

# A Novel Transcription Factor, T-bet, Directs Th1 Lineage Commitment

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## Summary

Naive T helper cells differentiate into two subsets, Th1 and Th2, each with distinct functions and cytokine profiles. Here, we report the isolation of T-bet, a Th1-specific T box transcription factor that controls the expression of the hallmark Th1 cytokine, IFN $\gamma$ . T-bet expression correlates with IFN $\gamma$  expression in Th1 and NK cells. Ectopic expression of T-bet both transactivates the IFN $\gamma$  gene and induces endogenous IFN $\gamma$  production. Remarkably, retroviral gene transduction of T-bet into polarized Th2 and Tc2 primary T cells redirects them into Th1 and Tc1 cells, respectively, as evidenced by the simultaneous induction of IFN $\gamma$  and repression of IL-4 and IL-5. Thus, T-bet initiates Th1 lineage development from naive Thp cells both by activating Th1 genetic programs and by repressing the opposing Th2 programs.

## Introduction

T helper (Th) lymphocytes differentiate into two distinct subsets, Th1 and Th2, as defined by functional abilities and cytokine profiles (Mosmann and Coffman, 1989; Seder and Paul, 1994). Th1 cells mediate delayed type hypersensitivity responses and provide protection against intracellular pathogens and viruses. Th2 cells provide help to B cells and eradicate helminthes and other extracellular parasites. The functional differences between Th subsets are explained primarily through the activities of the cytokines they secrete. IFN $\gamma$  is the signature cytokine of Th1 cells, which also produce IL-2 and TNF $\beta$  (LT). IL-4 is the corresponding signature cytokine of Th2 cells, which also secrete IL-5, IL-6, IL-9, and IL-13.

Upon encountering antigen, the naive CD4<sup>+</sup> T helper precursor (Thp) cell enacts a specific genetic program

that results in differentiation toward the Th1 or Th2 lineage. Although this differentiation program can be influenced by the antigen concentration or through ligation of costimulatory molecules, the most potent regulators of Th cell differentiation are undoubtedly cytokines (reviewed in Abbas et al., 1996; Constant and Bottomly, 1997; O'Garra, 1998). The critical Th2-inducing cytokine is IL-4, which mediates its effects on naive Thp cells through the Stat6 signaling pathway. Mice lacking either IL-4, IL-4 receptors, or Stat6 fail to develop Th2 cells (reviewed in Nelms et al., 1999). IL-12, acting via the Stat4 signaling pathway, is the primary inducer of Th1 development. Mice deficient in IL-12, IL-12 receptor  $\beta$ 1, or Stat4 have markedly reduced Th1 responses (reviewed in Gately et al., 1998). While mice lacking IL-18 or IFN $\gamma$  have defective *in vivo* Th1 responses, these cytokines do not directly induce Th1 differentiation but rather augment IL-12-induced Th1 development (O'Garra, 1998).

Th1 and Th2 cytokines both promote the growth/differentiation of their subset and inhibit the growth/differentiation of the opposing subset. For example, IL-4 produced by Th2 cells acts in a positive manner to activate IL-4 receptors present on naive Thp cells to initiate the Th2 differentiation process (Seder and Paul, 1994). In addition, IL-4 acts in a negative manner to extinguish IL-12 receptor expression on the developing cells, committing them to the Th2 lineage (Szabo et al., 1997; Ouyang et al., 1998). Moreover, IL-10, a cytokine preferentially expressed by Th2 cells, inhibits IL-12 production by activated macrophages, thus indirectly inhibiting Th1 development (Fiorentino et al., 1991). Th1-produced IFN $\gamma$  promotes Th1 development by inducing IL-12 production from activated macrophages and IL-12 receptor expression on antigen-activated Thp cells (Ma et al., 1996; Szabo et al., 1997), while directly inhibiting the growth of Th2 cells (Gajewski et al., 1989). Thus, the cytokines produced by each Th subset form positive and negative feedback loops that drive T helper cell polarization. The dual activity of Th cytokines results in the predominance of a particular Th phenotype as exemplified by the Th polarity seen in chronic infectious diseases (Romagnani, 1994).

Significant progress has been made in identifying the transcription factors that control the transition of a Thp to a Th2 cell. The Th2-specific transcription factor, c-Maf, along with NFAT and NIP45 (an NFAT interacting protein), confers on a non-T cell the ability to produce endogenous IL-4 (Hodge et al., 1996), and c-maf-deficient mice have severely impaired IL-4 production (Kim et al., 1999). GATA-3, like c-maf, is Th2 specific and upregulated during Th2 differentiation (Zhang et al., 1997; Zheng and Flavell, 1997). However, GATA-3 appears to regulate a broad spectrum of Th2 cytokine genes as evidenced by the ectopic expression of GATA-3 or dominant-negative GATA-3 in transgenic and retroviral transduction studies (Zheng and Flavell, 1997; Ouyang et al., 1998; Ferber et al., 1999; Zhang et al., 1999). Additionally, Stat6 was necessary for the chromatin remodeling of the IL-4/IL-13 locus found to occur in developing Th2 cells (Agarwal and Rao, 1998). Thus,

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GATA-3 and Stat6 appear to have a more global influence on Th2 cytokine gene expression and may mediate this effect by directly altering the chromatin structure of Th2-specific gene loci.

In contrast to Th2 differentiation, very little is known about the molecular basis of Th1 differentiation. The only known transcription factors whose absence results in a failure to generate Th1 cells are Stat4 (Kaplan et al., 1996; Thierfelder et al., 1996) and IRF-1 (Lohoff et al., 1997; Taki et al., 1997), neither of which is Th1 restricted. The lack of Th1 cells in IRF-1-deficient mice is likely due to its direct effect in controlling transcription of the IL-12 gene although IRF-1 was shown to be a Stat4 target gene in T cells (Coccia et al., 1999), and thus a defect in the T cell compartment may also contribute to this phenotype in vivo. The Ets family member ERM, which is induced by IL-12 in a Stat4-dependent manner, has recently been reported to be Th1 specific, but it does not affect the production of Th1 cytokines (Ouyang et al., 1999).

To isolate transcription factors that account for tissue-specific expression of Th1 type cytokines, we used a yeast expression cloning strategy in combination with RDA (representational difference analysis). This methodology resulted in the isolation of a novel protein belonging to the T box family of transcription factors. Members of this family, whose founding member is the *brachyury* gene, play critical roles in diverse developmental processes (Papaioannou, 1997; Smith, 1997). We have named our gene *T-bet* (*T*-box expressed in *T* cells) since it is expressed selectively in thymocytes and Th1 cells. T-bet is a Th1-specific transcription factor that plays a central role in Th1 development. T-bet transactivates the IFN $\gamma$  gene, induces IFN $\gamma$  production in retrovirally transduced primary T cells, and redirects polarized Th2 cells into the Th1 pathway.

