT1* locus was determined by the Jackson Laboratory interspecific backcross panel (C57BL/6JEi × SPRET/Ei)F1 × SPRET/Ei, known as the Jackson BSS cross [26]. *Pst*I digested DNAs from the 94 backcross animals of the Jackson BSS cross were hybridized with probe B (a 347 bp probe synthesized by PCR with forward primer: TCAGAGCCTGAGGAGTCC and reverse primer: TC-CTGGGGTGATTGTCCAAG) in Rapid-Hyb (Amersham) at 65 °C for 4 h, washed twice with 2 × SSC containing 0.2 % (w/v) SDS at room temperature for 15 min and then washed twice at 65 °C with 0.5 × SSC containing 0.2 % (w/v) SDS for 15 min. The allele pattern for the *FUT1* locus was compared with the 2300 other loci previously mapped in the Jackson BSS cross.

Northern-blot analysis

Total RNA was extracted from C57BL/6 mouse tissues by the guanidinium thiocyanate method (TRIzol Reagent, Gibco BRL). Poly(A)$^+$ RNA samples were prepared by oligo(dT) column chromatography, subjected to gel electrophoresis and transferred to Hybond-N as described previously [16]. Pre-hybridization and hybridization were performed at 42 °C in 50 % (w/v) formamide, 5 × SSC, 1 × PE [0.25 M Tris/HCl, pH 7.5/0.025 M EDTA/0.5 % (w/v) sodium pyrophosphate/5 % (w/v) SDS/1 % (w/v) poly(vinylpyrolidone)/1 % (w/v) Ficoll/1 % (w/v) BSA] and 150 mg/ml of sheared salmon sperm DNA [20]. Blots were hybridized for 24–48 h at 42 °C using probe B labelled with [α-^{32}P]dCTP to a specific radioactivity of 1×10^9–2×10^9 c.p.m./μg DNA using the Rediprime DNA labelling kit (Amersham). Blots were then washed twice in 2 × SSC containing 0.2 % (w/v) SDS at room temperature for 15 min, twice in 0.5 × SSC containing 0.2 % (w/v) SDS at 65 °C for 15 min and then subjected to autoradiography for up to 14 days at −70 °C. Blots were stripped with 50 % (w/v) formamide/0.5 × SSC at 65 °C for 1–2 h and then 0.5 × SSC at room temperature for 30 min, and were re-probed with a glyceraldehyde-3-phosphate dehydrogenase probe [27] to confirm that the poly(A)$^+$ RNA had been loaded and transferred equivalently amongst all lanes on the blot.

Hybridization analysis *in situ*

For hybridization analysis *in situ*, a murine FUT1 gene-specific probe containing T7 and Sp6 promoters (probe C, shown in Figure 1) was synthesized using the PCR. The forward primer with *Bam*HI site in bold and T7 core sequence underlined: C**GGATCC**<u>AATACGACTCACTATAGGGAGG</u>CCTGAAT GGAGGAAACTTCTTTCG; and reverse primer with *Xba*I site in bold and Sp6 core sequence underlined: GC**TCTAGA**G-<u>CCATACGATTTAGGTGACACTATAG</u>GCCAGCAATGT AGCATACTGCC. The resulting 228 bp PCR probe was subcloned into pCRII with the TA Cloning Kit (Invitrogen). The insert was re-excised with *Eco*RI, gel-purified, digested with either *Bam*HI or *Xba*I and then used as a template for single-strand RNA sense and antisense probe synthesis using a Riboprobe Systems kit (Promega, Madison, WI, U.S.A.) and [α-^{35}S]UTP (3000 Ci/mmol; Du Pont/NEN, Boston, MA). Hybridization *in situ* was performed as described previously on 10-μm frozen sections of mouse tissues [28]. After hybridization under glass cover-slips overnight, slides were washed under high stringency [twice with 50 % (w/v) formamide/2 × SSC/20 mM dithiothreitol at 65 °C for 30 min] then dipped in photographic NTB-2 emulsion (Kodak, Rochester, NY, U.S.A.) and exposed for 2–4 weeks. Slides were developed in D19 developer (Kodak), stained briefly in haematoxylin reagent (Sigma), and examined using dark-field microscopy.

RESULTS

Molecular cloning of a murine DNA segment homologous to the human *H* blood group α(1,2)FT *FUT1*

A hybridization screen of a murine genomic library with a human FUT1 probe yielded 15 independent hybridization-posi-

