Interaction of GATA-3/T-bet transcription factors regulates expression of sialyl Lewis X homing receptors on Th1/Th2 lymphocytes

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Communicated by Sen-itiroh Hakomori, Pacific Northwest Research Institute and University of Washington, Seattle, WA, September 11, 2006 (received for review April 12, 2006)

Selectin-dependent cell adhesion mediates inflammatory extravasation and routine homing of lymphocytes. Most resting peripheral T lymphocytes lack expression of sialyl Lewis X, the carbohydrate ligand for selectins, and are induced to strongly express it upon activation. T helper 1 (Th1) cells are known to more preferentially express sialyl Lewis X as compared with T helper 2 (Th2) cells upon activation. The molecular basis for this preferential expression, however, has not been elucidated to date. Here we show that the gene for fucosyltransferase VII (*FUT7*), the rate-limiting enzyme for sialyl Lewis X synthesis, is a unique example of the human genes with binding sites for both GATA-3 and T-bet, two opposing factors for Th1 and Th2 development, and is regulated transcriptionally by a balance of the two interacting transcription factors. T-bet promotes and GATA-3 represses *FUT7* transcription. Our results indicated that T-bet interferes with the binding of GATA-3 to its target DNA, and also that GATA-3 significantly interferes with the binding of T-bet to the *FUT7* promoter. T-bet has a binding ability to GATA-3, CBP/P300, and Sp1 to form a transcription factor complex, and GATA-3 regulates *FUT7* transcription by phosphorylation-dependently recruiting histone deacetylase (HDAC)-3/HDAC-5 and by competing with CBP/P300 in binding to the N terminus of T-bet. Suppression of GATA-3 activity by dominant-negative GATA-3 or repressor of GATA (ROG) was necessary to attain a maximum expression of *FUT7* and sialyl Lewis X in human T lymphoid cells. These results indicate that the GATA-3/T-bet transcription factor complex regulates the cell-lineage-specific expression of the lymphocyte homing receptors.

fucosyltransferase | helper memory T cells | lymphocyte homing | selectin | GATA-3/HDAC-3 interaction

Selectin-mediated cell adhesion figures heavily in the regulation of lymphocyte homing and inflammatory recruitment of leukocytes (1–3). Sialyl Lewis X determinant, the specific carbohydrate ligand for selectins, is constitutively expressed on granulocytes and monocytes and mediates inflammatory extravasation of these cells (4–6). Most resting peripheral T and B lymphocytes, on the other hand, lack its expression, and are induced to strongly express sialyl Lewis X upon activation (7, 8). Among T lymphocytes, the sialyl Lewis X determinant is known to be preferentially expressed on activated Th1 cells but not on Th2 cells (9).

The induction of sialyl Lewis X on lymphocytes accompanies transcriptional activation of fucosyltransferase VII gene (*FUT7*), which is involved in the last step of sialyl Lewis X synthesis (8, 10–15). Previously, we have shown that the human *FUT7* promoter is equipped with binding sites for the cAMP response element (CRE)-binding protein (CREB)/activating transcription factor (ATF) family, myeloid zinc finger protein 1 (MZF-1) and Sp1 (16, 17), and also GATA and T-bet (3). In this study, we attempted to clarify how *FUT7* transcription and sialyl Lewis X expression among human leukocyte subpopulations are regulated by these transcription factors.

Results

Role of GATA-3 in FUT7 Transcription. Because MZF-1 is expressed preferentially in myelomonocytic lineage (18), and GATA-3 is known to be specific to lymphoid lineage cells (19–21), we first studied the roles of MZF-1 and GATA-3 in *FUT7* transcription. In the reporter assay using the *FUT7* promoter, the introduction of mutation in the MZF-1 binding site abrogated the transcriptional activity, whereas transfection of the MZF-1 expression vector showed enhanced transcription in the HL-60 cell line, which belongs to myelomonocytic lineage (Fig. 1*A*). Introduction of mutation in the GATA binding site showed only a minimal reduction of transcriptional activity in HL-60 cell line.

On the other hand, the same mutation of the GATA binding site resulted in an apparently enhanced transcription in lymphoid cells such as Jurkat cells (Fig. 1*B*). This effect of the GATA binding site suggested that endogenous GATA binding factor(s) in Jurkat cells inhibit(s) *FUT7* transcription. In support of this, cotransfection of GATA family transcription factors in the reporter assays indicated that only GATA-3 suppresses the transcription, whereas GATA-1 and GATA-2, which are expressed on granulocytic cell lineage, do not (Fig. 5, which is published as supporting information on the PNAS web site).

In Jurkat cells, transcription of *FUT7* also was enhanced by the addition of a histone deacetylase (HDAC) inhibitor, trichostatin A (Fig. 1*B*). The finding that the effect of GATA mutation and trichostatin A was not additive suggested the possibility that the recruitment of HDAC by GATA-3 could be the mechanism for the suppression of the *FUT7* transcription. Immunoprecipitation of nuclear extracts of Jurkat cells with antibodies directed to isoenzymes of HDAC indicated that endogenous GATA-3 interacts specifically with HDAC-3 and HDAC-5 (Fig. 1*C*).