## Results

### Cloning of a Novel T box Transcription Factor, T-bet

We reasoned that Th1-specific transcription factors should control the expression of the IL-2, IFN $\gamma$ , or TNF $\beta$  genes. Several transcription factors are important for IL-2 gene transcription, including NFAT, AP-1, NF- $\kappa$ B, HMG, and Oct proteins, but none of these accounts for the tissue-specific expression of IL-2 in Thp and Th1 cells. However, since the Th1-specific region of the IL-2 promoter had been well localized (Brombacher et al., 1994; Lederer et al., 1994), we chose a yeast one hybrid approach using this region of the IL-2 promoter (-400 to -40) linked to the *HIS3* reporter gene. The EGY48 yeast strain was stably integrated with the IL-2 promoter/HIS3 construct and transformed with an activated Th1 cell library of cDNAs fused to the B42 activation domain. Of  $5.6 \times 10^6$  transformants, 488 grew on agar plates containing the selection media. Of the 210 clones tested during the secondary screen, 72 were induced by galactose, indicating promoter-induced specificity. We then utilized a screening procedure using Th1- and Th2-specific radiolabeled probes generated via RDA from mRNA from anti-CD3-activated Th1 (PL17) and Th2 (D10) clones. Of the 16 clones screened, 8 were positive with the Th1- but not the Th2-specific probe (data not shown).

Restriction enzyme analyses and sequencing revealed that all eight clones were related or identical. Sequence comparison of these clones with the NCBI GenBank sequence database yielded homology with the T box family of transcription factors (Figure 1A). We named our novel gene *T-bet*, for T box expressed in T-cells. T-bet is a 530-amino acid protein with a 189-amino acid T box DNA-binding domain (underlined). The human T-bet ortholog was subsequently cloned and shares 88% amino acid identity to murine T-bet (data not shown). The T box family of transcription factors is defined by homology among family members in a 200-amino acid DNA-binding domain called the T box (reviewed in Papaioannou, 1997; Smith, 1997). The T-bet DNA-binding domain (residues 138–327) is most similar to murine T brain and *Xenopus* eomesodermin and thus places T-bet in the Tbr1 subfamily of the T box gene family. These three proteins share 69% amino acid identity in the T box region (Figure 1B). Outside of the T box domain, T-bet bears no significant sequence homology to other T box family members or to any other known gene.

### T-bet Expression Is Restricted to the Th1 Subset and Is Regulated by Signals Transmitted via the T Cell Receptor

T-bet was isolated from a Th1 cDNA library and hybridized to a Th1-specific probe. To confirm the apparent Th1 restricted nature of T-bet, we examined T-bet expression in T helper cell clones. Northern blot analysis of RNA isolated from Th1 cell clones (PL17, OF6, and AE7) or Th2 clones (CDC35 and D10) revealed T-bet transcripts only in the Th1 clones (Figure 2A). Further, T-bet expression was augmented by signals transmitted via the TCR as evidenced by the induction of T-bet transcripts by anti-CD3 at 6 and 24 hr. T-bet transcripts were not detected in untreated or PMA plus ionomycin-treated EL4, a thymoma, M12, a B cell lymphoma, or Jurkat, a T cell lymphoma. To examine T-bet protein expression, we generated monoclonal (mAb) and polyclonal antisera specific to T-bet. Western blot analysis revealed an approximately 62 kDa protein present in nuclear extracts prepared from resting and PMA/ionomycin-activated (1 hr) AE7 cells but not activated D10 cells (Figure 2B, left). T-bet protein was also directly visualized by FACS analysis of permeabilized cells using the T-bet-specific mAb 4B10 (Figure 2B, right). T-bet protein was undetectable in D10 cells and present at low levels in unstimulated AE7 cells and at increased levels in stimulated AE7. Taken together, these experiments demonstrate that in T cell clones, T-bet is selectively expressed in Th1 cells and its level of expression is augmented by signals mediated through the TCR.

A multiple organ Northern blot revealed T-bet transcripts only in lung, thymus, and spleen, a distribution suggestive of T cell restricted expression (Figure 3A). To examine the kinetics of T-bet protein expression during primary stimulation of T cells, DO11.10 TCR transgenic splenocytes were activated in the presence of Th1- or Th2-inducing conditions and nuclear extracts prepared on day 0, 2, 3, 4, 6, and 8 (Figure 3C). T-bet protein was not expressed in resting splenocytes but was rapidly and selectively induced in Th1- but not Th2-driven cells. Since naive Thp cells were not purified, NK cells and