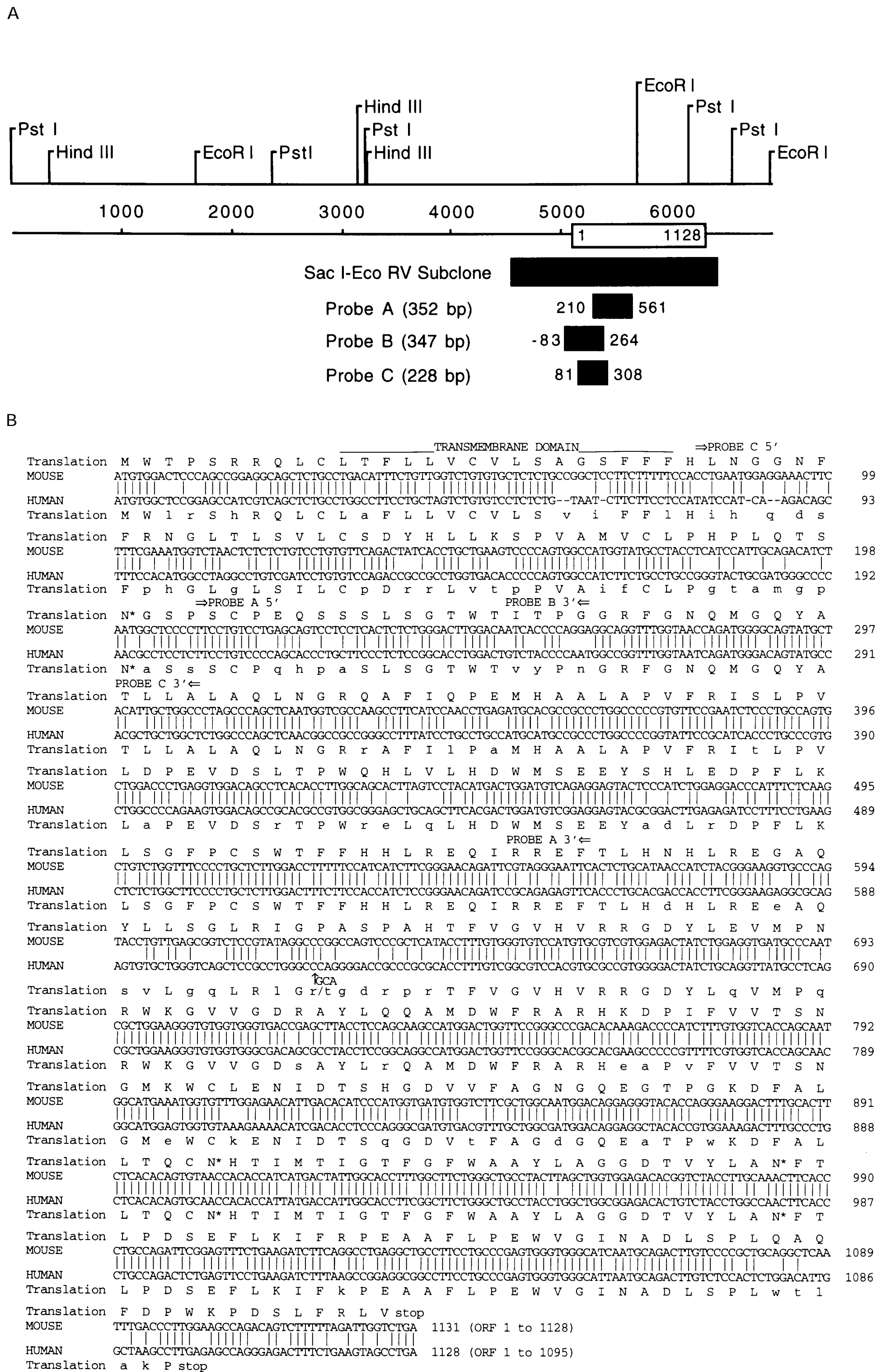

Figure 1 Restriction map and DNA sequence analysis of murine FUT1 α(1,2)FT

(**A**) Schematic diagram of the mouse *FUT1* locus. The open box indicates the position of the open reading frame sequence, which is detailed in (**B**). The position of restriction endonucleases used in Southern blotting and probes for Southern (probe A), Northern and chromosomal mapping (probe B) and hybridization *in situ* (probe C) analyses are shown as closed boxes. The small print adjacent to the closed boxes corresponding to each probe shows the base pairs of the end of each probe relative to the start codon of the open reading frame. The intron/exon borders are not known, however the open reading frame is believed to be encoded on a single exon whose 5′ border is 3 nt before the initiation ATG, analogous to human FUT1 [6]. (**B**) Comparison of DNA and derived amino-acid sequences of the mouse and human FUT1 open reading frames. Vertical lines denote identical nucleotides. Single letter designations for amino acids are shown above and below the DNA alignment. The putative transmembrane region is overlined. The 5′ and 3′ locations of probes described in the text are shown above the deduced amino-acid sequence. Potential Asn-linked glycosylation sites are indicated with an asterisk.

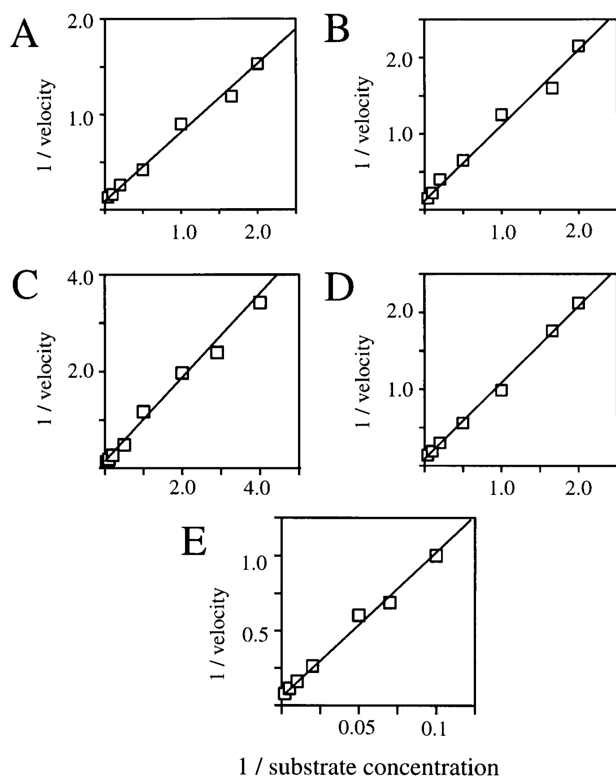

Figure 2 Fucosyltransferase activity of extracts from COS-7 cells transfected with a mouse FUT1 expression vector

Cell extracts containing 1% (v/v) Triton X-100 and 10% (v/v) glycerol were prepared from pcDNAI-mα(1,2)FT transfected COS-7 cells. Extracts were used in α(1,2)FT activity assays as described in the Materials and methods section, using various oligosaccharide acceptors: (**A**) phenyl-β-D-galactoside; (**B**) lactose (Galβ1-4Glc); (**C**) lacto-*N*-biose I (Galβ1-3GlcNAc); (**D**) *N*-acetyl-β-lactosamine (Galβ1-4GlcNAc); (**E**) GDP-fucose. One unit of activity is defined as 1 pmol of product formed/h. GDP-fucose K_m was determined in the presence of 25 mM phenyl-β-D-galactoside.

