Defining the Functional Boundaries of the Murine α1,3-Fucosyltransferase Fut7 Reveals a Remarkably Compact Locus*

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The selectins are a family of three carbohydrate binding adhesion molecules that play critical roles in leukocyte traffic both during inflammation and under normal homeostasis. Work by numerous investigators has definitively shown that L-selectin is essential for normal lymphocyte recirculation by mediating binding of blood-borne lymphocytes to high endothelial cells (HEC)2 of postcapillary venules of secondary lymphoid organs (1–4). E- and P-selectin are crucial for homing of hematopoietic stem cells to their niches in bone marrow (5, 6) as well as migration of dendritic cells into skin (7). All three selectins participate in recruitment of neutrophils, monocytes, and dendritic and T cells to sites of inflammation (8–15).

The α1,3 fucosyltransferase FucT-VII, encoded by Fut7, is well established as critical for the biosynthesis of ligands for all three selectins on all cell types on which these ligands are expressed, including L-selectin ligands on HEC and E- and P-selectin ligands on all classes of leukocytes (16–18). Fut7 gene expression is found in all types of blood-borne mature myeloid cells, including monocytes, dendritic cells, and neutrophils, in activated but not naive T cells (19, 20), in IgG plasma cells (21), and in HEC of lymph nodes and Peyer’s patches (18), mirroring expression of selectin ligands on these cell types. Fut7 expression is not detected in normal non-malignant epidermal or mesenchymal cells or tissues. The restricted expression of Fut7 to cell types that express glycan ligands for one or more selectins and the absence in Fut7 null mice of any phenotypes unrelated to defects in selectin ligand expression strongly suggests that this enzyme is specialized for selectin ligand formation.

Despite the critical importance of FucT-VII, little is known regarding transcriptional regulation of Fut7. In murine CD4+ T cells, T-bet expression is essential for maximal, IL-12-driven expression of Fut7 (22). Experiments in Jurkat T cells indicated that Fut7 promoter activity is positively regulated by a complex of T-bet, Sp1, and p300 (23). Fut7 is also transcriptionally induced by the HTLV-1 tax protein (24) via a mechanism involving CREB (cAMP-response element (CRE)-binding protein)/ATF proteins, possibly CREB1 and/or CREMα, binding with tax and CBP (CRE-binding protein) to a CRE element, which is close to the T-bet and Sp1 binding sites (23, 25). In contrast, Gata3, whose expression is driven by IL-4, repressed Fut7 promoter activity via recruitment of HDACs and interference with T-bet binding (23), which may account for the ability

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2 The abbreviations used are: HEC, high endothelial cell(s); PEC, peritoneal exudate cell(s); Tg, transgenic; LN, lymph node; qPCR, quantitative PCR; CMFDA, 5-chloromethylfluorescein (CMF) diacetate; TCR, T cell receptor.
of IL-4 to inhibit Fut7 expression (26). However, the activities of these transcription factors cannot fully explain the pattern of expression of Fut7 even in T cells, and no transcription factors that control Fut7 expression in myeloid cells or in HEC have been identified. Moreover, no cis-acting genetic elements that govern Fut7 expression, apart from the promoter, have been characterized.

A detailed understanding of transcriptional mechanisms that govern Fut7 expression requires, among other things, comprehensive identification of cis-acting elements that are responsible for expression or repression of Fut7 in different cell types. The most stringent and definitive test to determine if all essential cis-acting regulatory regions of a gene are contained within a given sequence of genomic DNA is the ability of that DNA, in the form of a transgene (Tg), to restore physiologically accurate expression and appropriate function in mice in which harbor homozygous null mutations in the endogenous gene of interest. Therefore, a transgenic, gain-of-function, genetic complementation approach in mice was used to define the functional boundaries of the murine Fut7 locus; this primer pair gives a band of 434 bp only from Tg + mice but not WT or Fut7 null mice. The third primer pair (neo primers) is completely within the neo cassette originally used to disrupt the Fut7 locus to produce Fut7 null mice (16) and gives a 248-bp band from Tg + and Fut7 null mice but not from WT mice. All primer sequences for this entire study are given in Table 1, and their locations (for those that amplify Fut7 sequences) are depicted in Fig. 1. Tg + founders, which by definition are homozygous for the endogenous Fut7 null allele (because they were produced with fut7 null eggs), were mated with (non-Tg) Fut7 null mice, and Tg + pups were identified as above. Mice derived from two independently derived founders, designated SN1.1 and SN1.2, were produced and analyzed in this study.