This specific interaction was further ascertained by immunoprecipitation of nuclear extracts of COS7 cells transfected with flag-tagged HDAC-3 and myc-tagged GATA-3 (Fig. 6*A*, which is published as supporting information on the PNAS web site). Experiments using truncated GATA-3 showed that GATA-3 binds to HDAC-3 through the zinc finger region (Fig. 6*B*). The

Author contributions: R.K. designed research; G.-Y.C. performed research; H.O. and L.F.S.-B. contributed new reagents/analytic tools; and G.-Y.C. and R.K. wrote the paper.

The authors declare no conflict of interest.

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Abbreviations: Th1, T helper 1; Th2, T helper 2; HDAC, histone deacetylase; ROG, repressor of GATA; CRE, cAMP response element; CREB, CRE-binding protein; MZF-1, myeloid zinc finger protein 1; NK, natural killer; TPA, phorbol 12-tetradecanoate 13-acetate.

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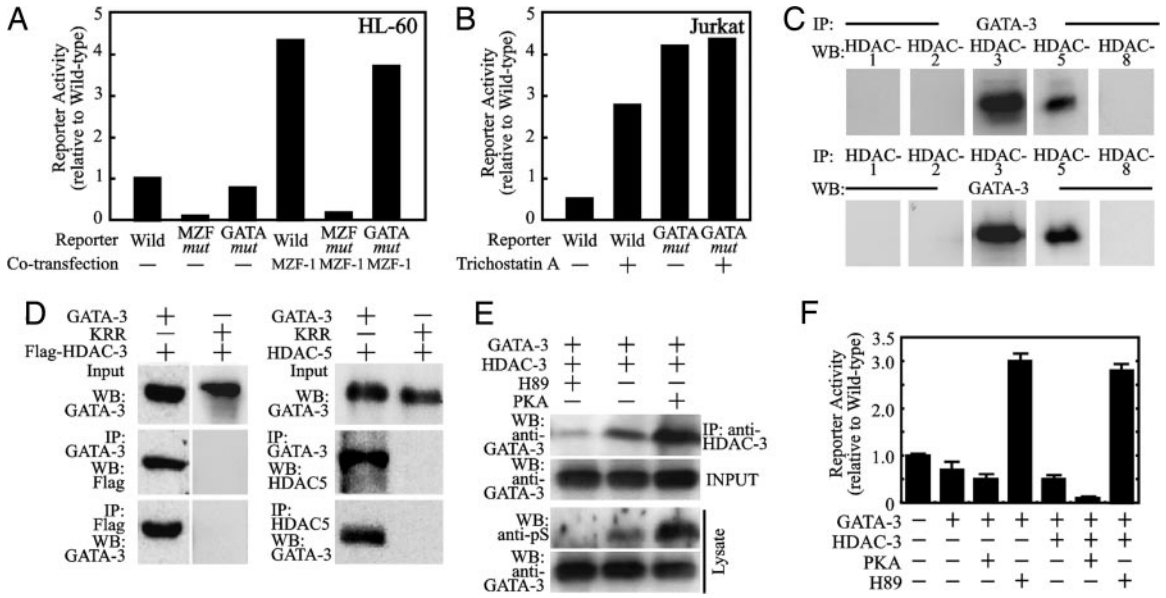

Fig. 1. Association of GATA-3 with HDAC-3/HDAC-5 and phosphorylation-dependent repressive effect of GATA-3 on *FUT7* transcription. (*A*) Reporter assays indicating enhancing effect of MZF-1 on *FUT7* transcription in HL60 cells. (*B*) Reporter assay indicating repressing effect of GATA-3 on *FUT7* transcription in Jurkat cells. (*C*) Immunoprecipitation analysis indicating interaction of endogenous GATA-3 with HDAC-3 and HDAC-5 in Jurkat cells. Lysates from Jurkat cells were immunoprecipitated with anti-GATA-3 or indicated anti-HDAC antibodies, and coimmunoprecipitated proteins were visualized by Western blot analysis using the corresponding antibodies. (*D*) Roles of KRR domain in the interaction of GATA-3 with HDAC-3 and HDAC-5 in COS7 cells. COS7 cells were transfected with the GATA-3 or its dominant-negative form, KRR, together with flag-HDAC-3 or HDAC-5, and they were subjected to immunoprecipitation analyses. (*E*) Enhancing effect of GATA-3 phosphorylation on its interaction with HDAC-3 in COS7 cells transfected with GATA-3, HDAC-3, and/or PKA constructs and cultured for 48 h in the absence or presence of 20 μM H89. (*F*) Reporter assays indicating enforced repression of *FUT7* transcription by GATA-3 phosphorylation in Jurkat cells transfected with GATA-3, HDAC-3, and/or PKA constructs and then incubated for 40 h in the absence or presence of H89 before luciferase assay.

KRR sequence in the zinc finger region of GATA-3 was found to be crucial for the binding to HDAC-3 and HDAC-5 (Fig. 1*D*). PKA is known to phosphorylate the serine residue in the sequence KRRLSA in the GATA-3 zinc finger (22). PKA-mediated GATA-3 phosphorylation facilitated its binding to HDAC-3 and led to a more pronounced repression of *FUT7* transcription, whereas H89, a specific inhibitor for PKA, abrogated HDAC-3 binding of GATA-3 and strongly enhanced *FUT7* transcription (Fig. 1 *E* and *F*).

