Involvement of Transcription Factors TCF-1 and GATA-3 in the Initiation of the Earliest Step of T Cell Development in the Thymus

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Summary

Flow cytometric and immunocytochemical analyses of murine fetal thymus (FT) cells with antibodies to various surface markers and transcription factors reveal that the synthesis of TCF-1 and GATA-3 proteins begins simultaneously in a fraction of the most immature population of FT cells, which have the phenotype of $CD4^-CD8^-CD44^+CD25^-$. No TCF-1–producing cell is found in the fetal liver (FL). In $CD44^+CD25^-$ FT cells, the production of TCF-1 is immediately followed by intracellular expression of CD3ε. It is also found that the T cell development from FL, but not FT, progenitors in the FT organ culture system is severely inhibited by the addition of antisense oligonucleotides for either TCF-1 or GATA-3. These results strongly suggest that TCF-1 and GATA-3 play essential roles in the initiation of the earliest steps of T cell development in the thymus.

T cells are produced mainly in the thymus from progenitors emigrated from extrathymic hematopoietic organs (1–3), although it is still controversial whether these progenitors are prethymically committed to give rise to lymphocytes or T cells (2–4). In the murine fetuses, it is known that progenitors derived from fetal liver (FL)[1] or other fetal hematopoietic organs colonize the thymus around day 11 of gestation (5, 6), and that the differentiation and growth of T cells from these progenitors are induced or supported by the thymic microenvironment (7). The mechanisms of TCR gene rearrangement and the interaction of maturing T cells with the thymic microenvironment through TCR, so-called positive and negative selection, are becoming clearer (8, 9). On the other hand, our knowledge about the earlier stages, during which the TCR and coreceptors CD4 and/or CD8 have not yet been expressed on the cell surface, is still fragmental.

T lineage cells in the fetal thymus (FT) that are negative for CD4 and CD8 can be divided into four subpopulations on the basis of CD44 and CD25. These are $CD44^+CD25^-$, $CD44^+CD25^+$, $CD44^-CD25^+$, and $CD44^-CD25^-$, and it is well known that the differentiation proceeds in this order (10, 11). It has been shown that progenitors capable of generating B and myeloid cells are present in fetal as well as adult thymuses (12–15). These B and myeloid progenitors have not been well characterized, and it is unclear whether thymic B and myeloid cells are derived from a common progenitor or stem cell that is also able to generate T cells. In the murine fetus, the presence of B and myeloid progenitors has been shown in the FT at day 12 of gestation (15), and the phenotype of day 12 FT is exclusively $CD44^+CD25^-$ (16). On the other hand, progenitors in the $CD44^+CD25^+$ population of FT, which is equivalent to the c-*kit*$^+CD25^+$ population, are committed to T lineage (17). With adult thymocytes, it has been shown that the mRNA for pre-TCRα (pTα) chain and all members of the CD3 complex are expressed at the $CD44^+CD25^-$ stage (18, 19), and it has been suggested by Christiaan et al. (20) that CD3ε proteins are present in the cytoplasm, but not on the surface of cells in this population. Although the exact role of intracellular CD3ε (IC-CD3ε) in T cell differentiation has not been clarified so far, the importance of this molecule is implicated by the finding that overexpression of human CD3ε in the cytoplasm of murine thymus cells causes a differentiation arrest before entering into the $CD44^+CD25^+$ stage (21). These findings collectively suggest that if thymic T cell progenitors are derived from a multipotent stem cell that has immigrated into the thymus, commitment to the T cell lineage should occur during the $CD44^+CD25^-$ stage.

Whether the thymic microenvironment is inductive or supportive, stem cells or progenitors initiate differentiation into T cells through interaction with thymic stromal cells.

[1] *Abbreviations used in this paper:* AGPC, acid guanidinium thiocyanate-phenol-chloroform; AS, antisense; dGuo, deoxyguanosine; FL, fetal liver; FT, fetal thymus; HOS, high oxygen submersion; IC, intracellular; Lin, lineage markers; RT, reverse transcription; S, sense.

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Although the exact mechanism of interactions leading to the initiation of T cell development is still unclear, involvement of both cell-to-cell and factor-mediated interactions has been shown by a variety of in vivo and in vitro experiments (17, 22–27). It is probable that after interaction with thymic stromal cells, the progenitors begin to produce transcription factors that are required for the expression of TCR and related genes, such as CD3ε. Investigation of the production of lymphocyte-specific transcription factors may provide a clue to elucidate the mechanism of the earliest step of T cell development. In the present study, we show that TCF-1 and GATA-3 are produced by a subpopulation of $CD44^+CD25^-$ cells shortly before the production of IC-CD3ε, and that the addition of antisense (AS) oligonucleotides specific for these transcription factors in a FT organ culture system prohibits the T cell development from prethymic progenitors. These findings strongly suggest that the production of these transcription factors is indispensable for commitment of stem cells to the T lineage or for setting toward the earliest differentiation of T cells that occurs at the $CD44^+CD25^-$ stage.

Materials and Methods

Mice and Organs

C57BL/6 (B6) mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and maintained in our animal facility. FT and FL were obtained from time-mated pregnant mice on various days of gestation. The date of finding the vaginal plug was taken as day 0. Thymuses were also obtained from newborn and 4-wk-old B6 mice. B6Ly5.1 mice (maintained in our animal facility) were also used in organ culture experiments as the source of FT lobes into which B6 (Ly5.2) progenitors were seeded.

Cell Lines

Mouse teratoma line F9, mouse fibroblast line NIH3T3, and SV-40–transformed monkey kidney cell line Cos-7 were grown in DMEM supplemented with 10% FCS. Mouse T lineage lines EL4, BW5147, BgV (28), YT5 (cloned by Dr. H. Nariuchi, Tokyo University, Tokyo, Japan), mouse B lineage lines 70Z/3 and A20.2J, mouse monocyte line P388, and mouse myeloblast line M1 were cultured in RPMI 1640 medium supplemented with 10% FCS.

Reverse Transcription-PCR Assay

RNA Isolation. Total RNA was isolated from fetal, newborn, and adult thymuses by a modification of the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method (29). Thymus cells were washed with PBS and suspended into solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium lauryl sarcosine, and 0.1 M 2-ME), and sheared by passage through a 21-gauge needle. The sheared lysate was mixed with 0.1 vol of 2 M sodium acetate, pH 4.0, an equal volume of water-saturated phenol, and a 0.2 vol of phenol-chloroform-isoamyl alcohol. After centrifugation, the RNA in the supernatant was precipitated with isopropanol. Pelleted RNA was lysed again in solution D and then precipitated with isopropanol, followed by washing with 70% ethanol and drying up. The extracted RNA was dissolved in water, and the concentration was determined by spectrophotometric measurement.