Construction of the SN1 Tg.—The BAC clone RP23–273F21, which contains ~197 kb of genomic DNA from mouse chromosome 2 with the Fut7 locus approximately centered, was obtained from Invitrogen. A ~12.7-kb SpeI/NotI fragment (see Fig. 1) containing virtually all genomic sequence between the nearest upstream (Npdc1) and downstream (Abca2) loci was subcloned into the SpeI/NotI sites of pBS/SK (Promega, Madison, WI). The insert, designated SN1, was excised with Clal and SacII, gel-purified, and used for microinjection into Fut7 null fertilized eggs.

Flow Cytometry.—Expression of E- and P-selectin ligands was quantitated using supernatants of COS cells transfected with expression plasmids expressing E-selectin/IgM and P-selectin/IgM fusion proteins (16), exactly as described (19, 22), using Cy5- or AF647-coupled goat anti-human IgM (BIOSOURCE) IgM fusion proteins (16), exactly as described (19, 22), using Cy5- or AF647-coupled goat anti-human IgM (BIOSOURCE) as the second step. FITC- or phosphatidylethanolamine-conjugated anti-Gr1 or CD4 were obtained from ebioscience. Data were gathered on a FACS Calibur or FACS Canto (BD Biosciences) and analyzed by Flojo software.

WBC Counts and Differential.—Approximately 50 μl of blood was collected from the tail vein in EDTA-coated tubes and analyzed on a Hemavet 950 (Drew Scientific, Oxford, CT) complete blood counter.

Peritonitis Assay.—Mice were injected intraperitoneally with 1 ml of 4% thioglycollate in PBS. After 4 h, mice were sacrificed, and cells were collected from the peritoneum by thorough lavage after injection of ~10 ml of PBS/EDTA and counted. In
all cases, recovered cells consisted of ~90% neutrophils, as assessed by flow cytometry.

Genomic qPCR—DNA from tail snips for four mice of each genotype was analyzed by SYBR Green qPCR according to the manufacturer’s instructions using a Bio-Rad iQ5 thermal cycler and two different oligonucleotide pairs within the catalytic domain of Fut7. Oligonucleotides for pgk2 were used as the normalization controls. Gene copy number was calculated by the standard 2^−ΔΔCt method. Results were normalized by first standardizing the quantified PCR product from fut7-specific primers to that of the pgk2 gene for each individual mouse, deriving a mean ± S.D. for each genotype and primer and then normalizing the mean of each group for each primer pair to WT mice = 2. Results are reported as means ± S.D. for each primer set and each genotype.

qRT-PCR—Total RNA was isolated from various cells and tissues using TRIzol (Invitrogen). 1 μg of total RNA was treated with DNase (Roche Applied Science) for 1 h at 37 °C, precipitated, and reverse-transcribed using the SuperScript II kit (Invitrogen). Equal volumes of cDNA were then amplified using SYBR Green qPCR as above for genomic DNA. Oligonucleotides for Hprt were used to assess RNA and cDNA integrity and as normalization controls. Normalized fold values were calculated as above and were expressed relative to the mean for WT (for neutrophils) or WT without IL-12 or TGFβ1 (for activated CD4 cells).

End Point RT-PCR—RNA and cDNA from various tissues were prepared as above, and conventional end point PCR was carried out using standard conditions and primers for Fut7 and Hprt and cycle numbers that gave robust bands for the positive controls. The entire PCR reaction was run on standard 1% agarose gels and visualized with ethidium bromide.