A

GCCGGGGTGCAGAGAAGC GCCCCAGCCCTAAGACCTT	CCAGGAGCAGCTGCGCGCT CGGGTCTCTCGAGCGCTGC	ATCCAACGGCGCTCAGGAG TGGAAAGCGCCAGCCCGC	CCAAGCTCCAGCTCCCGC TCGGATGGGCATCGTGAGC M G I V E	TCCAGTGAAGTTTCATGGT CGGGCTCGGAGACATGTGT P G C G D M L	CTTCGGAGCCGGCCCCGTC ACCGACCGAGCCGATGCC T G T E P M P	120 240 19
GAGTGACGAGGGCCGGGGC S D E G R G G A L V P A GGGCTACCCGCGCTGGATG G Y P P V D GCTCAGCAACACCTGTTGT L S N H L L AAGCCATTACAGATGTTTG S H Y R M F TGTCACCCAGACTCCCCCA V H P D S P GCAGTCTCCACAAGTACC Q S L H K Y TGCCAGTACTGCTACCCAGA A V T A Y Q CTGCCACCTGAGCCCACT S P P G P N GATCTCAGACCTTACTGGC I S Q P Y W CCGGGCAACAGACTCTGG R G Q D V L GGGATCCTCAGAGAACAGG G S S E E Q CTATCTCCAGTGGCGACA Y P S S G D TGCCCACTAACTAGAAAAC TGTTCTCTGCCCAACAGGA ACTCCAGAGAGTTTGGACC GTCAGACCTGTGTTAATC GAGAGGGTGGGTGCAGAGGA TTTATTGTAGAGAGTGTGT	CCGAGCGGACCAACAGCAT P G A D Q Q H A P G R F L G GCTACCCGCGCTGACCCG G Y P A P D P GGTCCAAGTTCAACCAGCAC W S K F N Q H TGGATGTGGTCTTGGTGGAC V D V V L V D ACACCGGAGCCCACTGGATG N T G A H W M AGCCCGGCTGCACATCGTG Q P R L H I V ACGCAGAGACTCACTCAGCTG N A E I T Q L GTCAACTGCTTGGGGAGAC C Q I L G G D TGGGACACCTCGGAAACAC L G T P R E H CGCCTGGAGCTGGTTGGCC A P G A G W P GCTCCTCCCTCGCTGGG G S S P S L W GCTCCTCCCGCTGGGGCC S S S P A G A AGAGCGGGGCTGAGAGGCC AAATACGACAGGAGTCCCC CAGAGGGACTTCATGGCTTT TCTGACCTGAAAGTGAAGAT AGGCTTTGAAGGCTGCACAT CTGGATGTATTTCTCTGTT	CSTTTCTCTATCCCGAGCC R F F Y P E P TCCTTCGCTACCCGCGCCG S F A Y P P R CGCGGGGGCTCACCAGC R A G L Y P G CAGACAGAGATGATCATCAC Q T E M I I T CAGCACCCTGGCGTACCA Q H H W R Y Q CCCCAGGAAGTTTCATTTGG R Q E V S F G GAGGTGAATGATGGAGAGCC E V N D G E P AAAAATCGACAACAACCCCTT K I D N N P F CCCTACTCACCTCTCTATFC P F S P L L S AGTTATGAAGCGGAGTCCG S Y E A E F R GTGCCCTCAATACCCCGC V A P Q Y P P CCTGAGGTCACTCCCTCCA P E V T S L Q CCTTCTCCTTTGATAGGA P S P F D K E CGAGCTCTTCCCCATCCCTT TGCCCTTTCTCTGCCCCGAA CTGCGAGGTGAGGGGTCGG ACACGCATTTTACAACAGC TTACCCAGGCGAGGCTCACT TTGCATCACTTCTGGAAT	GGGGCACAGGACCCGACCC G A Q D P T GGCTCAGGTGGCTGGCTTTC A Q V A G F GCCGCGAGGACTACCGAT P R E D Y A TAAGCAAGGACGGCAATGT K Q G R R M GAGCGGCAAGTGGGTGAGT S G K W V Q GAAGCTAAAGCTCACAACA K L K L T N AGAGGCTGCTGCAGTGCTT E A A C S A TGCCAAAGGATCCGGGAGA A K G F R E CAACCAATATCTGTTCCTCA N Q Y P V P AGCTGTGAGCAAGGCCA A V S M K P CAAGATGAGCCAGCTGGCT K M S P A G CCCGGAGCCGAGGACTCAG P E P S D S AACCGAAGGCCAGTTTATA T E G Q F Y CCCTGTATAGTGATTGGTGT CTACAGTCAACAGCTGGTG GGTGGGAGTCCAGGAGAGC CAGCCAAACAGAGAAGACTC TGAACCGGTGTACACACA AAACATGGACCT	TCCAGTGAAGTTTCATGGT CGGGCTCGGAGACATGTGT P G C G D M L ATCGCCGCGCAGGTAGCAGC D R R A G S S CGGGCTGGCGAGTTCCTC P G P G E F F TGCCCGGGGGTGGAGGTG L P A G L E V TCCCATTCTGTCTTACC F P F L S F T GTGGAAGGCAGAGGCAGC C G K A E G S ACAAGGGGCTTCCAACAAT N K G A S N N CTAACACACAGCTCTTACT S N T H V F T ACTTTGAGTCCATGTACGCA N F E S M Y A CCCGTTCTACCCGACCTT S R F Y P D L CACTCCTACCTCTGCCCCG T L L P S A P GGTCCGCGCCATGCGAAGT W F R P M R T GACTAGGCGAAGGAGACT G L G E G D T ATATTTTCCCAACTGAGAA N Y F P N *	CTTCGGAGCCGGCCCCGTC ACCGACCGAGCCGATGCC T G T E P M P CTGGGAGCCCTACTCTGG L G T P Y S G CGGGCTGGCGAGTTCCTC P P P A G A E TCTGGAAGCTGAGAGTCCG S G K L R V A GTGGCCGGCTGGAGCCAC F A G L E P T ATGCCAGGAAACCGCTTATA V A G N R L Y GTGACCCAGATGATCGTCT N T Q M I V L VTTCAGAGACCCGATTTACT F Q E T Q F I CTGTGTGATACGAGTGTCCC S V D T S V P CCAGGCCAGCCCAAGGATAT P G Q P K D M GGGCCACTGTGCCCTACTA G P T V P Y Y CTGCCATGGACCCGGCCCT L P M D P G L AAGAGGAGGAGGATATCCCC K R R R I S P AATGCCCTGAATGGAAAG GGATTTGGGTTTACTTCT CCATGGAGAACCGAGAATGG TCAGTAACTTTCAACTGTG GGCCACTGGAGGAGACAA CTTCGGGAGGGGGAGGCTA	

B

	136	150	151	165	166	180	181	195	196	210	211	225
T-bet	-RVALSNHLLWSKFN		<b>QHQT</b> EMIITK <b>QGRM</b>		<b>FPFL</b> SFTV <b>AGLEPTS</b>		<b>HYRM</b> FVDV <b>VLVDQ</b> HH		<b>WRY</b> QSGK <b>WVCGKAE</b>		<b>GSM</b> PNR <b>LYVHPD</b> SP	
T-brain	-QVFLCNRPLWLK <b>FKH</b>		<b>RHQ</b> TEMIITK <b>QGRM</b>		<b>FPFL</b> SFNIS <b>GLDPTA</b>		<b>HYNF</b> FVDV <b>VLADPNH</b>		<b>WRF</b> QSGK <b>WVCGKAD</b>		<b>TNV</b> QGN <b>RVMYHPD</b> SP	
eomesodermin	-QVFLCNRPLWLK <b>FKH</b>		<b>RHQ</b> TEMIITK <b>QGRM</b>		<b>FPFL</b> SFNIS <b>GLNPTA</b>		<b>HYNF</b> FVEV <b>VLADPNH</b>		<b>WRF</b> QSGK <b>WVCGKAD</b>		<b>NM</b> QGN <b>KVYVHPD</b> SP	
	226	240	241	255	256	270	271	285	286	300	301	315
T-bet	<b>NTGA</b> HWMR <b>QEIS</b> FGK		<b>LKLT</b> NNKGA <b>SNNV</b> TQ		<b>MIVL</b> QSLH <b>YQPR</b> LH		<b>IVEV</b> NDG <b>PEA</b> ACSA		<b>SNTH</b> V <b>FTF</b> Q <b>ETQ</b> FA		<b>VTAY</b> Q <b>NAEIT</b> Q <b>LKID</b>	
T-brain	<b>NTGA</b> HWMR <b>QEIS</b> FGK		<b>LKLT</b> NNKGA <b>SNNN</b> Q		<b>MVVL</b> QSLH <b>YQPR</b> LH		<b>IVEV</b> NDG <b>TED</b> TSQP		<b>GRVQ</b> T <b>FTF</b> P <b>ETQ</b> FA		<b>VTAY</b> Q <b>NDIT</b> Q <b>LKID</b>	
eomesodermin	<b>NTGA</b> HWMR <b>QEIS</b> FGK		<b>LKLT</b> NNKGA <b>SNNN</b> Q		<b>MIVL</b> QSLH <b>YQPR</b> LH		<b>IVEV</b> SED <b>GVED</b> LNSD		<b>AKNQ</b> T <b>FTF</b> P <b>ENQ</b> FA		<b>VTAY</b> Q <b>NDIT</b> Q <b>LKID</b>	
	316	330										
T-bet	<b>HNPF</b> AK <b>GFR</b> ENF---											
T-brain	<b>HNPF</b> AK <b>GFR</b> DNF---											
eomesodermin	<b>HNPF</b> AK <b>GFR</b> DNF---											