Role of T-bet in FUT7 Transcription. Resting lymphocytes, except natural killer (NK) cells and a small subset of helper memory T cells (7, 8, 23), lack the expression of sialyl Lewis X, but the determinant is known to be strongly induced upon lymphocyte activation, which accompanies transcriptional induction of *FUT7*. Results of reporter assays using the deletion constructs of *FUT7* in phorbol 12-tetradecanoate 13-acetate (TPA)-activated Jurkat cells indicated that the −134 to −237 region (Fig. 7*A*, which is published as supporting information on the PNAS web site), which contains the CRE element, plays an important role in its induction, along with the Sp1 binding site located near the initiator sequence. Introduction of a mutation in the CRE element or Sp1 binding site resulted in the substantial reduction of transcriptional activity (Fig. 7*A*).

During the course of this study, we noticed that the GC-rich 5′ arm region of the CRE element also has an enhancing activity in Jurkat cells (Fig. 7*B*). We tried to identify the binding factors for this region by one-hybrid screening in yeasts using the sequence covering a half-palindromic CRE element and its GC-rich 5′ arm (GGCTGTGGGGGCGTCATATTGCCCT-GGGG) as a bait in the cDNA library of human lymphoid YT cells. A significant number of clones encoding T-bet were obtained (Fig. 8, which is published as supporting information on the PNAS web site).

Results of chromatin immunoprecipitation (ChIP) assays indicated a significant binding of T-bet, as well as that of GATA-3, CREB 1, and Sp1, to the promoter region of *FUT7* (Fig. 2*A*). *FUT7* is an example of a regulatory region with binding sites for both T-bet and GATA-3. Immunoprecipitation experiments using Jurkat nuclear extracts indicated that endogenous T-bet interacts with CBP/P300, Sp1, and GATA-3, but not with CREB 1 (Fig. 2*B*). The interaction was further confirmed by the immunoprecipitation analyses using appropriately tagged molecules in COS7 cells (Fig. 2*C*). The C-terminal region of T-bet was involved in the interaction of T-bet with P300 and GATA-3, whereas the T-box binding region was involved in the interaction of T-bet with Sp1 (Fig. 2*D*).

The interaction of T-bet with CBP/P300 and Sp1 is described in this article, whereas the interaction of T-bet and GATA-3 just recently has been described (24). These results, together with the interaction of GATA-3 with HDAC-3 and HDAC-5 (Fig. 1), raised the possibility that these six transcription factors (T-bet, GATA-3, Sp1, CBP/P300, HDAC-3, and HDAC-5) may form a transcriptional complex on the *FUT7* promoter in human lymphoid cells.

Modulation of Cell-Surface Expression of Sialyl Lewis X by T-bet and GATA-3. T-bet significantly promoted *FUT7* transcription in reporter assays, whereas GATA-3 was repressive (Fig. 3*A*). This finding prompted us earlier to suggest that T-bet would play an important role in *FUT7* transcription and sialyl Lewis X expression in T lymphocytes (3). This proposal was supported by a recent study reporting a significant reduction of selectin ligand activity in T-bet deficient mice (25). The transfection of T-bet gene alone to cultured lymphoid cells, however, failed to confer appreciable selectin ligand expression either in a murine (26) or human system (see below), and the exact role played by T-bet in regulation of *FUT7* transcription remains obscure. In this study, we found that a simultaneous suppression of GATA-3 activity, either by the use of GATA-mutated reporter construct or by the