T Cell Activation Cultures—CD4+ T cells were isolated from spleens of mice by positive selection using CD4 magnetic beads and columns (Miltenyi). T cells were activated by plate-bound anti-CD3/CD28 (eBioscience) for ~48 h either with or without recombinant IL-12 (Peprotech) or recombinant mouse L-selectin-human IgG chimera (R&D Systems) ± 10 mM EDTA in Block or with 1 μg/ml of MECA-79 (BioLegend) or rat IgM isotype control (eBioscience) in Block was applied to the sections for 1 h at room temperature. After washing in CMF, 1.5 μg/ml Cy3-conjugated goat anti-human IgG or 2 μg/ml goat anti-rat IgM (Jackson ImmunolResearch) in Block was added and incubated for 45 min at room temperature. The sections stained with the biotinylated secondary were washed in CMF, and 1.7 μg/ml Cy2-streptavidin (Jackson ImmunolResearch) in Block was added for 30 min at room temperature. Finally, all sections were washed in CMF, lightly counterstained with Harris hematoxylin (Sigma), and coverslipped with Fluoro-Gel (Electron Microscopy Sciences). Pictures were obtained with a Zeiss Axioscam attached to a Nikon Optiphot using Fluor 20× objective. The exposure time for bright field was 3 ms, for Cy2 was 800 ms, and for Cy3 was 5000 ms.

In Silico Analysis—Analysis of evolutionary conservation of Fut7 and surrounding regions was performed using the Vista Browser (available at genome.lbl.gov).

Statistical Analysis—All statistical analyses were carried out using the Mann-Whitney non-parametric test within the Prizm program. p values for pairwise comparisons are given in the figure legends.

RESULTS

Production of Mice Expressing the SN1 Tg—A 12.7-kb contiguous genomic segment flanked by locally unique SpeI and NotI restriction sites, encompassing nucleotides 25,271,404–25,284,140 (from the Vista Browser, mouse July 2007 build) on mouse chromosome 2, was subcloned into pBS from the BAC clone RP23-273F21. This DNA segment, which we have designated SN1, encompasses the entire transcriptional unit, including the major transcriptional start site, transcriptional termination site, and polyadenylation site along with ~7.4 kb of upstream sequence and ~2 kb of downstream sequence (Fig. 1) and represents essentially all of the sequence between the immediate upstream (Npdc1) and downstream (Abca2) genes. This genomic segment also contains a number of conserved noncoding sequences both upstream and downstream of the transcriptional unit (Fig. 1), suggesting their possible involvement in gene regulation.

The SN1 DNA was used as a Tg for pronuclear injection into fertilized eggs from Fut7 null mice. One founder was identified among pups born from each of two independent microinjections, and these two founder lines were designated SN1.1 and SN1.2. For both lines, ~50% of both male and female progeny were Tg+, indicating normal Mendelian inheritance. Analysis
of Tg copy number using qPCR with two independent primer sets within the single large exon, which encodes the glycosyltransferase domain, revealed that SN1.1 mice harbored ~3-fold higher gene copy numbers of the Tg compared with WT, whereas SN1.2 mice harbored ~2.5 copies fold higher gene copy numbers (Fig. 2). These Tg mice, therefore, harbor ~6 and ~5 copies of the SN1 Tg, respectively.

**Fut7 Gene Expression and Function in Neutrophils in Tg Mice**—Fut7 null mice display a spectrum of abnormalities in hematopoietic and lymphoid tissues. Within the myeloid compartment, these include a nearly complete loss of both E- and P-selectin ligands, significantly higher blood neutrophil counts, and sharply impaired neutrophil recruitment in acute peritonitis models (16). Expression of selectin ligands can be reliably quantitated on a per cell basis by flow cytometry with selectin-IgM chimeras (16, 19). We found that the SN1 Tg restored E- and P-selectin ligands to neutrophils in the blood (Fig. 3A) and bone marrow (data not shown). Levels of selectin ligands were slightly higher than WT on neutrophils from SN1.1 mice and slightly lower than WT on neutrophils from SN1.2 mice (Fig. 3A), and these levels of selectin ligands correlated roughly with Fut7 mRNA levels in these cells (Fig. 3B). Critically, the neutrophilia found in Fut7 null mice was eliminated in both Tg lines (Fig. 3C). Similarly, neutrophil recruitment into the peritoneum in response to thioglycollate was also restored to WT levels (Fig. 3D) and this level of Fut7 gene expression led to expression of functional selectin ligands on these cells.