Figure 1. Cloning of a Novel Transcription Factor, T-bet

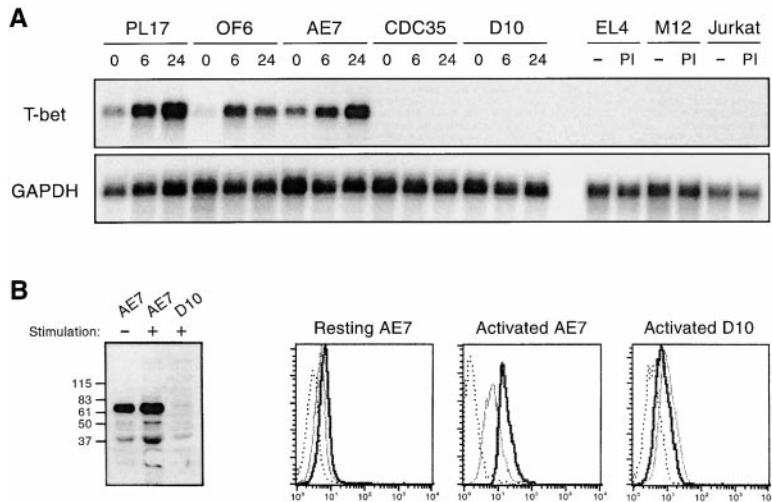
(A) Nucleotide and deduced amino acid sequence of murine T-bet. T-bet is a 530-amino acid protein with a 189-amino acid T box DNA-binding domain (underlined).  
(B) T-bet shares a region of homology with the T box family members T-brain and eomesodermin. Bold lettering indicates shared amino acid identity.

memory T cells along with other cell types could be contributing to T-bet expression. Therefore, we FACS purified naive DO11.10 cells and activated them with plate-bound anti-CD3 and anti-CD28 under Th1- or Th2-polarizing conditions and isolated RNA on day 0, 1, 2, 3, 5, and 8. In resting naive Thp cells, T-bet RNA was undetectable (Figure 3B). However, in T cells cultured under Th1- but not Th2-inducing conditions, abundant T-bet RNA expression was detected with maximal induction noted 24 hr after stimulation. Thus, T-bet expression is rapidly induced in primary T cells developing along the Th1 but not the Th2 pathway.

**T-bet Expression Correlates with IFN $\gamma$  Induction in NK and B Cells**

The T box domain of Xbra (a Brachyury ortholog) has been cocrystallized with DNA and demonstrates a novel sequence-specific DNA recognition architecture in which the protein contacts DNA in both the major and minor grooves (Müller and Herrmann, 1997). The nucleotides critical for binding are present within the proximal IL-2 promoter. Recombinant T-bet protein and a protein in

nuclear extracts from AE7 but not D10 cells that is supershifted by a T-bet mAb bind to a consensus T box site and to a T box site in the IL-2 promoter, and T-bet transactivates a multimerized T box site in transient transfection assays (data not shown). These data suggested that T-bet may activate IL-2 gene transcription. However, T-bet did not transactivate an IL-2 promoter/reporter either alone or in combination with other transcription factors (NFAT, AP-1, and NF- $\kappa$ B) (data not shown). Furthermore, Western blot analysis of nuclear extracts prepared from YT and NK3.3 (human NK), Jurkat, EL4, and M12 cells showed T-bet protein only in YT cells (Figure 3D). It was puzzling that two IL-2-producing cell lines, Jurkat and EL4, did not express T-bet, while the NK cell line YT, which does not produce IL-2, did express T-bet. One candidate gene that T-bet could potentially regulate, and which could explain its tissue distribution, was the cytokine gene IFN $\gamma$ . Th1 and NK cells express IFN $\gamma$ , which is induced upon TCR activation or cytokine stimulation, respectively. Additionally, IFN $\gamma$  is not expressed by Th2 cells, M12, EL4, or Jurkat cells.



**Figure 2. T-bet Expression Is Restricted to Th1 Clones**

(A) Expression of T-bet RNA in T helper cell clones. Th1 cell clones (PL17, OF6, and AE7) or Th2 clones (CDC35 and D10) treated with media or with plate-bound anti-CD3 for 6 and 24 hr, EL4, M12, and Jurkat untreated or PMA and ionomycin for 6 hr (PI).

(B, left panel) T-bet protein expression in Th cell clones. Western blot analysis was performed on nuclear extracts prepared from resting or PMA/ionomycin-stimulated (1 hr) bulk culture AE7 or D10 cells.