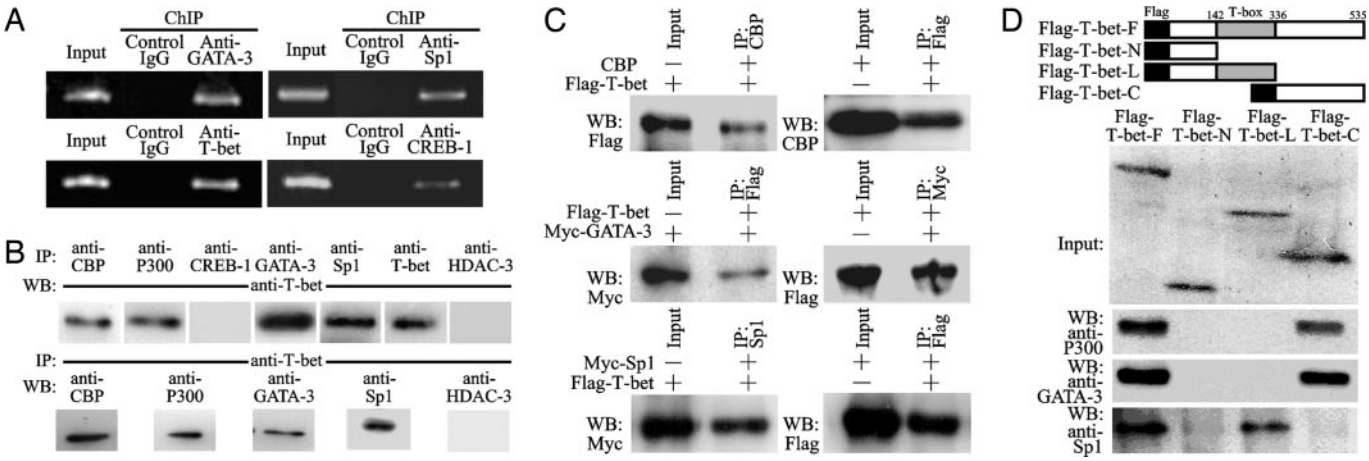

Fig. 2. Promoting effect of T-bet on *FUT7* transcription and association of T-bet with CBP/P300, GATA-3, and Sp1. (*A*) ChIP analysis showing binding of GATA-3, CREB 1, T-bet, and Sp1 on *FUT7* promotor region in Jurkat cells. (*B*) Immunoprecipitation analysis indicating interaction of endogenous T-bet with CBP/P300, GATA-3, and Sp1 in Jurkat cells. (*C*) Immunoprecipitation analysis indicating interaction of Flag-T-bet with CBP/P300, GATA-3, and Sp1 in COS7 cells. (*D*) Mapping of the T-bet domain interacting with CBP/P300, GATA-3, and Sp1 in COS7 cells.

transfection of KRR, a dominant-negative form of GATA-3 (27), confers a prominent promotion of *FUT7* transcription by T-bet (Fig. 3*A*).

Thus, we tested whether these transcription factors indeed affect cell-surface expression of sialyl Lewis X on lymphoid cells. The Jurkat cells, which do not express the determinant under the usual culture conditions, showed only minimal expression of sialyl Lewis X by the introduction of either T-bet or KRR alone (Fig. 3*B Left*). A significant sialyl Lewis X expression was obtained, however, when both T-bet and KRR were introduced (Fig. 3*B Left*). Further enhancement of sialyl Lewis X expression was observed when the cells transfected with T-bet and KRR constructs were activated with TPA (Fig. 3*B Right*).

Another T cell line, HUT78, also does not express the sialyl Lewis X determinant under the usual culture conditions. We previously isolated a spontaneously sialyl Lewis X-positive HUT78 clone (clone 9) (Fig. 3*C Left*) (28). *FUT7* reporter assays in these cells indicated a remarkably enhanced transcription in the clone 9 cells compared with parental HUT78 cells (Fig. 3*C Center*). The sialyl Lewis X-positive clone was characterized by an increased T-bet and decreased GATA-3 expression by RT-PCR (Fig. 3*C Right*). Introduction of GATA-3 in the clone 9 significantly reduced sialyl Lewis X expression (Fig. 3*D Left*), whereas cotransfection of T-bet and KRR to parental HUT78 cells induced significant sialyl Lewis X expression (Fig. 3*D Right*).

YT cells, which belong to the NK cell lineage, were characterized to have strong T-bet and *FUT7* expression (Fig. 3*E Left*). The cells expressed sialyl Lewis X, and transfection of GATA-3 significantly reduced sialyl Lewis X expression (Fig. 3*E Right*). Introduction of siRNA for T-bet to YT cells also significantly reduced sialyl Lewis X expression (Fig. 3*F*).

These results together supported the notion that both increased T-bet and decreased GATA-3 activities are requisite for full activation of *FUT7* transcription and maximum expression of sialyl Lewis X.

***In Situ* Interaction of GATA-3/T-bet with Other Transcription Factors on *FUT7* Promoter.** ChIP assays indicated that GATA-3 and HDAC-3 are the major transcriptional factors bound to the *FUT7* promotor region in Jurkat cells under normal culture conditions, whereas only a minimal binding of T-bet, CBP/P300, and POL II is detected. Thus, *FUT7* promotor region is associated with less acetylated histone H3 and poor POL II recruitment (Fig. 4*A*, lane 1).

Transfection of T-bet to Jurkat cells resulted in increased binding of T-bet, which is accompanied with decreased binding of GATA-3 to the *FUT7* promoter, which is compatible with the previously proposed concept (24) that T-bet inhibits DNA binding of GATA-3. This inhibition led to the concomitant decrease in HDAC-3 binding to the *FUT7* promoter. The increased binding of T-bet is associated with enhanced recruitment of CBP/P300 to the *FUT7* promoter, and the overall results are enhanced acetylation of histone H3 and recruitment of POL II (Fig. 4*A*, lane 3).

On the other hand, transfection of GATA-3 led to the strong binding of HDAC-3 and HDAC-5 and almost completely abrogated the binding of T-bet (Fig. 4*A*, lane 2), which had been visible, albeit weakly, in the control cells transfected with an empty vector. This result was associated with the complete disappearance of acetylated histone H3 and POL II recruitment. This finding raises the possibility that GATA-3 may inhibit the DNA binding of T-bet.