tive λ phage plaques. The phage designated E45 contained two strongly hybridizing *Eco*RI fragments, later found to be due to an internal *Eco*RI site within the open reading frame of a single gene. These fragments, and an overlapping *Pst*I fragment, were subcloned. Mapping and sequencing of these fragments yielded 6.9 kb of genomic sequence containing a single long open reading frame of 1128 nucleotides, encompassed by bp 5072–6202 (Figure 1A). DNA sequence comparisons between this open reading frame and previously documented α(1,2)FT loci revealed maximal sequence similarity with the human *FUT1* gene (Figure 1B). Conceptual translation of the open reading frame yields a 376 amino acid long polypeptide sequence that shares 75% overall amino acid sequence identity with the α(1,2)FT (FUT1) encoded by the human *H* blood group locus [5], and 96% identity within a 75 amino-acid-long region localized within the C-terminal, the presumed catalytic region of the enzyme. By contrast, the mouse polypeptide shares 55% amino acid sequence identity overall with the α(1,2)FT (FUT2) encoded by the human *Se* locus [8].

Hydropathy analysis (see the Materials and methods section) implies that this murine polypeptide maintains a type II transmembrane topology characteristic of other mammalian fucosyltransferases [29], reflected, in part, by a single 16-residue hydrophobic and presumably transmembrane segment that begins 10 residues from the N-terminus (Figure 1B). The initiation methionine codon predicted for this polypeptide is consistent with the Kozak rules for translation initiation [30]. The positions of three potential N-linked glycosylation sites are conserved between the mouse polypeptide and the human *H* blood group α(1,2)FT (Figure 1B). These considerations imply that this new mouse gene represents the orthologous homologue of the human *H* blood group α(1,2)FT FUT1.

Table 1 Oligosaccharide acceptor substrate specificity of recombinant mouse FUT1

Triton/glycerol extracts prepared from COS-7 cells transfected with pcDNAI-mα(1,2)FT were used in α(1,2)-FT activity assays as described in the Materials and methods section. The apparent K_m was determined by least-squares regression of inverse velocity versus inverse substrate concentration for each acceptor, as shown in Figure 2. *The K_m for GDP-fucose was determined with a saturating concentration of phenyl-β-D-galactoside (25 mM).

Acceptor	Apparent K_m (mM)
Phenyl-β-D-galactoside	7.6
Lactose (Galβ1-4Glc)	8.9
Lacto-*N*-biose (Galβ1-3Glc)	5.6
N-Acetyl-β-lactosamine (Galβ1-4GlcNAc)	14.3
GDP-fucose*	0.079

Expression of mouse FUT1 in COS-7 cells

To confirm that this murine sequence encodes a functional α(1,2)FT, a 1.7 kb *Sac*I–*Eco*RI fragment containing the open reading frame of the mouse FUT1 was cloned into the expression vector pcDNAI (see the Materials and methods section). The resulting plasmid [pcDNAI-mα(1,2)FT], or control plasmids [pcDNAI-hα(1,2)FT encoding the human H α(1,2)FT; pcDNAI negative control plasmid] were introduced by transfection into COS-7 cells, which are naturally deficient in endogenous α(1,2)FT activity [5]. The transfected cells were subsequently assayed for α(1,2)FT activity, and for vector-directed cell-surface expression of α(1-2)fucosylated antigens.

Extracts prepared from the transfected COS-7 cells were assayed for α(1,2)FT activity using several different oligosaccharide acceptor substrates previously employed to characterize the human H and Secretor enzymes [8]. Kinetic analysis yielded typical saturable, concentration-dependent Michaelis–Menten kinetics (results not shown). The apparent Michaelis–Menten constants for acceptor substrates were determined from least-squares regression of inverse velocity versus inverse substrate concentration for phenyl-β-D-galactoside, a substrate specific for α(1,2)FTs (Figure 2A), for lactose (Galβ1,4Glc) (Figure 2B), lacto-*N*-biose I (Galβ1-3GlcNAc) (Figure 2C), and N-acetyl-β-lactosamine (Galβ1-4GlcNAc) (Figure 2D), and for the donor substrate GDP-fucose in the presence of a saturating concentration of phenyl-β-D-galactoside (Figure 2E). The glycolipid lipid substrates GM1 and lactosylceramide were also tested as acceptor substrates in α(1,2)FT assays. These data are summarized in Tables 1 and 2. Use of the α(1,2)FT-specific acceptor substrate phenyl-β-D-galactoside demonstrates that the extracts contain α(1,2)FT activity, and indicate that the mouse sequence corresponds to an α(1,2)FT gene. Apparent K_ms obtained for each acceptor substrate (Table 1) fall within the ranges determined previously for these substrates when assayed with the human *H* and *Se* α(1,2)FTs [13], or obtained in assays using COS-7 extracts containing the human *H* α(1,2)FT with glycolipid acceptors (Table 2). These observations demonstrate that this murine enzyme can effectively utilize both type 1

Table 2 Glycolipid acceptor substrate specificity of recombinant human and mouse FUT1

Triton/glycerol extracts prepared from COS-7 cells transfected with pcDNAI-mα(1,2)FT (mouse FUT1) or with pcDNAI-hα(1,2)FT (human FUT1) were used in α(1,2)-FT activity assays as described in the Materials and methods section. The apparent K_m was determined by least-squares regression of inverse velocity versus inverse substrate concentration for each acceptor. *The K_m for GDP-fucose was determined with a saturating concentration of GM1 (300 μM).