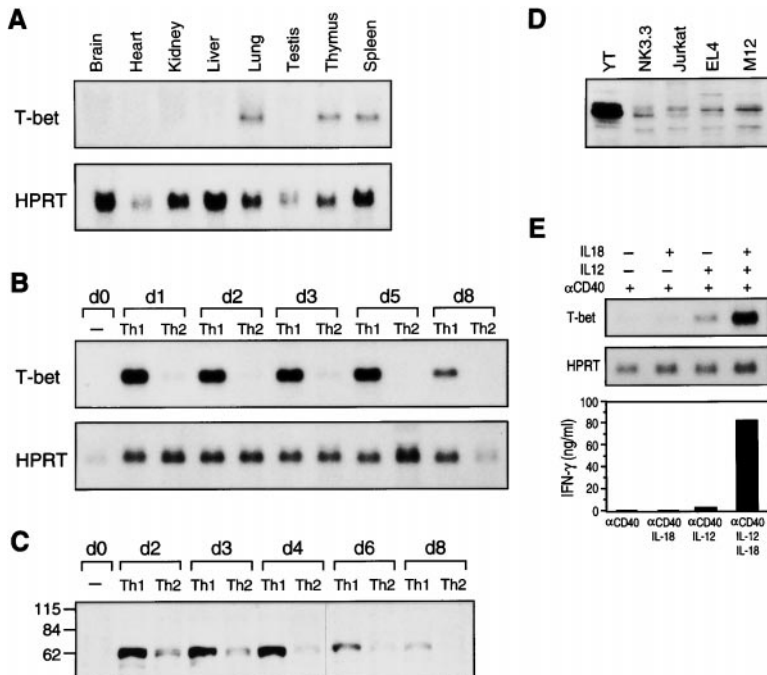
(B, right panel) Flow cytometric analysis of T-bet expression. AE7 or D10 were treated with media or PMA plus ionomycin for 5 hr, washed, fixed, and permeabilized, and stained with media (dashed line), an IgG1 isotype control antibody (dotted line), or an affinity-purified anti-T-bet mAb (4B10, solid line), followed by goat anti-mouse IgG1-PE.

We first attempted to correlate the expression of T-bet with  $IFN\gamma$  expression in non-T cells. Primary B cells, which do not produce  $IFN\gamma$  at baseline, can be induced to produce large amounts of  $IFN\gamma$  upon treatment with anti-CD40 antibody plus IL-12 and IL-18 (Yoshimoto et al., 1997). Purified B220<sup>+</sup> splenic B cells were treated for 72 hr with anti-CD40 mAb, rIL-12, and rIL-18, and RNA was isolated. Figure 3E shows induction of T-bet RNA in B cells treated with this combination of reagents, and the coordinate induction of  $IFN\gamma$ , but not IL-2, as measured by ELISA. During the B cell purification procedure, contaminating NK cells may be present, and thus under these culture conditions NK cells could contribute to the  $IFN\gamma$  production (Rolink et al., 1996). However, we have also observed the coordinate induction of T-bet

expression and secretion of  $IFN\gamma$  in NK3.3 cells that produce  $IFN\gamma$  only upon treatment with IL-2 and IL-12 (data not shown). In conclusion, while neither cell type expresses T-bet at baseline, both B cells and NK cells can be induced to express T-bet under conditions that result in  $IFN\gamma$  production. Thus, the pattern of T-bet expression correlates well with the production of  $IFN\gamma$ .

### T-bet Is a Potent Transactivator of the $IFN\gamma$ Gene

The regions of the  $IFN\gamma$  gene that direct its tissue-specific expression have not been identified either in vitro or in vivo. Reporter constructs containing 500 bp or 3 kb of upstream sequence are expressed in both Th1 and Th2 cells (Young et al., 1994). ATF-2, NF- $\kappa$ B, AP-1,



**Figure 3. T-bet Is Expressed Early in Developing Th1 Cells and NK Cells**

(A) Tissue distribution of T-bet. Northern analysis of an organ blot probed with the T-bet or HPRT cDNA probe.

(B) Kinetics of induction of T-bet transcripts in primary T cells. Naive Mel14<sup>+</sup> CD4<sup>+</sup> DO11.10 TCR transgenic T cells were activated under Th1- or Th2-polarizing conditions and RNA prepared on day 0, 1, 2, 3, 5, and 8 after primary activation.

(C) T-bet expression in antigen-APC-activated primary T cells. DO11.10 TCR transgenic splenocytes were activated with OVA peptide at  $3 \times 10^6$  cells/ml in the presence of Th1- or Th2-polarizing conditions and Western blot analysis performed on 30  $\mu$ g of nuclear extracts prepared on day 0, 2, 3, 4, 6, and 8 after primary activation and probed with polyclonal anti-T-bet antisera.

(D) T-bet protein expression in cell lines. Western blot analysis of YT, NK3.3, Jurkat, EL4, and M12 cells.

(E) T-bet expression correlates with  $IFN\gamma$  production in B cells. B220<sup>+</sup> splenic B cells purified by FACS and treated for 72 hr with various combinations of anti-CD40 mAb, rIL-12, and rIL-18 were subjected to Northern blot analysis using T-bet and HPRT probes.  $IFN\gamma$  levels in culture supernatants were measured by ELISA.



YY1, NF-AT, and Stat sites in the IFN $\gamma$  promoter or introns are functionally important but have not been shown to be responsible for the tissue-specific expression of IFN $\gamma$  (Young et al., 1994; Penix et al., 1996; Xu et al., 1996; Sica et al., 1997; Sweetser et al., 1998). Additionally, although Th1-preferential DNaseI hypersensitive sites have been noted both in the first and third introns, the relevant *cis* elements have not been identified (Young et al., 1994; Agarwal and Rao, 1998). The T box transcription factors have a consensus binding site (Figure 4A). Inspection of the IFN $\gamma$  gene revealed three potential T box binding sites, two located within the proximal promoter region and one in the third intron.

We therefore tested whether T-bet could transactivate a reporter construct containing the entire IFN $\gamma$  locus. This reporter construct contains 9.2 kb of genomic DNA, including the IFN $\gamma$  coding sequence with all three introns, 3 kb of upstream sequence, and 1.5 kb of downstream sequence (Figure 4B). Cotransfection of the mouse thymoma EL4, which produces IL-2 and IL-4 but not IFN $\gamma$ , with a T-bet cDNA expression plasmid and the IFN $\gamma$ -luciferase reporter construct resulted in substantial (20- to 40-fold) transactivation of the IFN $\gamma$  gene evident both in unstimulated and PMA/ionomycin-stimulated cells (Figure 4C). Further, EL4 cells were transiently transfected with T-bet or empty vector and endogenous IFN $\gamma$  production measured by ELISA at 24, 48, and 72 hr after PMA/ionomycin stimulation. Remarkably, ectopic expression of T-bet in EL4 cells led to substantial IFN $\gamma$  production (Figure 4D). These results are a novel demonstration of a Th1 restricted transcription factor capable of transactivating the IFN $\gamma$  gene and inducing endogenous IFN $\gamma$  production.

We then determined if ectopic expression of T-bet affected the transcription of other cytokine genes. Surprisingly, we found T-bet expression repressed the activity of an IL-2 promoter/reporter construct an average of 5- to 10-fold in PMA/ionomycin-stimulated EL4 cells (Figure 4C). T-bet did not repress basal IL-2 promoter activity in unstimulated EL4 cells. Additionally, T-bet expression had no effect on IL-4 promoter transactivation (Figure 4C) or a nonspecific control *Egr-3* promoter construct (data not shown). Thus, T-bet is a potent transactivator of the IFN $\gamma$  gene and can simultaneously repress IL-2 gene transcription in stimulated EL4 cells.