Reverse Transcription. A mixture of RNA (1.0 μg), 1 ml of 3 mg/ml random primers (GIBCO BRL, Gaithersburg, MD), and water (total volume 26 μl) was incubated at 65°C for 5 min. After chilling on ice, 8 μl of 5× reverse transcription (RT) buffer (0.25 M Tris-HCl, pH 8.3, 0.37 M KCl, and 15 mM $MgCl_2$), 4 μl of 0.1 M DTT, 1 μl of 100 mM dNTPs, and 1 μl of M-MLV reverse transcriptase (200 U/ml; GIBCO BRL) were added. This reaction sample was incubated at 37°C for 60 min and heated to 95°C for 5 min, then chilled on ice. To check for contamination of genomic DNA, the reaction was also performed without reverse transcriptase.

PCR. cDNA was amplified by PCR using various primers chosen on the basis of GENETYX-MAC program (Software Development Co., Tokyo, Japan). The primers used included: GATA-3 sense (S), 5′-TCGGCCATTCGTACATGGAA-3′; GATA-3 AS, 5′-GAGAGCCGTGGTGGATGGAC-3′; TCF-1 S, 5′-GCCAGCCTCCACATGGCGTC-3′; TCF-1 AS, 5′-CGGGTGAGGGATGGCTGCTG-3′; LEF-1 S, 5′-AACTCTGCGCCACCGATGAG-3′; LEF-1 AS, 5′-AGAAAAGTGCTCGTCGCTGT-3′; Ikaros S, 5′-AACGGCCTTTCCAGTGCAAC-3′; Ikaros AS, 5′-GTTGGCACTGTCATAGGGCA-3′; Sox-4 S, 5′-GAGAACACTGAGGCTCTGCT-3′; Sox-4 AS, 5′-GGGAGTCCGCACCTTGTAGA-3′; lck S, 5′-CATTCCCTTCAACTTCGTGG-3′; lck AS, 5′-TAATGGCGGACTAGATCGTG-3′; β-actin S, 5′-TCCTGTGGCATCCATGAACT-3′; β-actin AS, 5′-GAAGCACTTGCGGTGCACGAT-3′. The reaction volume was 10 μl containing 1 μl of cDNA sample, 1 μl of 10× PCR buffer (500 mM Tris-HCl, pH 8.3, 2.5 mg/ml BSA, and 30 mM $MgCl_2$), 200 μM dNTPs, 0.5 U Taq polymerase (Wako Pure Chemical Industries, Osaka, Japan), and 100 μM of each primer. PCR amplification was performed in the Air Thermo-Cycler™ (Idaho Technology, Idaho Falls, ID) under optimal rapid cycle PCR condition (30, 31). Cycling times and temperatures were as follows: denaturation at 94°C for 5 s, annealing at 57°C for 5 s, and elongation at 75°C for 30 s. The cycle number was 20 because the amplification was found to be within linear range (data not shown). Whole PCR product was electrophoresed through a gel containing 4% NuSieve GTG agarose (FMC Corp. BioProducts, Rockland, ME) and 1% agarose, and was blotted onto a nylon membrane.

Southern Hybridization. Hybridization was performed as previously described (32) with probes prepared from PCR products amplified with internally nested primers. The hybridized membranes were processed by an imaging analyzer (BAS 2000; Fuji Photo Film, Tokyo, Japan). Radioactivity of the band representing each PCR product was measured at the same time.

Northern Hybridization

All procedures were performed as previously described (32). Briefly, total RNA (20 μg) samples extracted from various cell lines and adult thymocytes by the modified AGPC method were subjected to electrophoresis, transferred onto a nylon membrane, and hybridized with TCF-1 or Ikaros cDNA probes labeled by random oligo priming (Megaprime DNA labeling system; Amersham International, Buckinghamshire, UK).

Transient Transfection

Coding regions of TCF-1 (33) and Ikaros (34) cDNA were inserted into the BamHI site of pNeoSRα (35), and these recombinant plasmids were transfected to Cos-7 cells by electroporation, as described (32). After incubation for 48 h in DMEM, whole-

cell lysates were prepared from these transfected cells and used for Western blot analysis.

Antibodies. The following antibodies were used in this study: anti–erythroid lineage cell mAb TER119 (produced by T. Kina, Dept. of Immunology), anti–Mac-1 (M1/70, Caltag Laboratories, San Francisco, CA), anti–Gr-1 (RA3-8C5, PharMingen, San Diego, CA), anti-B220 (RA-6B2; Caltag), anti-Thy1.2 (5a-8; Caltag), anti-CD4 (GK1.5; Caltag), anti-CD8 (YTS169.4; Caltag), anti–c-*kit* (ACK-2) (36), anti-CD44 (IM.7.8.1; PharMingen), anti-CD25 (PC61; PharMingen), anti-CD3ε (145-2C11; PharMingen), and anti–human-GATA-3, which cross-reacts with mouse GATA-3 (HG3-31; Santa Cruz Biotechnology, Santa Cruz, CA). In cases where biotinylated reagents were used as the first antibody, APC-conjugated streptavidin (PharMingen) was added as a second-step reagent.

Antibody Production, Western Blot Analysis and Immunocytochemistry. Polypeptides of TCF-1 (amino acids 1–253) (33) and Ikaros (amino acids 1–432) (34) were expressed in *Escherichia coli* using the pET vector system (37) and purified by SDS-gel electrophoresis. Rabbits were subcutaneously injected with one of these peptides emulsified with CFA, followed 4 wk later by a subcutaneous injection of the same antigen emulsified with IFA. Sera were harvested from these rabbits and checked for their antibody activity. The sera showing high antibody activity were used for Western blot and immunocytochemical analyses. For flow cytometric analysis, the Ig fraction was purified from the sera by using a protein G column (Hi TrapTM protein G; Pharmacia Biotech, Uppsala, Sweden), and was conjugated with FITC as described (38).

Nuclear extracts and cytoplasmic fractions of various cell lines and adult thymocytes, which were prepared according to Shreiber et al. (39), were subjected to Western blot analysis (38) using the rabbit antisera at a dilution of 1:500. Detection of anti–TCF-1 and anti-Ikaros antibodies was accomplished by incubation of the immunoblots with a horseradish peroxydase–conjugated goat anti–rabbit IgG antibody (Zymed Laboratories, South San Francisco, CA).