	Apparent K_m (μM)	
Lipid acceptor	Human FUT1	Murine FUT1
GM1	82	110
Lactosylceramide	68	52
GDP-fucose*	70	16

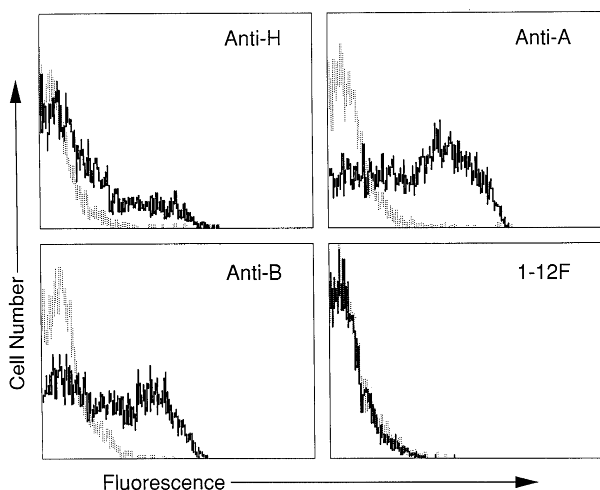

Figure 3 Flow-cytometry profiles of cell surface H, A, and B epitope expression by COS-7 cells transfected with a mouse FUT1 expression vector

COS-7 cells were transiently transfected with the expression vector pcDNAI-mα(1,2)FT, encoding mouse FUT1 (solid lines) or with the negative control vector pcDNAI (dotted lines). After a 72 h expression period, the cells were harvested, stained with IgM monoclonal antibodies to the H, A, B and sialyl Lewis x (1-12F) epitopes and analysed by flow cytometry as described in the Materials and methods section.

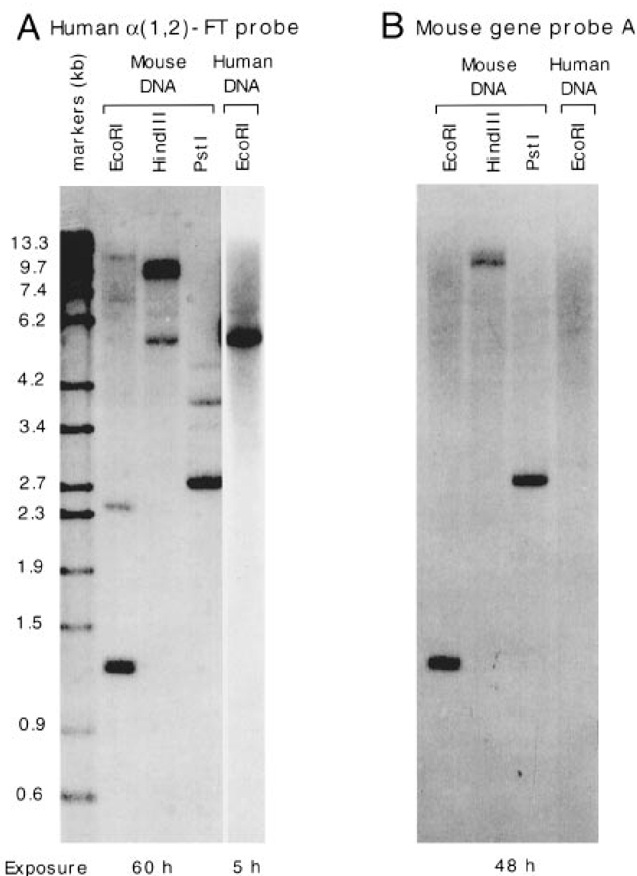

Figure 4 Southern-blot analysis of (A) human and (B) mouse genomic DNA with human and mouse FUT1 probes

Mouse and human genomic DNA were digested to completion with the indicated restriction enzymes and subjected to Southern-blot analysis (see the Materials and methods section). The autoradiogram shown in (**A**) was derived from the blot hybridized and washed at low stringency, using a *Hin*f I restriction fragment derived from the human α(1,2)FT gene. The same blot was stripped and re-hybridized at high stringency with probe A, derived from the 5′ end of the mouse gene (see Figure 1 and the Materials and methods section).

(Galβ1-3Glc) and type 2 (Galβ1-4Glc) soluble low-molecular-mass acceptors *in vitro*, and are largely consistent with previously published kinetic analyses for the human *H* locus (*FUT1*) [8].

Flow-cytometry analyses were used to determine if the α(1,2)FT activity detected *in vitro* was accompanied by construction of α(1-2)fucosylated glycans *in vivo*. Since COS-7 cells are known to express surface-localized type 2 oligosaccharide chains that are susceptible to modification by other transfected fucosyltransferases, we expected that expression of the mouse α(1,2)FT in COS-7 cells following transfection would yield cell surface H antigen expression. However, initial control experiments using an expression vector encoding the human *H* locus α(1,2)FT [plasmid pCDM7-α(1,2)FT] yielded low levels of cell surface blood group H antigen expression, when assayed using a monoclonal anti-H antibody, even though extracts prepared from the same plate of transfected cells contained substantial amounts of pCDM7-α(1,2)FT-derived α(1,2)FT (results not shown).