This notion was supported further by the finding that transfection of the repressor of GATA (ROG) (29, 30) facilitated the binding of endogenous T-bet to the *FUT7* promoter, leading to the accumulation of CBP/P300. Binding of GATA-3 and HDAC-3 was decreased, and, consequently, acetylation of histone H3 was markedly accelerated (Fig. 4*A*, lane 5). A similar finding was obtained with the cells transfected with KRR, the dominant-negative form of GATA-3, except that the decrease in GATA-3 binding was not apparent in this case because of the cross-reactivity of anti-GATA-3 antibody with KRR (Fig. 4*A*, lane 4).

GATA-3 inhibited binding of T-bet to *FUT7* promoter, and introduction of KRR-mutated GATA-3, or repression of DNA binding of GATA-3 by ROG-induced binding of T-bet to *FUT7* promoter (Fig. 4*A*). Repression of GATA-3 activity by introducing increasing amounts of ROG (Fig. 4*B*) stoichiometrically abrogates GATA-3 binding and induces a concomitant and progressive increase of T-bet binding to *FUT7* promoter. These findings indicate that GATA-3 interferes with the binding of T-bet to its target DNA. Moreover, GATA-3 phosphorylation-dependently recruits HDACs (Fig. 1 *E* and *F*) and competes with P300 in the binding to the N terminus of T-bet (Fig. 4*C*).

Discussion

Selectin-mediated cell adhesion mediates extravasation of leukocytes in inflammatory diseases as well as in routine homing of