FT cells and lineage marker negative (Lin^-) FL cells were immunocytochemically analyzed. Lin^- FL cells were obtained as follows: FL cells from day 13 fetuses were reacted with TER119, Mac-1, Gr-1, and B220, and the cells negative for these markers were sorted by a cell sorter (FACS® Vantage; Becton Dickinson & Co., Mountain View, CA). Lin^- FL cells and FT cells were settled onto slide glasses by cytocentrifugation (Cytospin 3; Shandon Scientific Ltd., Chesire, UK), fixed in 4% paraformaldehyde for 20 min, washed in PBS, blocked in PBS containing 10% goat serum for 30 min at room temperature, and incubated with the first antibody for 5 h in a humidified chamber at 4°C. An FITC-conjugated goat anti–rabbit IgG antibody (Organon Teknika, Durham, NC) was used for immunodetection.

Flow Cytometric Analysis

Surface staining and flow cytometric analysis of FT and FL cells were performed as previously described (26). In analyzing the expression of intracellular proteins, cells were fixed and permealized as described (40). For two-color analysis for TCF-1 and GATA-3, FT cells were fixed by incubating in 100 μl of reagent A (FIX & PERM cell permealization kit; Caltag) for 15 min at 20°C, washed in PBS, and incubated in 50 μl of permealization reagent B (FIX & PERM) containing FITC–anti–TCF-1 and biotinylated anti–GATA-3 for 15 min at 20°C. The cells were washed again and reacted with APC-conjugated streptavidin. For simultaneous detection of surface CD25 and intracellular proteins (TCF-1 and CD3ε), FT cells were incubated with PE–anti-CD25, washed in PBS, and fixed as described above. The cells were washed again and resuspended in 50 μl of permealization reagent B containing a combination of APC–anti–CD3ε and FITC–anti–TCF-1, or a combination of APC–anti-CD3ε and FITC–rabbit IgG. These stained cells were analyzed by a FACS® Vantage.

Sense and Antisense Oligonucleotides

AS phosphorothioate oligodeoxynucleotides were designed against the translational start region of TCF-1 and GATA-3 on the basis of the reported gene sequences (33, 41). As controls, complementary S oligonucleotides were created. The sequences are as follows: TCF-1 AS, 5′-CTCTTTGTACATGCCAC-TGGC-3′; TCF-1 S, 5′-GCCAGTGGCATGTACAAAGAG-3′; GATA-3 AS, 5′-CGCAGTCACCTCCATGTCCTC-3′; GATA-3 S, and 5′-GAGGACATGGAGGTGACTGCG-3′. To determine the optimal concentrations of oligonucleotides that are not nonspecifically toxic but reduce the production of transcription factors, we initially tested the effect of various concentrations of TCF-1 AS oligonucleotide on EL4 cells. A final concentration of 100 μg/ml was used in organ cultures because this concentration of oligonucleotide partially reduced the expression of TCF-1 protein in EL4 cells without affecting the growth of these cells (data not shown).

High Oxygen Submersion (HOS) Culture

The basic procedure for HOS culture has been described previously (42, 43). In short, RPMI1640 medium supplemented with 10% FCS, sodium pyruvate (1 mM), sodium bicarbonate (2 mg/ml), nonessential amino acid solution (1 mM), 2-ME (5×10^{-5} M), streptomycin (100 μg/ml), and penicillin (100 U/ml) was used as complete medium. FT lobes obtained from day 15 fetuses of B6Ly5.1 mice were cultured with deoxyguanosine (dGuo; 1.35 mM) for 6 d to deplete T lineage cells and their precursors from the lobe. Single dGuo-treated lobes were submerged in 0.2 ml of complete medium in a well of a 96-well U-bottom plate (Costar Corp., Cambridge, MA). Lin^-c-kit^+ FL cells (10^3) or c-kit^+ FT cells (10^3) isolated from B6 mice by sorting with a FACS® Vantage were inoculated into each well. S or AS oligonuculeotides (final concentration = 100 μg/ml) for TCF-1 and GATA-3, or the 1:1 mixture (total = 100 μg/ml) of these AS oligonucleotides, were added to each well. The plates were placed into a plastic bag (Ohmi Oder Air Service, Hikone, Japan), and the air was exchanged with a gas mixture of 5% CO_2, 70% O_2, and 25% N_2. The plastic bag was incubated in a 37°C incubator. On day 3 of culture, half of the medium was exchanged with fresh complete medium containing the corresponding oligonucleotides. Cells in the lobes were harvested on day 5 of culture, enumerated, and surface profiles of these cells were analyzed by a FACS® Vantage.

Results

Semiquantitative RT-PCR Analysis of the Transcription of Nuclear Protein Genes in FT. We first investigated the expression levels of several transcription factors that are known to be involved in the transcription of TCR and related genes. RNA was extracted from the thymus cells of fetuses at various gestational ages, and was used in a RT-PCR assay. Amplification was done for 20 cycles because prelimi-

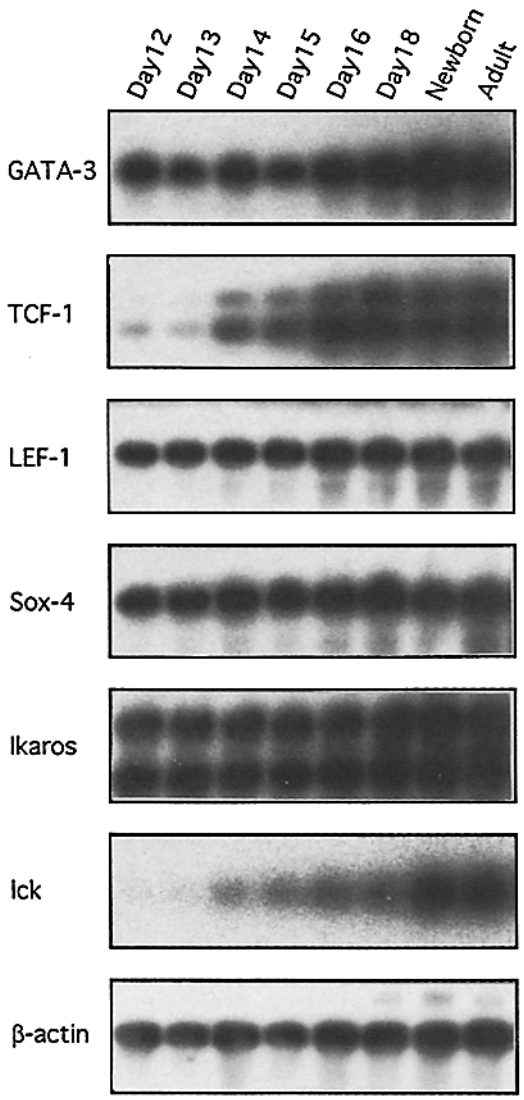

Figure 1. RT-PCR analysis of the expression of transcription factor genes in FT cells. RNA was prepared from thymus cells of fetuses at days 12, 13, 14, 15, 16, and 18 of gestation, as well as of newborn and 4-wk-old mice. 1 μg of RNA was reverse-transcribed, and the cDNA was amplified for 20 cycles with primers specific for each transcription factor gene. After RT-PCR, the products were subjected to Southern blot analysis. As controls, the expression of β-actin and *lck* genes was investigated with the same method.