An apparent discordance between glycosyltransferase activity *in vitro* and accumulation of its carbohydrate product *in vivo* has been seen in the human colon where abundant blood-group-A-like α(1,3)*N*-acetylgalactosaminyltransferase activity was found, but no A antigen was detected due to a lack of α(1,2)FT activity [31]. In the above experiments, we considered the possibility that the inability to easily detect cell surface blood group H antigen expression was due to masking of these determinants by the subsequent action of blood-group-A-like α(1,3)*N*-acetylgalactosaminyltransferase and/or blood-group-B-like α(1,3)galactosyltransferase activities, endogenous to COS-7 cells and capable of converting the newly synthesized H determinants to blood group A or B determinants respectively [21]. Indeed, COS-7 cell extracts were found to contain blood-group-A-like and blood-group-B-like transferase activity, using the H-type acceptor 2′-fucosyl-lactose (see the Materials and methods section and [21]), at levels approximating to 10 and 27 pmol/h per mg of extract protein respectively. The glycosyltransferase activities are also operative *in vivo*, since flow cytometry analyses demonstrate that the expression vector pcDNAI-mα(1,2)FT, encoding the mouse α(1,2)FT, can direct surface-localized expression of significant

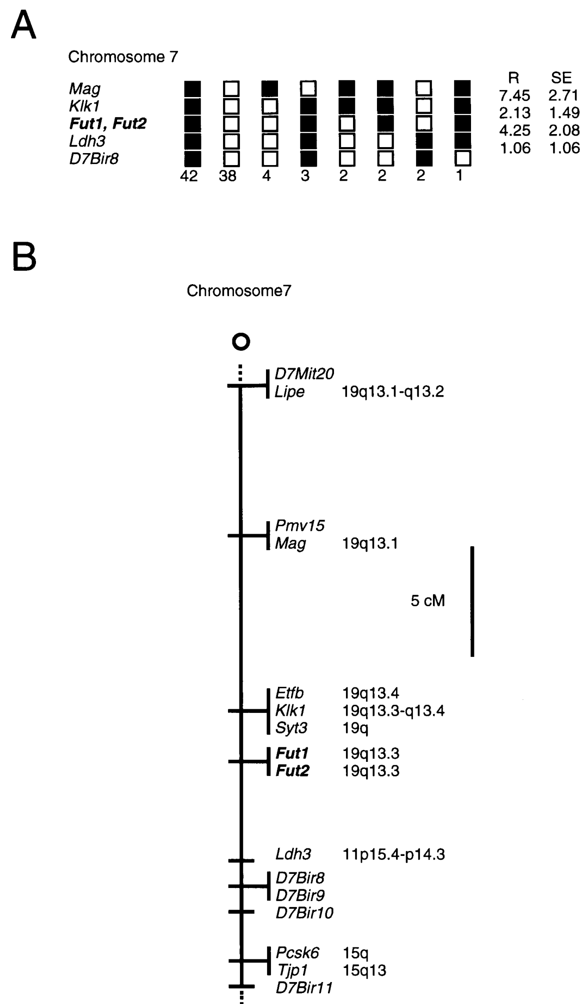

Figure 5 Chromosomal mapping of the mouse *FUT1* locus

(**A**) Haplotype distribution of 94 progeny of the Jackson BSS cross. The segregation patterns of *FUT1*, *FUT2* and flanking gene loci (Mag, myelin-associated glycoprotein; Klk1, kallikrein renal/pancreas/salivary; Ldh3, lactate dehydrogenase 3, C chain, sperm specific; D7Bir8, DNA segment, chromosome 7, Birkenmeier 8) are shown as heterozygous for C57BL/6JEi and SPRET/Ei alleles (filled boxes) or homozygous for the SPRET/Ei allele (open boxes). The number of progeny inheriting each type of chromosome is listed below the columns showing close linkage between these genetic loci. Recombination distances (R) and standard error (SE) in cM units between each genetic locus are listed to the right. (**B**) Genetic map of Chr 7 constructed from the haplotype distribution of the Jackson BSS cross. The map shows part of mouse chromosome 7 in relation to the *FUT1*/*FUT2* locus with the centromere oriented toward the top. Recombination distances are depicted to scale with a 5 cm (cM) scale bar at the right. Map positions of homologous human genes on human chromosome 19 are listed to the right of the locus name. Missing typings were inferred from surrounding data where assignment was unambiguous. Raw data from the Jackson Laboratory were obtained from the World Wide Web address http://www.jax.org/resources/documents/cmdata. Human homologues were obtained from The Mouse Genome Database at http://www.informatics.jax.org/homology.html

amounts of easily detectable blood group A and B determinants, with lesser amounts of H determinants that have presumably escaped modification and masking (Figure 3). Similar results were obtained with plasmid pCDM7-α(1,2)FT, encoding the human *H* locus α(1,2)FT (results not shown). By contrast, cells transfected with the negative-control vector pcDNAI did not express detectable amounts of A and B antigens (Figure 3). Considered together, these observations confirm the results obtained with the α(1,2)FT assays *in vitro*, and further demonstrate that this mouse sequence encodes an α(1,2)FT. These

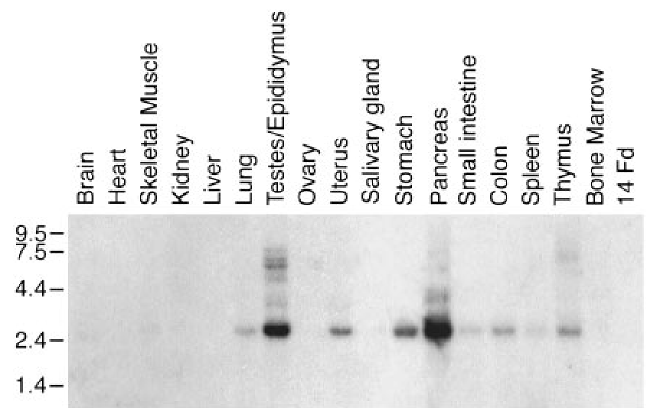

Figure 6 Northern-blot analysis of *FUT1* transcript expression in adult mouse tissues

Each lane contains 3 μg of poly(A)$^+$ mRNA isolated from the mouse tissues indicated above each lane. The Northern blot was prepared and hybridized as described in the Materials and methods section using probe B (see Figure 1). Transcript sizes may be estimated from the markers (kb) on the left. The Northern blot was re-probed for glyceraldehyde-3-phosphate dehydrogenase to confirm that the poly(A)$^+$ RNA was intact and that it had been loaded and transferred equally amongst all lanes on the blot (results not shown).

observations additionally demonstrate that COS-7 cells exhibit a glycosylation phenotype that is in some respects analogous to that of the 'Bombay', or non-secretor, blood group phenotypes [6,8], in that the cells are deficient in endogenous α(1,2)FT activity but can support blood group A and/or B antigen expression when genetically reconstituted with α(1,2)FT activity.