**Expression of Fut7 in Activated T Cells**—CD4 T cells activated via the TCR and cultured with IL-2 alone show low levels of E- and P-selectin ligands, whereas CD4 T cells activated via the TCR and cultured with IL-2 plus either IL-12 or TGFβ1 show significantly higher levels of selectin ligands (19, 29). CD4 T cells from WT, Fut7 null, SN1.1, and SN1.2 mice were isolated, activated by plate-bound anti-CD3/CD28 mAb, and cultured with IL-2 alone, with IL-2 plus IL-12, or with IL-2 plus TGFβ1 to determine if the SN1 Tg could restore the ability of these cells to express Fut7 and selectin ligands in response to signals emanating from the TCR, IL-12 receptor, and/or TGFβ receptor. Cells were analyzed every 2 days to ensure that any shift in the kinetics of expression would be detected. We found that CD4 T cells from both the SN1.1 and SN1.2 mice expressed WT levels of E- and P-selectin ligands in response to activation and culture in IL-2 alone or with inclusion of either IL-12 or TGFβ1 (Fig. 4A). For each cytokine condition, levels of Fut7 mRNA were similar to WT in both SN1.1 and SN1.2 CD4 cells (Fig. 4B). These data show that the SN1 Tg contains all cis-acting elements essential for induction of Fut7 gene expression in response to signals originating from the TCR, IL-12 receptor, and TGFβ receptor.

To determine whether restoration of Fut7 expression was restored on activated CD4 T cells in vivo, we injected each genotype of mice intraperitoneally with brain lysate prepared from WT mice chronically infected with *T. gondii*, which triggers a robust Th1 response (27, 28), and analyzed induction of selectin ligands on activated (CD44-hi) splenic CD4 T cells 7 days after infection. Selectin ligands are induced predominantly on the activated (CD44-hi) subset of cells (Fig. 4C). Induction of E- and P-selectin ligands on SN1.1 and SN1.2 CD4 + CD44+ cells was

![FIGURE 1. Structure and conservation of the Fut7 locus and the SN1 Tg.](image)
equivalent to that of WT (Fig. 4D). To determine if recruitment of T cells to the peritoneum in response to infection with T. gondii was selectin-dependent, we analyzed CD4 T cell PEC at day 7 in WT and Fut7 null mice. These data showed a 90% reduction of accumulated CD4+ PEC in the Fut7 null mice (Fig. 4E), indicating a strong requirement for selectins in T cell migration to the peritoneum, paralleling that of neutrophils (9–12). We, therefore, analyzed recruitment of CD4 T cells to the peritoneum of SN1 Tg mice infected with T. gondii. We found that the numbers of CD4 T cell recruited to the peritoneum of SN1 Tg mice were equivalent to WT (Fig. 4E). Taken together, these data demonstrate that the SN1 Tg restored Fut7 expression and functional selectin ligands on activated CD4 T cells in response to infection with T. gondii.

Restoration of Fut7 Expression in HEC of Tg Mice—Lymph nodes from Fut7 null mice display significantly lower total lymphocyte numbers compared with WT, concomitant with immunohistochemically undetectable L-selectin ligands, and support significantly lower levels of lymphocyte homing (16). We examined each of these aspects of Fut7 expression in our SN1 mice. Total cell numbers in peripheral LNs were restored to WT levels in both SN1.1 and SN1.2 mice (Fig. 5A), and these LNs contained normal ratios of T and B cells (data not shown). HEC in SN1 mice were fully able to support short term homing of CMFDA-labeled lymphocytes (Fig. 5B), consistent with restoration of immunohistochemically detectable L-selectin ligands on HEC (Fig. 5C). Direct analysis of Fut7 mRNA levels in HEC was not possible due to the inability to purify sufficient numbers of HEC but was clearly sufficient to restore expression and function of L-selectin ligands on these cells. These data demonstrate that physiologic levels of Fut7 gene expression are restored to HEC in SN1 Tg mice.