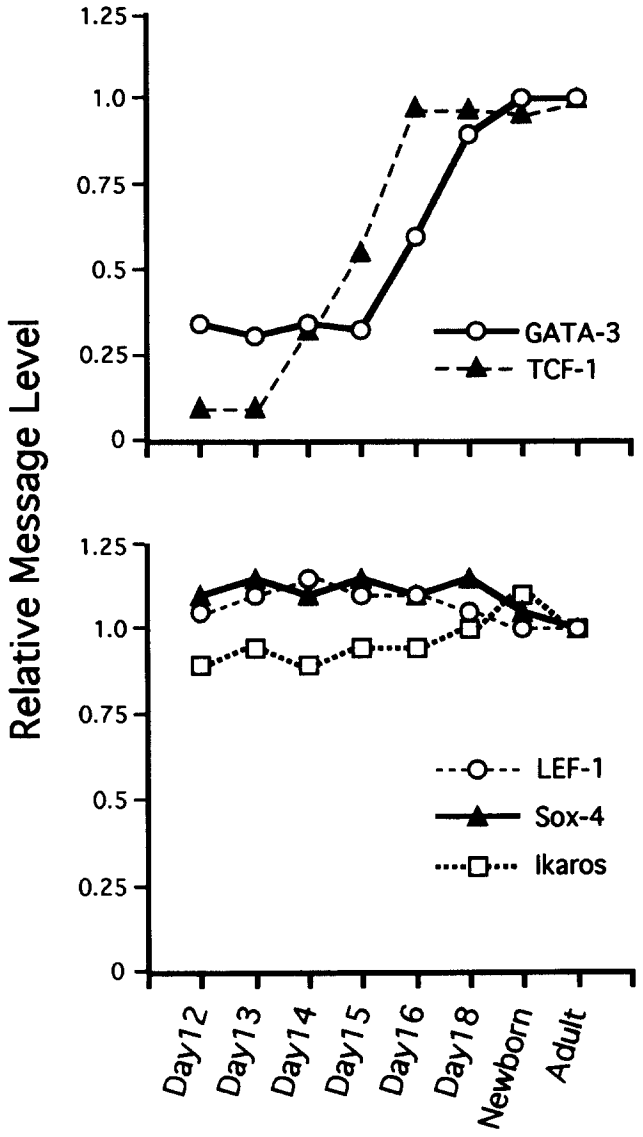

Figure 2. Relative message levels of various transcription factors expressed in thymus cels from fetuses at different gestational ages. The signal intensity of Southern blot analysis bands in Fig. 1 was measured by an imaging analyzer and normalized by that of β-actin. The normalized signal intensity corresponding to adult thymus cells in each transcription factor gene was set as 1.0, and the relative values of newborn and fetal mice were plotted.

nary experiments indicated that the amount of PCR product was approximately proportional to the amount of original mRNA with this cycle number (data not shown). RT-PCR products were hybridized with the probe specific for each gene (Fig. 1), and the relative amounts of the product determined as the signal intensity of bands in Fig. 1 are plotted in Fig. 2 in reference to the age of the mice. All the genes examined are more or less expressed in the thymus as early as day 12 of gestation. Two major bands, each seen in cases of TCF-1 and Ikaros, are caused by the design of PCR primers encompassing an exon that can be spliced out. According to the expression profile in FT, these transcription factors can be divided into two groups. The first group comprises GATA-3 and TCF-1, which have previously been shown to be T lineage specific (33, 44, 45). The expression levels of these two genes steeply increase during early- to mid-gestational age, suggesting that they play some roles in the early phase of T cell differentiation. The second group comprises LEF-1 (46, 47), Sox-4 (48, 49), and Ikaros (34), and these genes are expressed at high levels as early as day 12 of gestation, and the levels are constantly maintained until adult age.

Rabbit Antibodies against TCF-1 and Ikaros. Although the RT-PCR experiments indicated that TCF-1 and GATA-3

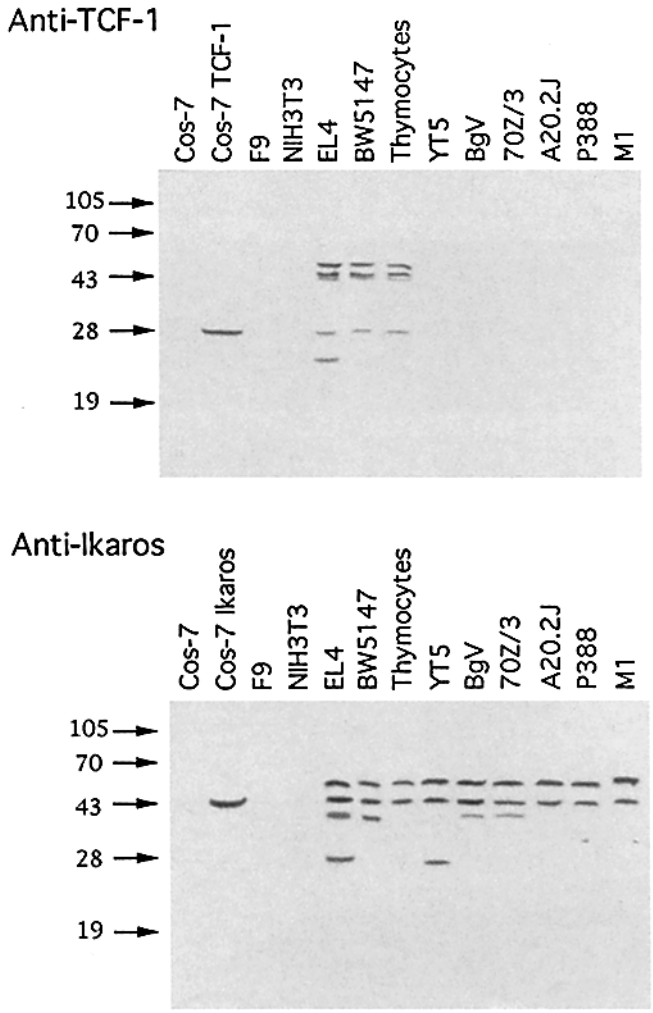

Figure 3. Western blot analysis of various cell lines and adult thymocytes with anti–TCF-1 and anti-Ikaros. Whole-cell lysates of Cos-7, TCF-1–transfected Cos-7 (*Cos-7 TCF-1*) and Ikaros transfected Cos-7 (*Cos-7 Ikaros*), and nuclear extracts from a teratoma cell line (*F9*), a fibroblast cell line (*NIH3T3*), T lineage cell lines (*EL4*, *BW5147*, *YT5*, and *BgV*), B lineage cell lines (*70Z/3* and *A20.2J*) and myeloid cell lines (*P388* and *M1*), and adult thymocytes were size-fractionated by SDS-PAGE. The electrophoresed lysates or nuclear extracts were transferred onto a nylon membrane, and were reacted with anti–TCF-1 or anti-Ikaros serum at a dilution of 1:500. The sizes of molecular mass markers are indicated in kilodaltons. Preimmune rabbit sera do not show any band (data not shown).