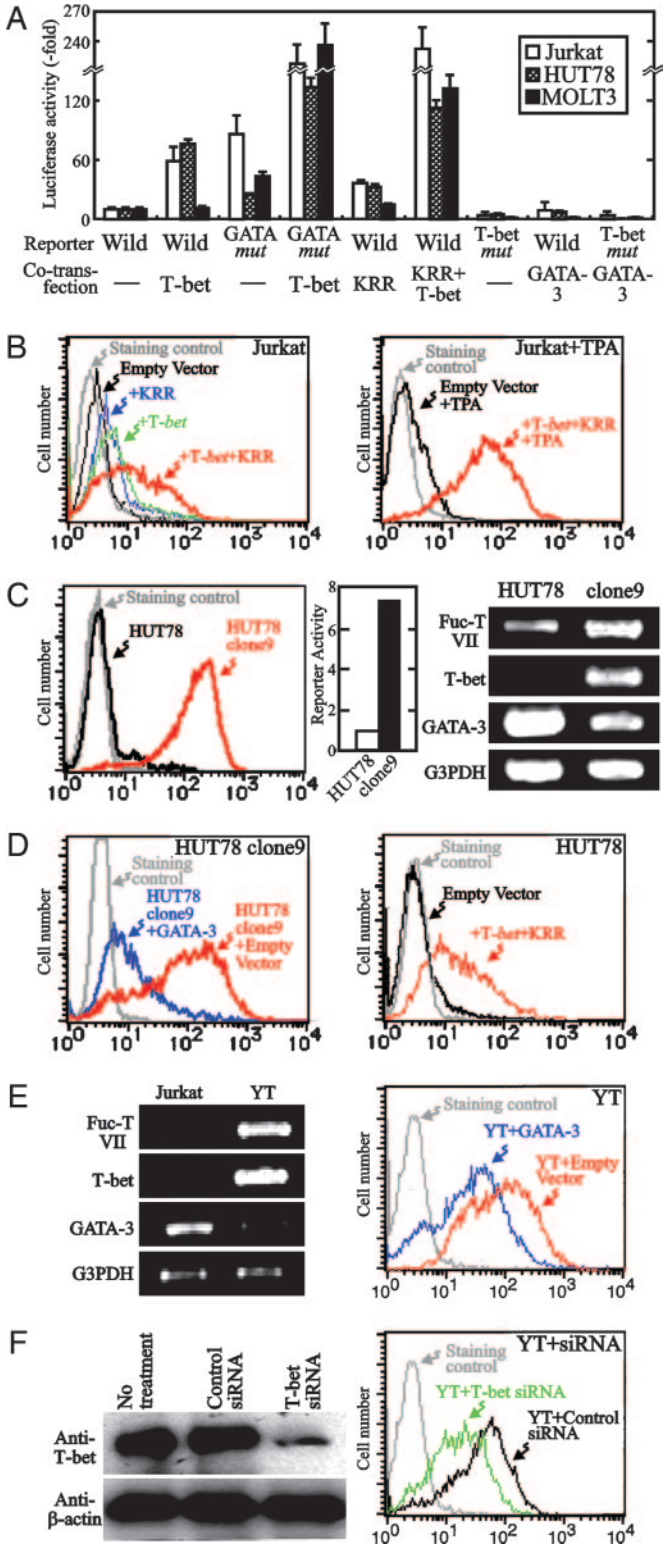

Fig. 3. Involvement of GATA-3 and T-bet in *FUT7* transcription. (*A*) Reporter assays showing promoting effect of T-bet on *FUT7* transcription. Results indicate the need for GATA-3 suppression to achieve maximum induction of *FUT7* transcription. (*B*) Flow-cytometric analysis of sialyl Lewis X expression on Jurkat cells transfected with expression vectors for T-bet and KRR-mutant GATA-3 in the absence (*Left*) or presence (*Right*) of TPA and ionomycin. (*C*) Expression of sialyl Lewis X in HUT78 cells and HUT78 clone 9 cells. HUT78 clone 9 cells, high expressors of sialyl Lewis X on flow-cytometric analysis (*Left*), supported accelerated transcription of *FUT7* in reporter assays (*Center*) and were shown to have increased T-bet and decreased GATA-3 by RT-PCR (*Right*). (*D*) Flow-cytometric analysis of sialyl Lewis X expression on HUT78 clone 9 cells transfected with or without GATA-3 expression vector (*Left*), and that on parental HUT78 cells transfected with expression vectors for T-bet and KRR-mutant GATA-3 (*Right*). Expression of sialyl Lewis X on HUT78 clone 9 was suppressed by the transfection of GATA-3 (*Left*). In contrast, introduction of T-bet and KRR-mutant of GATA-3 conferred significant sialyl Lewis X expression. (*E*) YT cells were shown in RT-PCR analyses (*Left*) to strongly express T-bet and *FUT7*, but GATA-3 only weakly, compared with Jurkat cells. Expression of sialyl Lewis X on YT cells was suppressed by transfection of GATA-3 (*Right*). (*F*) Introduction of siRNA for T-bet to YT cells significantly reduced the protein level of T-bet in Western blot analyses (*Left*), and this led to suppression of sialyl Lewis X expression on the cells as ascertained by flow cytometry (*Right*).

lymphocytes, and it is regulated by expression levels of selectins and their carbohydrate ligand, sialyl Lewis X. Most resting peripheral T lymphocytes lack sialyl Lewis X expression but are induced to strongly express it upon activation. The induction of sialyl Lewis X depends on transcriptional activation of fucosyltransferase VII gene (*FUT7*), the rate-limiting enzyme for sialyl Lewis X synthesis (8, 10–15). Th1 cells are known to more preferentially express sialyl Lewis X as compared with Th2 cells upon activation (9). The molecular basis for this expression, however, has not been elucidated to date. Here we showed that *FUT7* is unique in having binding sites in its promoter region for both GATA-3 and T-bet, two opposing factors for Th1 and Th2 development (31, 32), and is transcriptionally regulated by a balance of the interacting two transcription factors. GATA-3 represses *FUT7* transcription through phosphorylation-dependent interaction with HDAC-3 and HDAC-5, whereas T-bet promotes its transcription through recruiting CBP/P-300. These factors form a transcription factor complex on *FUT7* promoter, and the GATA-3/HDAC-3, GATA-3/HDAC-5, T-bet/CBP/P300, and T-bet/Sp1 interactions described here, as well as recently described T-bet/GATA-3 interaction (24), dynamically regulate composition of the transcriptional complex on the *FUT7* promoter. T-bet interferes with binding of GATA-3, which in turn interferes with binding of T-bet to the *FUT7* promoter as evidenced by ChIP assays. Suppression of GATA-3 activity by dominant-negative GATA-3 or ROG, in addition to T-bet, was necessary to attain full activation of *FUT7* transcription and maximum sialyl Lewis X expression in human T lymphoid cells. Regulation of *FUT7* transcription by GATA-3/T-bet interaction well explains the lineage-specific expression of sialyl Lewis X among lymphocyte subsets.

These findings indicate that not only T-bet but also GATA-3 figures heavily in regulating *FUT7* transcription, and that a balance of the two interacting transcription factors, GATA-3 and T-bet, regulates *FUT7* transcription, which well explains Th1-dominant expression of sialyl Lewis X (9) among T lymphocytes (Fig. 9, which is published as supporting information on the PNAS web site). Another subset of lymphocytes that significantly express sialyl Lewis X is NK cells, and T-bet is known to be active in NK cells (33, 34). We propose that transcriptional regulation of *FUT7* plays important roles in lineage-specific expression of selectin ligands among lymphocyte subpopulations.

Proteins and nucleic acids generally are believed to be indispensable functional molecules, but the role of glycoconjugates as the third type of biofunctional molecules has long remained obscure. Most genes for cell-surface carbohydrate synthesis have been regarded as housekeeping genes, and dynamic and multifarious regulation at their transcription levels has not been expected. Here we show that a gene for glycosyltransferase responsible for synthesis of a carbohydrate lymphocyte homing receptor is elegantly and exquisitely regulated by a complex of intriguing transcription factors, including T-bet and GATA-3, to attain cell lineage-specific expression in lymphocyte subsets such as Th1 and Th2 cells. This is an outstanding example of the most