Absence of Fut7 Expression in Inappropriate Tissues in Tg Mice—A striking aspect of Fut7 expression is its highly restricted pattern of expression, which is in contrast to most other glycosyltransferases, including those involved in selectin ligand biosynthesis. Therefore, to determine whether any silencers or other regulatory elements essential to tissue-specific expression of Fut7 were absent from the SN1 Tg, we analyzed Fut7 mRNA expression in a panel of non-hemato-

**FIGURE 3.** The SN1 Tg restores Fut7 and functional selectin ligand expression in neutrophils. A, flow cytometry analysis of E- and P-selectin ligand expression on blood neutrophils. Neutrophils were identified by characteristic forward and side scatter properties (gating not shown). Total blood cells were stained with anti-Gr-1, which identifies neutrophils, and E- or P-selectin/IGM chimera. B, qRT-PCR of Fut7 mRNA levels in neutrophils. For each mouse, results were first normalized to hprt for that mouse and then to the WT (=100%). One of two similar experiments is shown. C, absolute neutrophil numbers in peripheral blood of each genotype of mice. Each symbol represents a single mouse. D, total PEC recovered from the peritoneum of mice 4 h after intraperitoneal injection of 4% thioglycollate. Depicted is the mean ± S.D. for n = 12–16 for each genotype. For C and D: *, different (p < 0.01) from all other groups; **, no difference between groups.
poietic tissues that do not normally express Fut7. The results showed that Fut7 mRNA was undetectable in any of the tissues examined, including brain, lung, liver, kidney, skeletal muscle, and small intestine (Fig. 6). Fut7 expression is, therefore, absent from inappropriate cell types in these Tg mice. Taken together with the data presented above, the lack of detectable Fut7 expression in cell types that do not normally express this gene demonstrates that all essential cis-acting genetic elements involved in Fut7 gene expression, both positive and negative, are contained within the 12.7-kb SN1 Tg.
DISCUSSION

Complex and highly restricted patterns of gene expression are determined in part by an array of functionally distinct cis-acting genetic elements, including enhancers, silencers, locus control regions, and insulators. An essential prerequisite for comprehensive and unbiased identification of cis-acting elements that control expression of any particular gene is the delineation of the functional boundaries of the locus under study, defined as any contiguous stretch of genomic DNA that contains all cis-acting genetic elements essential for accurate physiologic expression. Here, we have used a genetic complementation/gain-of-function approach in Tg mice to define the functional boundaries of the murine Fut7 locus. We found that a 12.7-kb contiguous genomic region, which lies completely between the flanking genes Npdc1 and Abca2, fully restores normal levels of Fut7 expression and selectin ligands in neutrophils, activated CD4 T cells, and HEC when analyzed as a Tg on a Fut7 null background. In addition, no aberrant expression of Fut7 was observed in a panel of tissues that do not normally express this gene. These findings, obtained in mice derived from two independently generated Tg founder mice, convincingly demonstrate that this 12.7-kb Tg incorporates all essential cis-acting genetic elements required for accurate physiologic expression of Fut7.