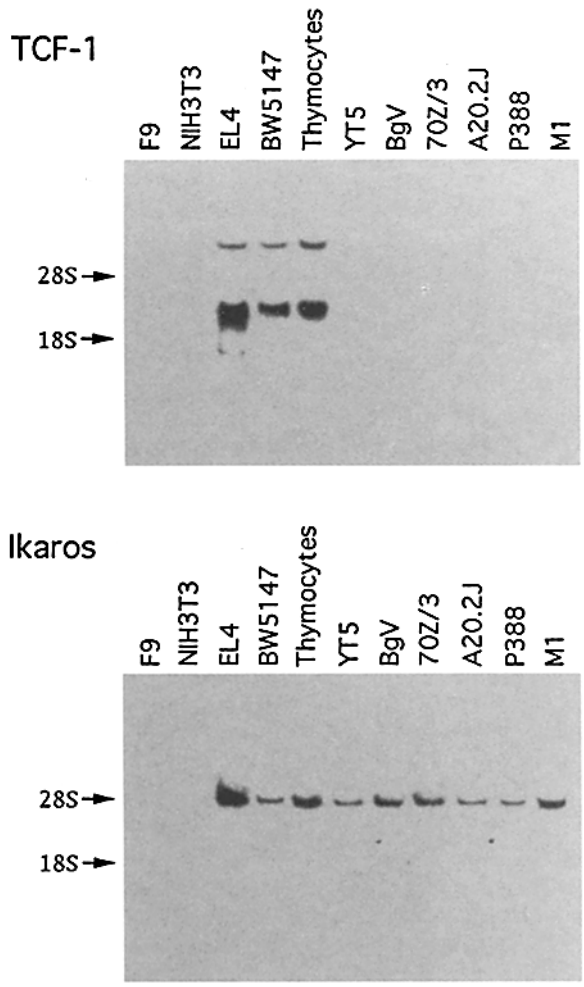

Figure 4. Northern blot analysis for expression of TCF-1 and Ikaros in various cell lines and adult thymocytes. Total RNA (20 μg) from various cell lines and adult thymocytes was size-fractionated by gel electrophoresis, transferred onto a nylon membrane, and hybridized with a probe of the coding region of TCF-1 or Ikaros cDNA.

began to be produced at a very early stage of T cell development, these experiments could not clarify whether these nuclear proteins were produced by a small number of day 12 FT or day 13 FT cells, or they were produced in small amounts by all the FT cells. Immunocytochemical and flow cytometric investigations would be effective in clarifying this point and also identifying the differentiational stage of thymocytes at which these nuclear factors are first synthesized. Anti–human GATA-3 mAb can be used to detect mouse GATA-3 and is commercially available, but anti–TCF-1 is unavailable so far.

We raised rabbit antibodies to TCF-1 and also Ikaros (for details see Materials and Methods), the latter of which has been shown to be expressed by all lymphocytes, including FT cells and lymphoid progenitors (34). Western blot analysis was performed with whole-cell lysates or nuclear extracts of various cell lines and adult thymus cells (Fig. 3). 28- and 48-kD bands are clearly seen in Cos-7 cells transfected with TCF-1 and Ikaros cDNA, respectively. No anti–TCF-1–reactive band was observed in untransfected Cos-7 cells, a teratoma line F9, a fibroblast line NIH3T3, B lineage lines 70Z/3 and A20.2J, a monocyte line P388, or a macrophage/granulocyte precursor line M1. On the other hand, the anti–TCF-1 antiserum detected several bands in two (EL4 and BW5147) out of four T lineage lines and adult thymocytes. The 28-kD band represents the same protein as that produced in Cos-7 cells transfected with TCF-1 cDNA. On the other hand, it has not exactly been