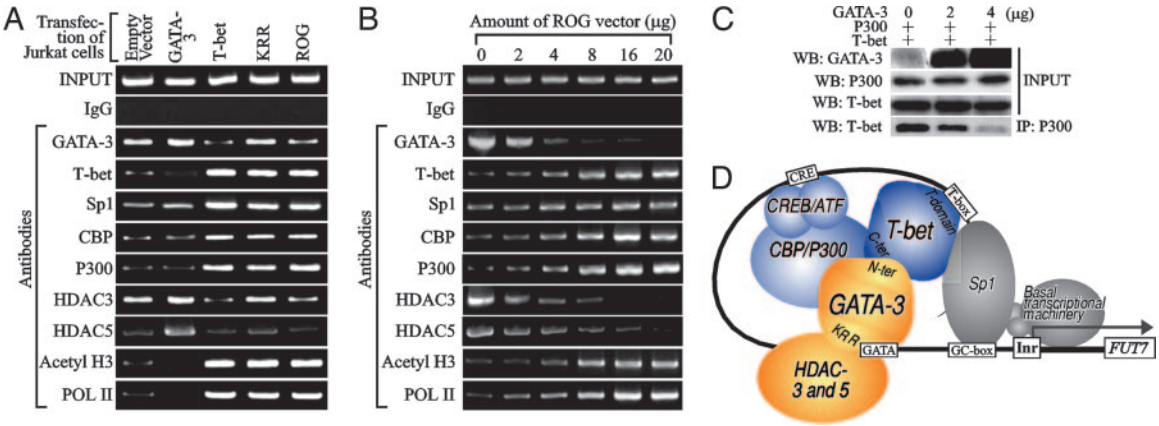

Fig. 4. Interaction of transcription factors present in *FUT7* promotor region. (*A*) ChIP analysis on the effect of GATA-3, T-bet, KRR, and ROG on the assembly of transcription factors on *FUT7* promotor region in Jurkat cells. (*B*) ChIP analysis on the effect of increasing amount of ROG on the assembly of transcription factors on *FUT7* promotor region in Jurkat cells. (*C*) Immunoprecipitation analysis of competition of GATA-3 in the binding of P300 to T-bet. COS7 cells were transfected with p300, T-bet, and increasing amounts of GATA-3 expression vector, and the presence of T-bet in p300 immunoprecipitates was assessed by using an anti-T-bet antibody. Expression of transfected proteins was monitored by direct Western blotting. (*D*) Schematic diagram showing interaction of GATA-3, T-bet, and other transcription factors in FUT7 promotor region.

sophisticatedly regulated glycogenes, and the expression of cell-surface glycoconjugates coordinated with other cell lineage-specific proteins may usher in an altogether new concept in functional glycomics.

Materials and Methods

Cells, Antibodies, and Flow-Cytometric Analysis. Human cell lines Jurkat, HL-60, HUT78, HUT78 clone9, and MOLT 3 were maintained in RPMI medium 1640 supplemented with 10% FCS. COS7 cells were cultured in DMEM supplemented with 10% FCS. A monoclonal anti-sialyl Lewis X antibody (2H5, IgMκ) was prepared as described (35). Anti-T-bet (H-210, sc-21003), anti-GATA-3 (H-48, sc-9009), anti-CREB 1 (24-H4B, sc-271), anti-Sp1 (H-225, sc-14027), anti-CBP (C-20, sc-583), anti-p300 (C-20, sc-585), anti-HDAC-3 (H-99, sc-11417), anti-HDAC-5 (H-74, sc-11419), and anti-POL II (H-224, sc-9001) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-acetyl-histone H3 and anti-myc tag, clone 9E10 were from Upstate Biotechnology (Lake Placid, NY). Anti-FLAG M2 monoclonal antibody was from Sigma (St. Louis, MO). For flow-cytometric analysis, Jurkat, HUT78, or HUT78 clone9 cells were harvested at a semiconfluent stage and stained with the respective antibody by using purified antibody at 1 μg/ml or culture supernatant at a dilution of 1:5. Cells then were stained with a 1:100 dilution of FITC-conjugated second antibody and analyzed with FACScalibur (Becton Dickinson, Franklin Lakes, NJ). In some experiments, the cells were stimulated with TPA (100 ng/ml) and ionomycin (1 μM) for 48 h and then harvested for luciferase report assay or flow-cytometric analysis.

Plasmid Constructs. The FucT VII luciferase report plasmid constructs and mutant derivatives, as well as the CREB and PKA expression constructs, have been described (17). For mammalian expression constructs of T-bet, the ORF was amplified by PCR and was subcloned into pCMV-Tag 2A (Sigma, St. Louis, MO) in-frame with an amino-terminal Flag tag. GATA-1, GATA-2, and GATA-3 expression constructs were prepared as described in ref. 36. KRR expression construct, a GATA-3 dominant-negative mutant, was a gift from Aster Winoto (27), and ROG expression vector was from Tang K. Tang (37). HDAC-1, HDAC-2, and HDAC-3 expression constructs were prepared as described in ref. 38. HDAC-5 expression construct was obtained from Origene (Rockville, MD), whereas p300 expression construct was obtained from Upstate Biotechnology (Lake Placid, NY). Standard molecular cloning techniques described by Maniatis *et al.* (39) were used for preparing the deletion mutant of T-bet and GATA-3 expression constructs. All constructs were verified by restriction enzyme digestion and DNA sequencing.