Chromosomal mapping of the murine *FUT1* locus

Southern-blot analysis of human and mouse genomic DNA was used to define the cross-hybridization patterns of the mouse *FUT1* locus and related sequences in the mouse genome, and to assist in efforts to assign the mouse *FUT1* locus to a specific mouse chromosome and to delineate the tissue-specific expression patterns of this locus by Northern blot and *in situ* hybridization analyses. Southern-blot analyses were completed using a hybridization probe (probe A) derived from the 5′ portion of the open reading frame at a region where the human *FUT1* and *FUT2* genes share less than 60% identity [5,7]. Low hybridization stringency Southern-blot analysis with a human FUT1 probe (Figure 4) reveals a single hybridizing band in human genomic DNA and four bands of varying intensity in murine genomic DNA (Figure 4A). Re-probing the same Southern blot at high stringency with the FUT1-specific mouse probe A disclosed single hybridizing bands in murine genomic DNA for each of three different restriction endonuclease digests (Figure 4B). The single bands correspond to the most strongly hybridizing bands observed with the human FUT1 probe, providing additional support for assigning this mouse sequence as mouse FUT1 (Figure 4). The simple pattern observed is consistent with the small sizes of the mouse and human *FUT1* genes.

A mouse chromosomal map analysis of the *FUT1* locus was completed to further confirm this gene as a homologue of human *FUT1*. A *Pst* I restriction site polymorphism in the murine *FUT1* locus between the mouse strain C57BL/6JEi (2.9 kb hybridizing band) and the strain SPRET/Ei (2.5 kb hybridizing band; results not shown) was used for this purpose. *Pst*I-digested DNAs from 94 backcrossed animals were hybridized with a FUT1 probe (probe B; see Figure 1). Comparisons of the allelic patterns for

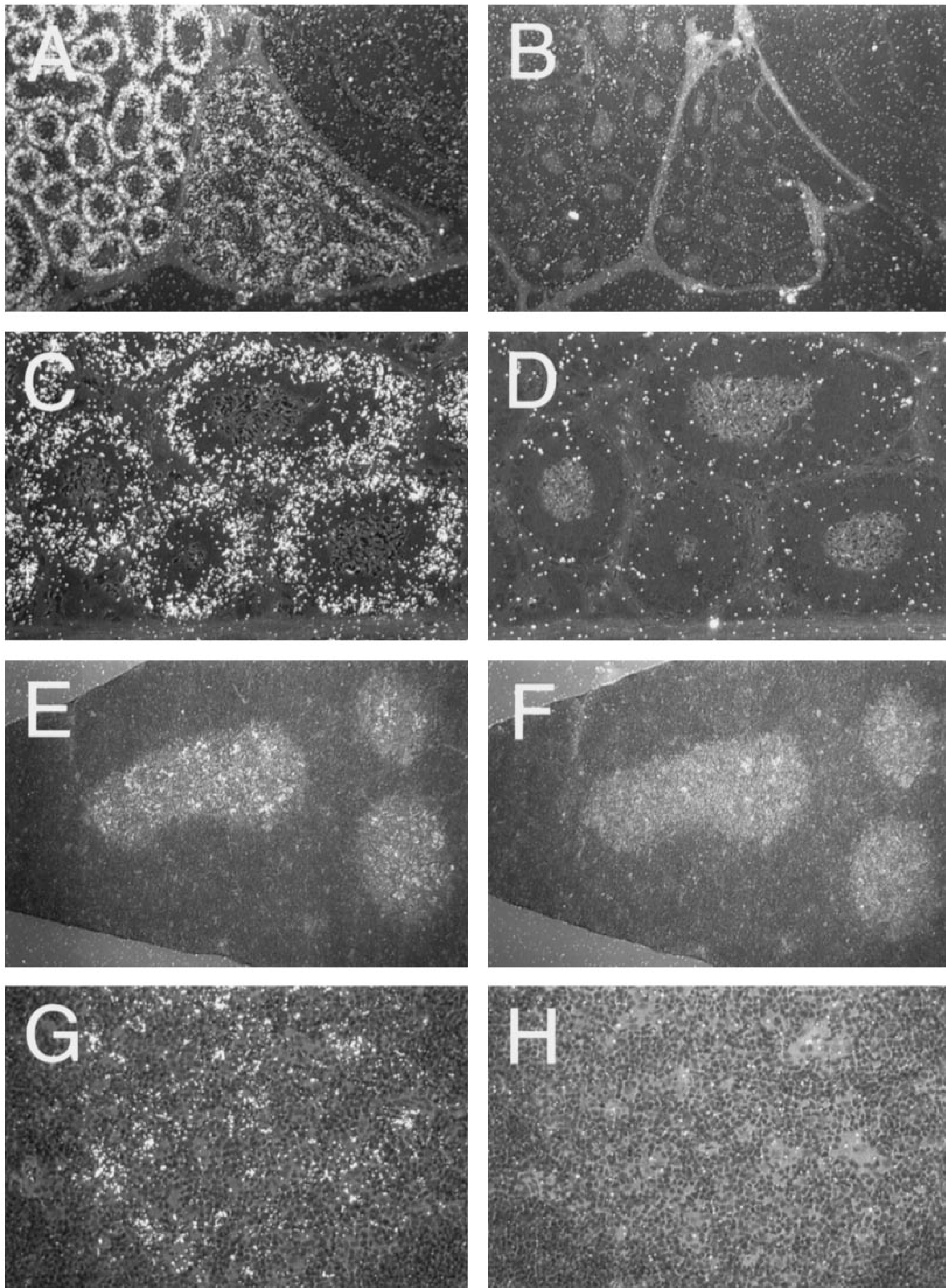

Figure 7 Cell-type expression of FUT1 mRNA transcripts in epithelial cells of thymus and epididymis by hybridization *in situ*