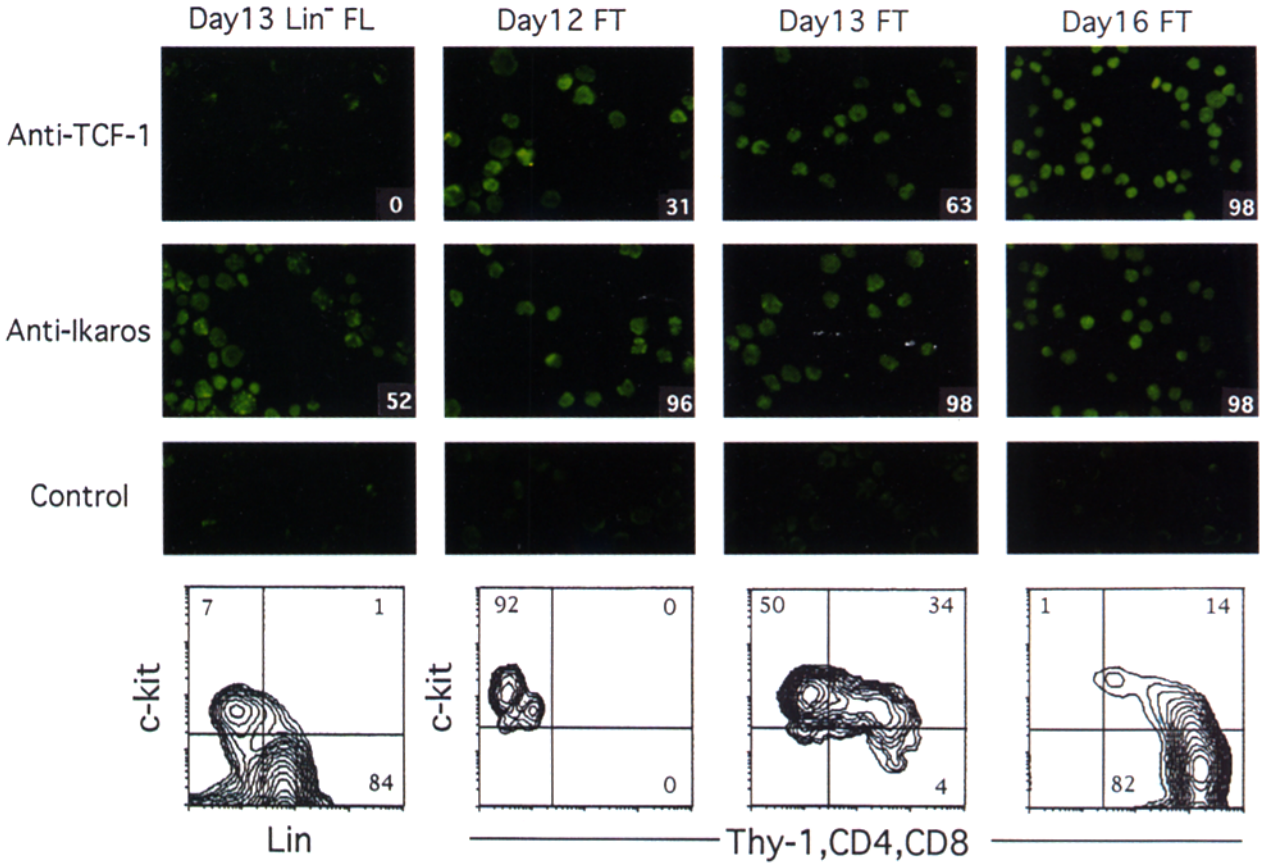

Figure 5. Immunocytochemical staining of FT cells and Lin⁻ FL cells with anti–TCF-1 and anti-Ikaros. FT cells from days 12, 13, and 16 fetuses and FACS®-sorted Lin⁻ FL cells from day 13 fetuses were reacted with anti–TCF-1, anti-Ikaros, or normal rabbit serum (*Control*) at a dilution of 1:500, and stained by FITC-conjugated goat anti–rabbit IgG. Photomicrographs: ×400. Percentages of TCF-1⁺ and Ikaros⁺ cells, as determined by inspection of >1,000 cells, is demonstrated at the lower right edge of each photograph. Flow cytometric profiles for c-*kit* vs Lin markers (TER119, Mac-1, Gr-1, and B220) on day 13 FL cells, and those for c-*kit* vs T lineage markers (Thy-1, CD4, and CD8) on FT cells, are shown at the lowest lane. The percentage of cells that fall into each quadrant is indicated.

determined whether other bands represent the products of alternative splice forms of mRNA or other unknown cross-reactive proteins. However, circumstantial evidence including the fact that protein bands are observed only in cells positive by Northern blot analysis (Fig. 4) strongly suggests that the larger and smaller proteins are the products of alternative splice forms of TCF-1 (see Discussion).

Proteins reactive with anti-Ikaros are produced by all hematopoietic cell lines and adult thymocytes, and one of these proteins is the same as that produced in cDNA transfected Cos-7 cells (Fig. 3). One to three other bands are also seen in all these cell lines and thymocytes. Because no band is observed in nonhematopoietic lines that are negative by Northern blot analysis (Fig. 4) and the molecular weights of the detected proteins correspond to those of alternative splice forms of Ikaros (50), it is highly probable that every band represents a product of the Ikaros gene.

Detection of TCF-1–producing Cells in FT by Immunocytochemical Staining. FT cells from fetuses of various gestational ages and FACS®-sorted Lin⁻ FL cells from day 13 fetuses were settled on slide glasses by cytocentrifugation. Cells were indirectly stained with anti–TCF-1 and anti-Ikaros antisera, as described in Materials and Methods, and the fluorescence micrographs are shown in Fig. 5. Flow cytometric profiles of surface staining of the same cells with anti–c-*kit* and anti-Lin markers are also shown. The expression of the Ikaros protein was observed in about half of the Lin⁻ FL cells and in virtually 100% of FT cells. On the other hand, no TCF-1–positive cell was found in FL. About 30% of day 12 FT cells were positive for TCF-1 expression, and the proportion TCF-1⁺ cells increased with gestational age to reach ~100% on day 16. The expression of TCF-1 in FT cells is not directly correlated with the surface expression of Thy-1, CD4, and CD8.

Expression of TCF-1 and GATA-3 before Intracellular Expression of CD3ε. As has previously been characterized (16), all day 12 FT cells show a phenotype of CD4⁻CD8⁻ c-*kit*⁺ CD44⁺CD25⁻ and do not express CD3ε on their surface. Day 13 FT cells are CD3ε⁻ and a large majority of these cells are still c-*kit*⁺, whereas about a quarter of them express CD25. The data confirming these previous findings are shown in Fig. 6 *A* and Fig. 7, *A* and *B*. Since immunocytochemical experiments in the preceding section indicated that ~30% of day 12 FT cells were positive for TCF-1

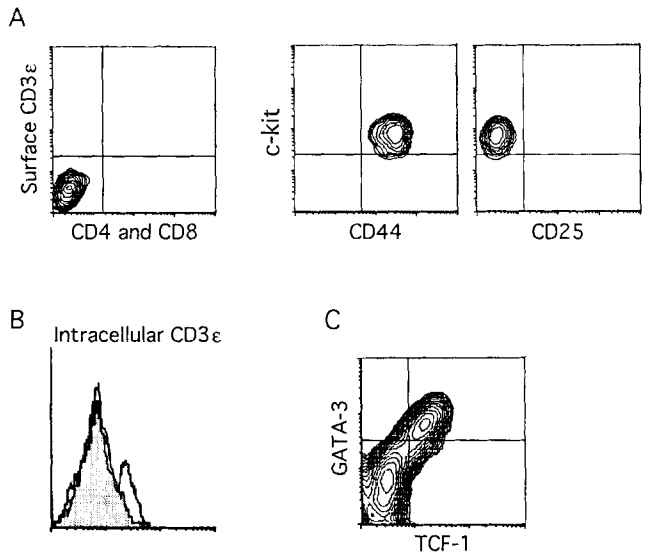

Figure 6. Flow cytometric analysis for the intracellular expression of TCF-1, GATA-3, and CD3ε in day 12 FT cells. (*A*) Surface expression profiles for CD3ε vs CD4 and CD8, c-*kit* vs CD44, and c-*kit* vs CD25. (*B*) Intracellular expression of CD3ε. The shaded histogram represents background staining with APC-streptavidin. (*C*) Two-color staining profile for TCF-1 vs GATA-3. In this experiment, fixed and permealized cells were reacted with biotinylated anti–GATA-3 followed by staining with APC-conjugated streptavidin and FITC–anti–TCF-1.

(Fig. 5), day 12 FT cells were thought to be useful for comparing the expression of TCF-1 with that of GATA-3 or IC-CD3ε. Day 12 FT cells were fixed, permealized, and stained with anti-CD3ε or dual stained with anti–TCF-1 and anti–GATA-3. The results are shown in Fig. 6, *B* and *C*. About 20% of the cells were positive for IC-CD3ε. The profile of double staining with anti–TCF-1 and anti–GATA-3 indicated that these two transcription factors were produced in the same cells of ~30% of day 12 FT.