Transfection and Luciferase Assay. Transfection and luciferase assay were performed as described in ref. 17. The luciferase reporter plasmids (1 μg) and pRL/CMV plasmid (50 ng) used as an internal standard for normalization of the transfection efficiency were cotransfected. After 40 h, cell extracts were prepared and used for luciferase assay (Dual-Luciferase Reporter Assay system; Promega, Madison, WI). All experiments were performed in triplicate. In experiments using human myeloid HL-60 cells, the cells were transiently transfected with expression vectors for MZF-1 and luciferase reporters linked to the wild-type *FUT7* promoter or the promoter with a mutation in the MZF-1 or GATA site. Values are expressed as fold activation relative to wild type. MZF-1-mut had a mutation at bases −77 to −70 (from GGTGGGGA to GGTGGGCT) in the MZF-1 consensus element. For Jurkat cells, the cells were cotransfected with luciferase reporters linked to the wild-type *FUT7* promoter or the promoter with a mutation in the GATA or T-bet site and the indicated expression vectors. GATA-mut had a mutation at bases −86 to −78 (from CGAGATGGG to CGACTAGGG) in the GATA consensus element. After 40 h of incubation, luciferase activity in the extracts was measured. In some experiments, the cells were transfected with the luciferase reporters and then incubated (for 40 h) in the absence or presence of trichostatin A before luciferase activity in the extracts was measured. For siRNA experiments, T-bet siRNA(h) (sc-36598) and control siRNA-A (sc-37007) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The siRNA was electroporated into YT cells with the Si PORT Electroporation Buffer (8990G; Ambion, Austin, TX). After 72 h, the cells were collected for flow cytometric and Western blot analyses.

Immunoprecipitation and Western Blot Analysis. Whole Jurkat cell lysates were prepared in the buffer [0.5% Nonidet P-40, 50 mM Tris (pH 7.2), 0.1 mM EDTA, 150 mM NaCl, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride)] for determination of interactions of endogenous transcription factors. An equal amount of the extract was incubated with 4 μg of the respective antibody with gentle shaking overnight at 4°C. Samples then were mixed with 60 μl of protein A-conjugated

agarose beads (Upstate Biotechnology, Lake Placid, NY) for 2 h at 4°C, and immunoprecipitates were washed four times with PBS and resuspended in SDS sample buffer for Western blot analysis. Proteins in the immunoprecipitates were separated by SDS/PAGE and transferred onto a nitrocellulose membrane. The concentration of running gel was 10% unless otherwise specified. After blocking, the blots were incubated with respective primary antibody (1:1,000 dilution). After incubation with the second antibody (HRP-conjugated goat anti-rat IgG, rabbit anti-goat IgG, or goat anti-mouse IgG) (1:5,000 dilution), the signal was detected with an ECL kit (Amersham Biosciences, Piscataway, NJ). In experiments for analysis of GATA-3 interaction with HDAC-3 and HDAC-5, the COS7 cells were cotransfected with the constructs for Flag-tagged HDAC-3 or HDAC-5 together with Myc- or Flag-tagged GATA-3 constructs, and cell extracts were subjected to immunoprecipitation with indicated antibodies followed by immunoblotting analysis. Mutation constructs of Flag-tagged GATA-3 were used for mapping of the GATA-3 domain interacting with HDACs. COS7 cells were transfected with the indicated constructs and cultured for 48 h in the presence of 20 μM H89 in some experiments. In experiments for analysis of T-bet interaction with GATA-3, CREB 1, T-bet, and Sp1, the COS7 cells were cotransfected with the construct for CBP/P300, Myc-GATA-3, or Myc-Sp1 together with the Flag-tagged T-bet construct before extraction. Mutation constructs of Flag-tagged T-bet were used for mapping of the T-bet domain interacting with P300, GATA-3, or Sp1.

ChIP Assay. Analysis was performed following a kit protocol (Upstate Biotechnology, Lake Placid, NY). Chromatin samples were isolated from Jurkat cells. The antibodies used for ChIP were anti-T-bet, anti-GATA-3, anti-CREB 1, anti-Sp1, anti-CBP, anti-p300, anti-HDAC-3, anti-HDAC-5, anti-acetyl-histone H3, and anti-POL II. Chromatin from Jurkat cells was precipitated with these antibodies, and precipitated *FUT7* promoter DNA was amplified by PCR. The primers used for detecting were: FucT F (5′-CTGCCCAGCTGGACATCTCT 3′) and FucT R (5′-CACAGCTACGCCCTAGCTCAG-3′). When indicated, the Jurkat cells were transfected with expression vectors for GATA-3, T-bet, KRR, or ROG and incubated for 48 h before ChIP. In some experiments, the cells were transfected with the increasing amount of ROG expression vector, and ChIP assays were performed after cells were incubated for 48 h.

We thank Dr. Astar Winoto (University of California, Berkeley, CA) for the gift of GATA-3 and KRR mutant vector; Drs. Takao Hamakubo, Tatsuo Kodama, and Masayuki Yamamoto (all at University of Tokyo, Tokyo, Japan) for the gifts of other GATA series vectors; Dr. Tang K. Tang (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan) for ROG vector; and Dr. Nozomu Hiraiwa for his help in performing the yeast one-hybrid assays. This work was supported in part by Grant-in-Aid 17590258 and on priority areas 17015051 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, grants-in-aid for the Third-Term Comprehensive Ten-Year Strategy for Cancer Control from the Ministry of Health, Labor, and Welfare of Japan, and a grant from the Core Research for Evolutionary Science and Technology (CREST) program of the Japan Science and Technology Agency.

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