#### Retroviral Gene Transduction of T-bet into Primary T Cells Results in Increased IFN $\gamma$ Production

The experiments described above argue strongly for a critical role of T-bet in controlling IFN $\gamma$  transcription. To further test whether T-bet is responsible for the tissue-specific expression of IFN $\gamma$ , retroviral gene-mediated transfer of T-bet into primary T cells was performed. We generated two different bicistronic retroviral vectors expressing both T-bet and GFP (Figure 5A). The retroviral vectors containing GFP only (GFP-RV and pGC) were used in all experiments to control for the effects of retroviral infection. BALB/c CD4<sup>+</sup> T cells were activated with anti-CD3 and anti-CD28 and infected with retrovirus 36 hr after primary activation. After 7 days of culture, GFP-positive cells were isolated by FACS, rested overnight in IL-2, and stimulated with PMA/ionomycin and cytokine production was measured by intracellular

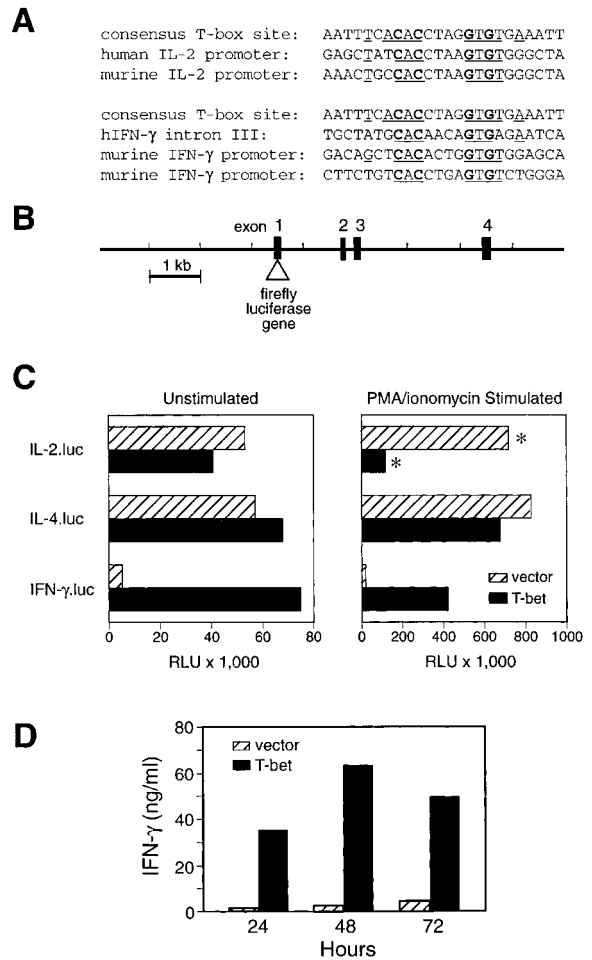


Figure 4. T-bet Transactivates the IFN $\gamma$  Gene and Represses the IL-2 Gene in EL4 Cells

(A) Putative T box sites in the IL-2 and IFN $\gamma$  genes as compared to a consensus T box sequence.

(B) Schematic drawing of the 9.2 kb IFN $\gamma$  reporter construct.

(C) Analysis of IL-2, IL-4, and IFN $\gamma$  promoter transactivation in EL4 cells. The following plasmids were used: an IL-2 promoter/reporter construct (IL-2.luc, containing -2060 to +40 of the IL-2 promoter), an IL-4 promoter/reporter construct (IL-4.luc, containing -760 to +68 of the IL-4 promoter), and the IFN $\gamma$  reporter construct described above (IFN $\gamma$ .luc). Striped bars are empty pCDNA vector, and solid bars are the T-bet expression vector. Left panel is unstimulated cells, and right panel is stimulation with PMA plus ionomycin for 5 hr. The luciferase units in the bars marked with an asterisk represent 1/20 of actual activity. The data shown are representative of three independent experiments.

(D) Endogenous IFN $\gamma$  production in EL4 cells transiently transfected with T-bet. The striped bars are pCDNA vector alone and the solid bars are T-bet expression plasmid. PMA plus ionomycin was added 12 hr after transfection and the level of IFN $\gamma$  produced at 24, 48, and 72 hr measured by ELISA. The data shown are representative of three independent experiments.

staining. Transduction of primary CD4<sup>+</sup> T cells with T-bet resulted both in a dramatic increase in the number of cells producing IFN $\gamma$  (74% versus 4%) and in the amount of IFN $\gamma$  produced per cell as compared to cells transduced with GFP alone (Figure 5B). Interestingly, IL-2 production remained unchanged. The effect of T-bet in Th differentiation was also assayed using an antigen-specific TCR transgenic system. MBP TCR transgenic