Antisense (**A**, **C**, **E**, **G**) and sense (**B**, **D**, **F**, **H**) probes corresponding to probe C (see Figure 1) were prepared and were hybridized to adjacent frozen sections as described in the Materials and methods section. Representative sections are shown of epididymis (**A**–**D**) and thymus (**E**–**H**) at $\times 40$ magnification (**A**, **B**, **E**, **F**) and $\times 250$ magnification (**C**, **D**, **G**, **H**).

FUT1 to the 2300 other loci previously mapped in the Jackson BSS cross revealed a genetic linkage profile that defined a new genetic locus 2.1 cM from the *Klk1* locus on chromosome 7 (Figure 5). These data confirm and extend the region of similarity between mouse chromosome 7 and the long arm of human chromosome 19. A second mouse fucosyltransferase gene, *FUT2* (S. E. Domino, N. Hiraiwa and J. B. Lowe, unpublished work), obtained in the hybridization screen of the murine phage library also displays a *Pst*I restriction polymorphism between mouse strains C57BL/6JEi (1.9 kb) and SPRET/Ei (5.5 kb) and shows linkage pattern identical to the *FUT1* locus. These data imply that the mouse *FUT1* and *FUT2* loci may maintain a close tandemly-linked physical relationship analogous to that observed for the human *FUT1* and *FUT2* loci at chromosome 19q13.3 [7].

Tissue-specific expression of murine *FUT1* transcripts

The tissue-specific expression pattern of murine *FUT1* transcripts was defined in adult tissues by Northern-blot analysis and explored in detail for selected tissues by hybridization *in situ*. Northern-blot analyses, using a probe from the 5′ region of the open reading frame (probe B), reveals a major transcript of 2.8 kb that is most abundant in pancreas and testes/epididymis, but that is also present in thymus, lung, stomach, small intestine, colon, spleen and uterus (Figure 6). In tissues where accumulation of the message was most abundant, additional fainter bands at higher molecular masses were seen (see testes/epididymis, pancreas and thymus). These may represent partially processed transcripts, variants derived from alternative transcription initiation and termination sites or alternative splicing events. FUT1 mRNA transcripts were not detected by Northern-blot analysis in adult mouse brain, heart, skeletal muscle, kidney, liver or bone marrow.

To delineate which cells within these tissues express FUT1, hybridization analysis *in situ* was performed on all tissues shown to be positive on Northern-blot analyses. *FUT1* transcripts were specifically detected in medullary epithelial cells of the thymus and in epithelial cells lining the epididymis (Figure 7). The thymic hybridization *in situ* pattern is most consistent with FUT1 expression in type-6 thymocytes, a specific histologically defined type of thymic epithelial cell exhibiting a patchy distribution in the thymic medulla, and of unknown function [32]. Although present in epididymis, transcripts were not detected within the testes proper (results not shown). Other tissues that were positive on Northern blots did not reveal specific hybridization patterns *in situ* (results not shown), perhaps due to technical limitations in preserving intact transcripts in tissues with abundant RNase levels (e.g. pancreas), or because levels of message in these tissues were below the level of detection by this *in situ* hybridization method.

DISCUSSION

To further define the enzymes and mechanisms that express cell surface murine Fucα(1-2)Galβ carbohydrate epitopes in mammals, we have isolated and characterized a murine gene corresponding to the human H blood group α(1,2)FT *FUT1*. The mouse gene maintains a high degree of sequence similarity to the human *FUT1* gene and maps to a region of mouse chromosome 7 that is homologous to human chromosome 19q13.3 occupied by the human *FUT1* locus. Furthermore, like the human H α(1,2)FT, the murine FUT1 enzyme utilizes type 1 [Galβ(1-3)Glc] and type 2 [Galβ(1-4)Glc] acceptors *in vitro* and elaborates H blood group epitopes when expressed in COS-7 cells. A survey of the tissue-specific expression pattern of this gene reveals an unexpectedly wide distribution, including expression in gastrointestinal tissues, male and female reproductive tract, lung, spleen and thymus. Whether this expression pattern is similar to that of human *FUT1* remains to be determined.

While our characterization of this murine gene was in progress, three independent laboratories have reported the use of PCR probes or cDNA fragments derived from the human *FUT1* gene to identify and characterize rat, rabbit and pig genes with sequence similarity to human *FUT1*. Two partial rat DNA clones, called *FTA* and *FTB*, share 92% and 65% sequence identity, respectively, with mouse *FUT1*. These rat sequences were found to be expressed differentially throughout the rat colon, as is mouse FUT1, though nothing further is known about the expression pattern of these rat genes, nor about the characteristics of the presumably corresponding enzymes [33]. Likewise, while the pig α(1,2)FT shares 72% sequence identity with mouse *FUT1*, the tissues where this pig locus is expressed have not been defined [34].