A more detailed investigation to identify the phenotypes of cells expressing TCF-1 was performed with day 13 FT cells, because day 13 FT cells are still $CD4^-CD8^-CD3\epsilon^-$, but they begin to express CD25 (Fig. 7, *A* and *B*). For investigating TCF-1 expression, three-color analysis was performed with FITC–anti–TCF-1, APC–anti-CD3ε, and PE–anti-CD25. The results shown in Fig. 7 *C* strongly suggest that CD3ε proteins are produced after the production of TCF-1. The expression of IC-CD3ε, however, is not necessarily related to the surface expression of CD25 (Fig. 7 *D*), and day 13 FT cells can be divided into four populations of $CD25^-$IC-$CD3\epsilon^-$, $CD25^+$IC-$CD3\epsilon^-$, $CD25^-$IC-$CD3\epsilon^+$, and $CD25^+$IC-$CD3\epsilon^+$. The expression of TCF-1 in these populations is shown in Fig. 7 *E*. It can be seen that a small proportion of $CD25^-$IC-$CD3\epsilon^-$ cells express TCF-1 at low levels, whereas all cells in the other populations express this nuclear protein at high levels. We also found that the expression profile of GATA-3 in these four subpopulations was virtually the same as that of TCF-1 (data not shown).

Inhibition of T Cell Development in FT Organ Cultures by AS Oligonucleotides for TCF-1 and GATA-3. Experiments were undertaken to examine whether TCF-1 and GATA-3 play any role in T cell development. Lin^-c-kit^+ cells from day 13 FL or c-kit^+ cells from day 13 FT were isolated and cultured with dGuo-treated FT lobes in the presence of S or AS oligonucleotides for these transcription factors. Groups given no oligonucleotides or S oligonucleotides were also set up in parallel. Cells were harvested on day 5 of culture, enumerated, and assayed for expression of surface markers. The results shown in Table 1 indicate that T cell development from FL progenitors is severely inhibited by the addition of AS but not S oligonucleotides for either TCF-1 and

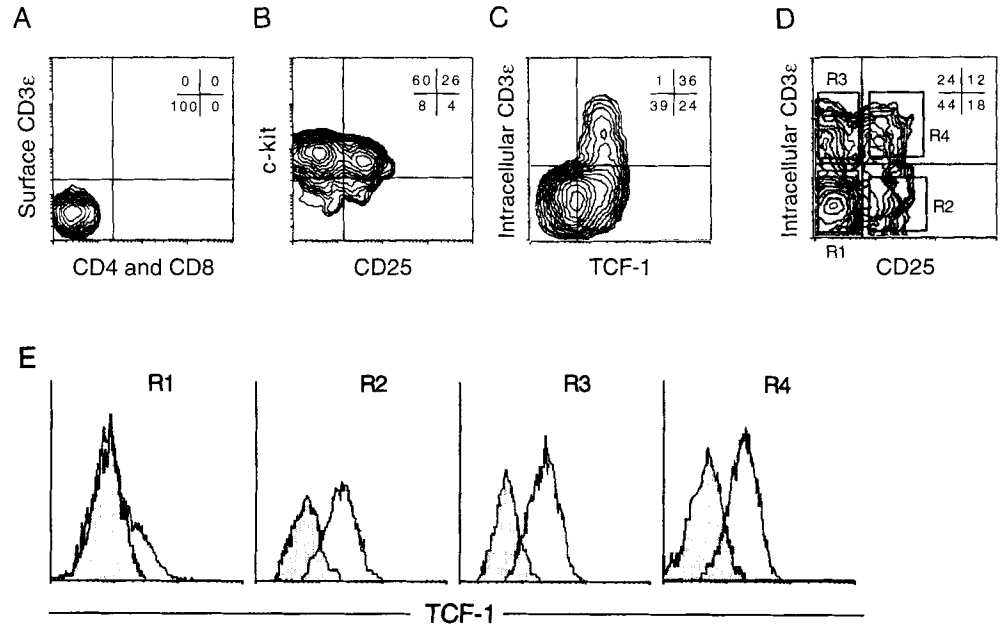

Figure 7. Flow cytometric analysis for comparing the expression levels of TCF-1 and IC-CD3ε in day 13 FT cells. (*A*) Surface expression profile for CD3ε vs CD4 and CD8. (*B*) Surface expression profile for c-*kit* vs CD25. (*C*) Two-color staining profile for IC-CD3ε vs TCF-1. (*D*) Two-color staining profile for surface CD25 vs IC-CD3ε. The percentage of cells falling into each quadrant is indicated in the inset of each panel (*A–D*). (*E*) Expression profiles of TCF-1 in cells included in areas R1, R2, R3, and R4 gated on D. Shaded histograms represent background staining with FITC-conjugated normal rabbit IgG.

Table 1. *Inhibition of T Cell Development in Organ Cultures by AS Oligonucleotides for GATA-3 and TCF-1*

Progenitor cells*	Oligonucleotide‡	No. of recovered Thy-1$^+$ cells per lobe ($\times 10^3$)	Percentage in Thy-1$^+$ cells	
			CD4$^-$CD8$^-$	CD4$^+$CD8$^+$
FL Lin$^-$c-*kit*$^+$ cells (10^3)	None	5.0	98	0
	GATA-3(S)	4.5	96	0
	GATA-3(AS)	0.8	96	0
	TCF-1(S)	4.8	98	0
	TCF-1(AS)	1.0	97	0
	GATA-3(S)+TCF-1(S)	4.0	98	0
	GATA-3(AS)+TCF-1(AS)	0.1	98	0
FT c-*kit*$^+$ cells (5×10^2)	None	24.0	44	24
	GATA-3(S)	20.0	40	21
	GATA-3(AS)	10.0	64	11
	TCF-1(S)	21.0	42	22
	TCF-1(AS)	12.0	51	11
	GATA-3+TCF-1(S)	19.0	43	20
	GATA-3(AS)+TCF-1(AS)	9.5	56	9

*Lin$^-$c-*kit*$^+$ population of day 13 FL cells or c-*kit*$^+$ population of day 13 FT cells were cultured under HOS conditions together with a dGuo-treated FT lobe in a U-bottom well for 5 d. The number of progenitors cultured with a lobe is shown in the parenthesis.
‡S or AS oligonucleotides were inoculated into the well at the final concentration of 100 μg/ml. In cases where S or AS oligonucleotides for both GATA-3 and TCF-1 were added, the final concentration of each oligonucleotide was 50 μg/ml.

GATA-3. Moreover, T cell development is completely abolished by the addition of both AS oligonucleotides. On the other hand, the effect of these AS oligonucleotides is only slight, if any, on T cell generation from day 13 FT c-*kit*$^+$ cells, which contain cells already expressing TCF-1 and GATA-3. Similar results were obtained when the organ culture was continued for 8 d (data not shown). These results strongly suggest that TCF-1 and GATA-3 are indispensable for the very early stages of differentiation and growth of T lineage cells.