This 12.7-kb genomic segment, therefore, encompasses genetic elements required to respond to an array of distinct signals in diverse cell types. In HEC, Fut7 expression may be controlled by dendritic cell-triggered LTβR signaling (30),
which classically involves NF-κB and TRAF (TNR receptor-associated factor) adaptor proteins (31, 32). In myeloid cells, G-CSF is capable of inducing E-selectin ligands on mobilized peripheral blood progenitors (33), but no signaling pathways or transcription factors that control Fut7 expression or selectin ligand expression in response to G-CSF or other cytokines involved in myelopoiesis have thus far to our knowledge been identified. In CD4 T cells, signals initiated by the TCR evoke modest levels of Fut7 expression and selectin ligands, possibly mediated by Ras (34, 35), and these lower levels of Fut7 and selectin ligands are significantly enhanced by IL-12 or TGFβ1 (19, 29). The signaling and transcriptional pathways downstream of the TCR, IL-12 receptor, and TGFβ receptor, which are responsible for induction and/or up-regulation of Fut7 and selectin ligands, remain incompletely characterized. We previously uncovered a requirement for the Th1 transcription factor T-bet in IL-12-driven Fut7 and selectin ligand expression (22) and a requirement for p38 MAPK activity in TGFβ1-induced Fut7 and selectin ligand expression (29). Whether T-bet and/or p38 MAPK directly or indirectly control Fut7 expression and selectin ligand expression remains unclear. Despite this limited information, it nonetheless seems likely that T lymphocytes, myeloid cells, and HEC use distinct transcriptional mechanisms for induction or maintenance of Fut7 gene expression. Our results show that all cis-acting genetic elements required for each of these signals to act are present within the 12.7-kb SN1 Tg. Thus, complex inputs from multiple distinct pathways converge in a relatively small genomic space.

Expression of Fut7 is not detected in a wide variety of cells and tissues that do not express selectin ligands. Crucially, we also found that Fut7 expression in our Tg mice was undetectable in tissues that do not normally express Fut7. The absence of Fut7 expression in epithelial and mesenchymal tissues could be due to the absence of appropriate transcription factors, the presence of negative cis-acting genetic elements, silencing epigenetic chromatin modifications, or a combination of these. Our results indicate that if any negative regulatory elements such as silencers, boundary elements, or insulators are required to maintain the absence of Fut7 expression in epithelial and mesenchymal tissues, these regulatory elements must also be present in the 12.7-kb SN1 Tg.

As analyzed using the Vista Browser, the Fut7 locus is nestled within a large, evolutionarily conserved array of genes whose pattern of expression is quite distinct both from that of Fut7 and from each other. In particular, the presence, order, sequence, genomic spacing, and overall arrangement of all of the genes surrounding Fut7 are evolutionarily highly conserved among human, dog, horse, and mouse for at least 466 kb upstream and 278 kb downstream of Fut7, indicating strong evolutionary pressure to maintain this higher order genomic arrangement. This large region contains at least 46 genes, each of which is found and conserved in its exon/intron organization in all four of the species examined. This finding makes it likely that mechanisms to prevent inappropriate expression or silencing of neighboring loci, including insulators and boundary elements, are also conserved within this overall genomic arrangement and is consistent with our results in this report that no essential cis-acting elements required for physiologically accurate Fut7 expression lie within the boundaries of the upstream or downstream genes.

Other investigators have carried out similar experiments to define the functional boundaries of other mammalian genes. Engel and co-workers (37) have shown that the minimal functional boundaries of the Gata2 gene required for mouse viability and normal development was 413 kb (36). Lichtenheld and co-workers (37) identified an ~150 kb interval of the human Prf1 gene that conferred accurate lineage- and stage-specific expression of perforin in primary cytotoxic T and NK cells. Weaver and co-workers (38) utilized an ~160-kb “BAC-in” reporter gene strategy to delineate Ifng expression in Tg mice and achieved faithful expression of the reporter in T cells (i.e. expression of the reporter gene exclusively in cells that also expressed the endogenous Ifng gene). Each of these studies identified a genomic interval of at least 150 kb that conferred physiologically accurate expression. In contrast, the current study shows that physiologically accurate expression of Fut7 required a much smaller genomic region.

The comparatively small size of the Fut7 locus, coupled to its sharply restricted expression, should facilitate studies aimed at exploring how distinct classes of cis-acting genetic elements function to control Fut7 gene expression in distinct cell types and in response to distinct developmental signals. The work presented here, therefore, provides a platform for mechanistic studies of cis-acting genetic elements that control leukocyte traffic and suggests that further studies of this gene could yield insights into fundamental questions of gene regulation.

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