Three rabbit α(1,2)FT genes with sequence similarity to human *H* locus *FUT1* (*RFT-I*) and human *Se* locus *FUT2* (*RFT-II* and *RFT-III*) have been more completely studied than the rat and pig sequences [35,36]. RFT-I shares 75% amino acid sequence identity with mouse FUT1, whereas RFT-II and RFT-III share 53% and 57% identity with mouse FUT1 respectively. RFT-I is also most similar to human FUT1 [35,36], suggesting that RFT-I may represent the orthologous homologue [15] of human and mouse FUT1. Like murine FUT1, RFT-I exhibits a relatively low K_m for phenyl-β-D-galactoside, utilizes type 1, type 2 and glycolipid acceptors *in vitro*, and directs expression of blood group A epitopes when expressed in COS-7 cells. The tissue-specific expression pattern of RFT-I differs substantially from that exhibited by mouse FUT1, however, in that the rabbit sequence is restricted in its expression pattern to the cerebrum and cerebellum [35], whereas the mouse sequence is not expressed in these tissues but is widely transcribed in many others. It remains to be determined if these differences reflect species-specific differences in expression patterns of orthologous homologues, or whether rabbit RFT-I is a paralogous homologue [15] of human (and mouse) FUT1.

The function(s) of mammalian α(1-2)fucosylated glycans have not yet been illuminated by these early characterizations of these lower mammalian α(1,2)FT genes, nor by other studies of the human sequences. This is considered by us to represent the most important issue for future study with respect to these molecules. Possible functions for α(1-2)fucosylated glycans are implied by a rather wide variety of studies that have examined expression of α(1,2)FT activity, and/or α(1-2)fucosylated glycans, in specific biological contexts. For example, in the context of our observation that FUT1 transcripts localize to thymic medullary epithelial cells, it is interesting to note previous studies demonstrating [^{3}H]fucose labelling of medullary thymic epithelial cells in the rat [37] and histochemical identification of α(1-2)fucose epitopes in the medullary area of the murine thymus, using the α(1-2)fucose-specific lectin *Ulex europaeus* agglutinin-I [38]. These observations together imply that thymic epithelial cell expression of α(1-2)fucosylated epitopes is directed by the mouse *FUT1* locus. Nonetheless, a specific role for α(1-2)fucosylated epitopes in thymus is not yet known, even though carbohydrate–lectin interactions have been implicated in several immune-related activities, including thymic epithelial cell antigen capture by a receptor with homology with a mannose receptor (DEC-205; [39]), thymic T-lymphocyte apoptosis stimulated by a thymic stroma cell-surface lectin (galectin-1) [40], expression of a host-defence lectin (serum mannose binding protein) on immature thymocytes [41], and identification of a cell surface

natural killer cell lectin (Ly-49C) with proposed but as yet uncharacterized α(1-2), α(1-3), α(1-4) or α(1-6)fucosylated carbohydrate ligands [42].

Similar uncertainties regarding function exist with α(1-2)fucosylated epitopes and development of spermatozoa. Thus, although it has been observed that lactosaminoglycans synthesized by mouse male germ cells are fucosylated by a partially characterized epididymal fucosyltransferase [43] and that mouse caput spermatozoa contain significantly more fucosyltransferase activity than do spermatozoa from the cauda epididymis [44], the functional relevance of these observations is not yet apparent. Similarly, the developmental variation observed in the activities of spermatogenic and Sertoli cell α(1,2), α(1,3) and α(1,4)fucosyltransferases is without a functional correlate [45]. Uncertainty also exists with the functional relevance of expression of α(1-2)fucosylated epitopes such as Le^b, Le^y and H antigens or A and B antigens in other cell types, including the acinar epithelial cells of the pancreas [46] and pancreatic tumours [47]. This uncertainty extends to adenomas and adenocarcinomas in general, where expression of α(1-2)fucosylated epitopes is associated with tumour progression, with an implied though not yet demonstrated relationship with pathogenesis in malignancy [48,49].

In the context of tumorigenesis, it is important to note that the α(1-2)fucosylated ganglioside fucosyl-GM1 can be constructed by human and murine FUT1 and their rabbit homologues, and has been identified in the serum and on the tumour cells in patients with small-cell lung cancer [50]. Given the proposed neuro-ectodermal origin of some small-cell lung carcinomas [51], it is possible that this can be accounted for by observations that fucosyl-GM1 is a normal constituent of sensory nerves [52] and dorsal root ganglia in humans [53], and is regulated in its localization during the development of dorsal root ganglia and peripheral nerves in the rabbit [54,55]. These observations are consistent with the possibility that expression of fucosyl-GM1 in small-cell lung cancer may reflect the re-emergence of an embryonic gene expression programme, including α(1,2)FT gene expression, associated with malignant transformation in this disease. Again, however, the functional significance of these observations is not apparent. While it has been reported that axonal outgrowth of a cultured neuroblastoma cell line is suppressed by transfection with the rabbit fucosyltransferase RFT-I and that suppression is reversed by addition of inhibitors of fucosyl-GM1 expression [56], these observations have yet to be extended to an intact developing mammalian nervous system, and their physiological relevance remains to be definitively demonstrated. Similar considerations apply to inducible α(1,2)FT gene expression [57] and cognate expression of fucosyl-GM1 [58] upon conventionalization of ex-germ free mice.

Each of these will be important areas for future study and should be aided by the availability of cloned DNA segments encoding α(1,2)FTs, because these segments can facilitate specific genetic modulation of expression of α(1-2)fucosylated epitopes in an effort to assign function. Specifically, approaches using transgenic- and gene-ablation technologies in the mouse represent a powerful approach to explore the function(s) of glycosyltransferase genes and their cognate oligosaccharide products. For example, targeted deletion of the murine Fuc-TVII α(1,3)fucosyltransferase locus has shown that this enzyme participates in an essential way in L-, E- and P-selectin ligand biosynthesis, thereby controlling lymphocyte homing and blood leucocyte emigration [59]. Molecular cloning of murine α(1,2)FT genes and an examination of their expression patterns and cognate enzymic activities is an indispensable first step to similar approaches in assessing the function of these genes and cognate α(1-2)fucosylated oligosaccharides in a mammalian organism.

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