Discussion

The present study showed that the transcription factors TCF-1 and GATA-3 were produced by ~30% of the cells in the CD44$^+$CD25$^-$ population of FT, which has been known as the most immature subset, and that the production of these nuclear proteins is immediately followed by the intracellular expression of CD3ε. It was further shown that the AS oligonucleotides for TCF-1 or GATA-3 severely inhibited the T cell development from progenitors in FL, but not FT, indicating that these transcription factors play a critical role in a very early phase of T cell development.

The finding that TCF-1 and GATA-3 begin to be produced by ~30% of the most immature subset of FT cells was made by flow cytometric analysis in which antibodies to these transcription factors were used for intracellular staining. Anti–GATA-3 mAb used in the present work has been produced by immunizing a mouse with recombinant human GATA-3 protein (51), and this mAb cross-reacts with mouse GATA-3. Anti–TCF-1 produced by immunizing a rabbit with the recombinant mouse TCF-1 protein exhibits several protein bands other than that corresponding to the protein used for immunization of a rabbit. It seems unlikely that these bands represent cross-reactive irrelevant proteins for the following reasons: (*a*) no reactive bands were seen in non–T lineage cell lines (Fig. 3); (*b*) none of the protein bands was detected in T lineage lines YT5 and BgV that showed no detectable band in the Northern blot analysis (Figs. 3 and 4); (*c*) the presence of several alternative splice forms of TCF-1 mRNA is known (reference 33 and see Fig. 4); and (*d*) addition of AS oligonucleotides for TCF-1 into the culture of EL4 cells resulted in a reduction of the density of all bands to the same extent (data not shown). For these reasons, we regard that all these protein bands represent the products of alternative splice forms of TCF-1. On the other hand, since our anti–TCF-1 does not discriminate between these proteins and we have not identified the protein(s) produced by FT cells, so far, it is unclear which of these proteins is critical for early T cell differentiation.

It has not been clarified whether the commitment of hematopoietic stem cells to the T lineage occurs prethymically or in the thymus. Previous studies using genetically marked bone marrow cells (52, 53) suggested a prethymic commitment to the T lineage. In these studies, however, a possibility cannot be excluded that genetically marked mul-

tipotent stem cells immigrated into the thymus are induced to develop exclusively into T cells. On the other hand, the presence in the thymus of progenitors capable of generating B cells (13, 14) and myeloid cells (12, 15) has repeatedly been shown, suggesting that the progenitors immigrating into the thymus retain the multipotent stem cell activity. These findings, however, do not formally preclude the possibility that T lineage commitment occurs before immigrating into the thymus because it is possible that all three types of progenitors precommitted to the T, B, or myeloid lineage immigrate into the thymus. Trials to discriminate between prethymic and intrathymic progenitors on the basis of their surface phenotype were unsuccessful, since it is hard to exactly identify or isolate these progenitors. It is only possible to compare progenitor cell–enriched populations from FL, adult bone marrow, FT, or adult thymus, and the phenotypes of these progenitor cell–enriched populations from different sources resemble each other quite well (14, 26, 54).

Regardless of the similarity in phenotypes of progenitors from different sources, the T cell–producing activity of these progenitors is markedly different from each other. For example, a nearly two times longer period is required for FL progenitors than for FT progenitors to give rise to T cells when cocultured with a dGuo-treated FT lobe (Kawamoto, H., N. Hattori, and Y. Katsura, manuscript in preparation). A larger difference has been shown to exist between progenitors from adult bone marrow and thymus (14, 55, 56). Since "thymus-type" progenitor cells, which are able to generate T cells within a few days, have never been detected in bone marrow or FL (56, 57; Kawamoto, H., N. Hattori, and Y. Katsura, manuscript in preparation), it is probable that a very small number of multipotent stem cells that constantly generate thymus-type progenitors exist in the thymus. If this is the case, the prethymic progenitors or stem cells that immigrate into the thymus may immediately be induced to generate thymus-type progenitors, although such a dramatic change of progenitor activity has not been correlated with a change in surface phenotype. The present study showed that synthesis of TCF-1 and GATA-3 occurred in a subpopulation of the most immature subset of FT cells before the production of the CD3ε protein. The production of these nuclear proteins could be used as the earliest differentiation markers. On the other hand, the subpopulation of $CD44^+CD25^-$ cells that have not yet expressed these transcription factors may be even more primitive. Further investigation is in progress to elucidate the relationship between the expression of these transcription factors and commitment to the T lineage.

AS oligonucleotides for either TCF-1 or GATA-3 inhibited T cell generation from FL progenitors in the FT organ culture system. The inhibitory effect of TCF-1 AS oligonucleotides conforms to the recent finding that in the TCF-1 null mutant mice, the numbers of thymocytes is reduced 10–100-fold, compared to wild type littermates (58). The importance of GATA-3 in the hematopoiesis was shown in GATA-3 knockout mice (59), in which the hematopoiesis in FL is severely impaired. The present finding that the suppressive effect was much stronger when AS oligonucleotides for both TCF-1 and GATA-3 were added strongly suggests that these two transcription factors cooperate in early T cell differentiation. On the other hand, the inhibitory effect of AS oligonucleotides on T cell development from FT progenitors was only slight, and the inhibition was not strengthened by the addition of AS oligonucleotides for both TCF-1 and GATA-3. As seen in Fig. 7, c-*kit*$^+$ FT cells used as progenitors in this experiment include cells already producing TCF-1 and GATA-3 proteins. These thymic progenitors may be resistant to treatment with the AS oligonucleotides for their differentiation and growth.

It has been shown that TCF-1 binds to the enhancer of CD3ε (33), whereas no GATA-3–binding site has been discovered in the enhancer region of the CD3ε gene. Although a GATA-3–binding site exists in the enhancers of TCRβ, TCRδ, TCRα, CD4, and CD8 genes (60, 61), it seems improbable that the production of GATA-3 protein is required at the $CD44^+CD25^-$ stage, since no transcript of these genes has ever been detected at this or the next $CD44^+CD25^+$ stage (10). Identification of the target(s) of GATA-3 as well as TCF-1 will be one of the most important steps for elucidating the molecular mechanism of early T cell differentiation. Various transcription factors, including LEF-1 (46, 47, 62), Sox-4 (49), and Ikaros (34) investigated in the present work, are expressed at very early FT. Although the expression of these molecules is not necessarily T lineage specific, it is probable that they cooperate with TCF-1 and GATA-3. Studies are in progress to clarify the cooperation of transcription factors in T cell development.

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