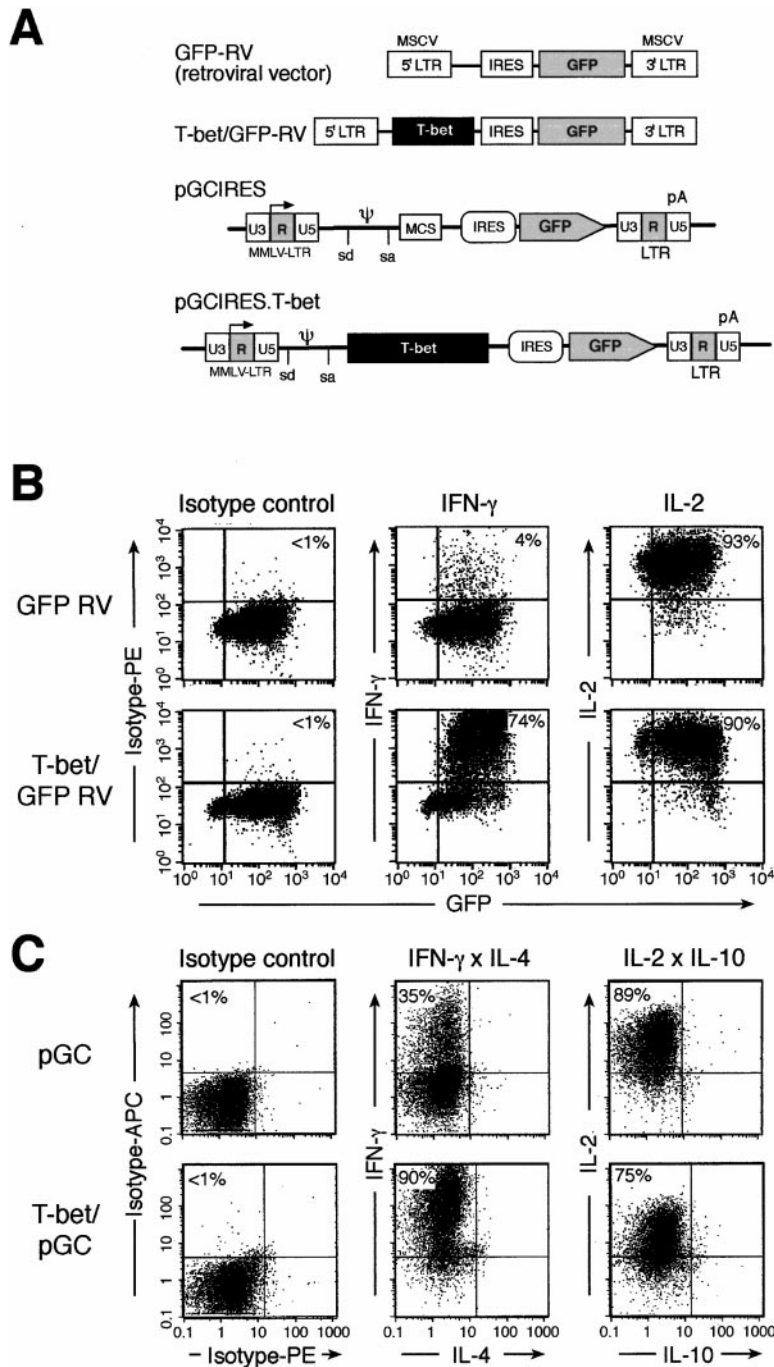


Figure 5. Retroviral Gene Transduction of T-bet into Primary CD4<sup>+</sup> T Cells Induces IFN $\gamma$  Production

(A) Schematic diagram of retroviral constructs.

(B) Retroviral transduction of primary CD4<sup>+</sup> T cells with control GFP-RV and T-bet/GFP-RV. FACS-purified BALB/c CD4<sup>+</sup> T cells were infected with the indicated retroviral construct 36 hr after primary activation by anti-CD3 plus anti-CD28. Cells were harvested on day 7, resorted for GFP expression, rested overnight in IL-2, and intracellular cytokine staining performed. Data are shown as two-color dot plots showing GFP expression (horizontal axis) versus intracellular cytokine (vertical axis) expression.

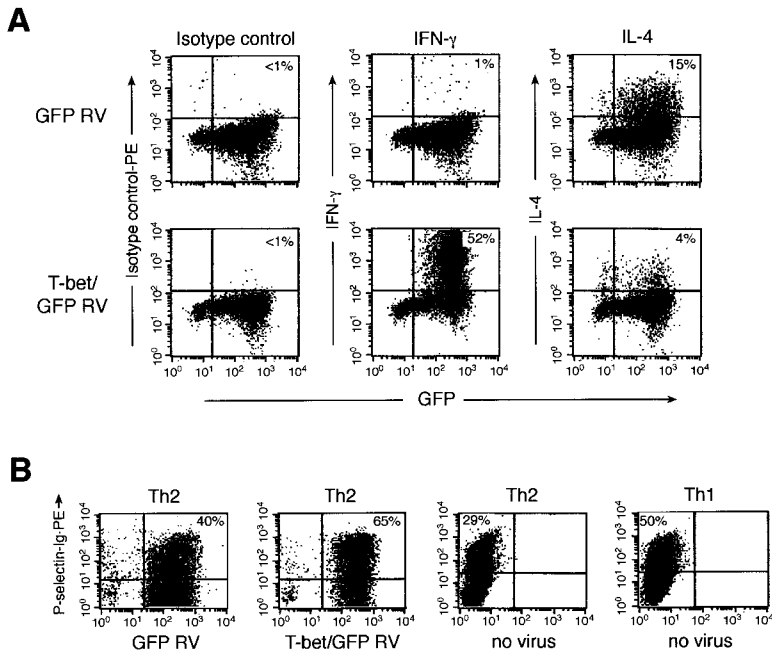
(C) Transduction of MBP TCR transgenic T cells. Splenocytes were stimulated with 6  $\mu$ M MBP and infected after 24 hr with GFP alone (pGC) or T-bet/GFP (T-bet/pGC). On day 7, cells were sorted for CD4 and GFP expression, rested overnight in IL-2, and intracellular cytokine analysis was performed. Data are shown as two-color dot plots showing a first intracellular cytokine staining (vertical axis) versus a second intracellular cytokine (horizontal axis) of events gated on GFP expression.

splenocytes were activated with MBP peptide for 24 hr and the cells infected with GFP alone (pGC) or T-bet/GFP (T-bet/pGC). On day 7, cells positive for CD4 and GFP were isolated by FACS and rested overnight in IL-2, and cytokine production was measured. Similar to the transduced polyclonally activated BALB/c T cells, a marked increase was seen in IFN $\gamma$  by MBP-TCR transgenic T cells transduced with T-bet/GFP (90%) compared to cells transduced with GFP alone (35%) (Figure 5C). Additionally, a modest repression in IL-2 production was noted in the transgenic T cells transduced with T-bet/

GFP (89% to 75%). Thus, in primary T cell cultures T-bet expression is a potent inducer of IFN $\gamma$  production.

#### T-bet Activates IFN $\gamma$ and Represses IL-4 Production in Developing Th2 Cells

We next asked whether T-bet could induce IFN $\gamma$  production in Thp cells activated in the presence of stimuli that would drive them toward the Th2 lineage. CD4<sup>+</sup> T cells were activated under Th2-inducing conditions, retroviral infection was performed at 36 hr, and cells were expanded with IL-2. The Th2 culture conditions were main-



**Figure 6. T-bet Activates IFN $\gamma$  and Represses IL-4 Production in Developing Th2 Cells by Invoking a Th1 Developmental Program**

FACS-purified BALB/c CD4<sup>+</sup> T cells were activated with anti-CD3 and anti-CD28 in the presence of Th2-inducing conditions, retroviral infection with either GFP alone (GRP RV) or T-bet (T-bet/GFP RV) was performed at 36 hr, and cells were expanded with IL-2 and maintained under Th2-polarizing conditions. On day 7, GFP-positive cells were sorted and rested overnight in IL-2.

(A) Intracellular cytokine staining analysis was performed after stimulation with PMA/ionomycin for 2 hr and an additional 2 hr incubation with 3 mM monensin.

(B) Surface expression of PSGL-1 was analyzed by FACS after 5 hr of stimulation with PMA/ionomycin.

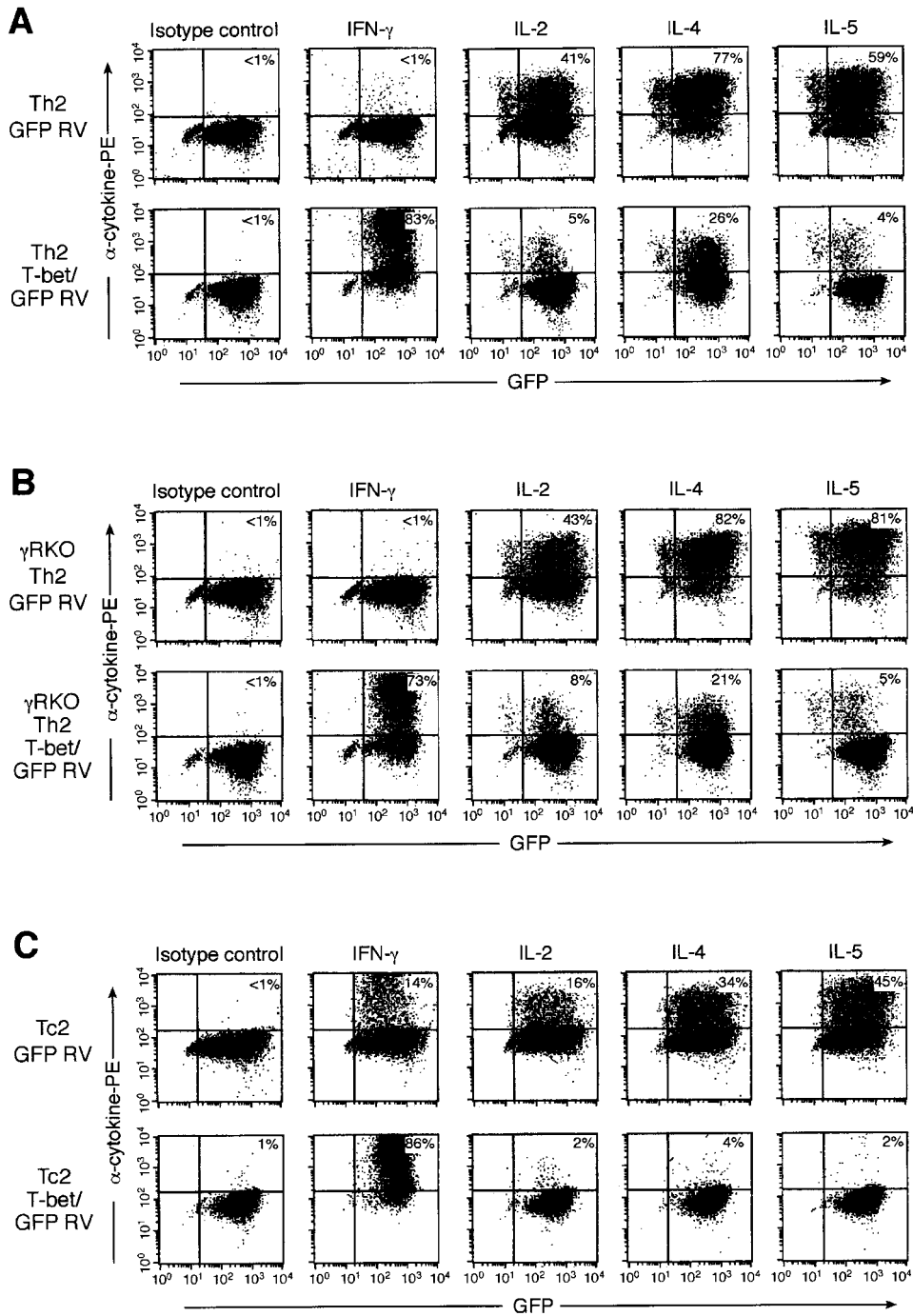
tained at each step throughout the 7 day experiment. On day 7, GFP-positive cells were sorted and rested overnight in IL-2, and cytokine production was measured. Transduction with control GFP-RV alone resulted in a population that contained 1% IFN $\gamma$  producers and 16% IL-4-producing cells (Figure 6A). Introduction of T-bet/GFP RV not only induced IFN $\gamma$  production but also induced a substantial shift in the Th2 developmental program of these Thp cells toward the Th1 phenotype as evidenced by the large number of IFN $\gamma$ -producing cells (53%) and the reduced number of IL-4-producing cells (4%). Remarkably, the redirection of the Th2 genetic program occurred in the continuous presence of conditions that inhibit Th1 differentiation.

To further address whether T-bet was inducing a Th1 developmental program in these differentiating Th2 cells, we analyzed the expression of Th1-specific genes other than IFN $\gamma$ . Several surface markers that distinguish fully differentiated effector Th1 and Th2 cells have been recently identified. Th1 preferentially expressed genes include the IL-12 receptor  $\beta$ 2 chain, IL-18 receptor, CCR5, and CXCR3 chemokine receptors, and a modified form of P-selectin glycoprotein-1 (PSGL-1) that can be detected using the P-selectin-Ig reagent (Borges et al., 1997). Thp cells under Th2-polarizing conditions were retrovirally transduced as above. On day 8, GFP-positive cells were stimulated with PMA/ionomycin for 5 hr, and surface expression of PSGL-1 and CCR5 were analyzed. T-bet/GFP caused a modest increase in the modified form of PSGL-1 (65%) expression compared to control GFP infected cells (40%) (Figure 7B). This relative difference was similar to the differences in PSGL-1 expression in nontransduced primary T cells differentiated along a Th1 (50%) versus Th2 (29%) pathway in vitro (Figure 6B). Additionally, the expression of CCR5 in T-bet/GFP-transduced cells was similar to levels expressed on nontransduced Th1 cells (data not

shown). We conclude that T-bet can overcome Th2-promoting signals to induce Th1 differentiation as evidenced by the increased expression of IFN $\gamma$ , PSGL-1, and CCR5.

### T-bet Redirects Effector Th2 Cells into the Th1 Pathway

A naive Thp has the potential to differentiate along the Th1 or Th2 pathway. This developmental window remains open during the first 48 hr of primary T cell activation (Nakamura et al., 1997). However, after 7 days of differentiation Th cells are referred to as effector cells, since the developmental program is complete and exogenous signals are not required to maintain the cell in its differentiated state (Huang et al., 1997). To determine if T-bet could redirect the commitment of effector Th2 cells, CD4<sup>+</sup> T cells were polyclonally activated and cultured under Th2-polarizing conditions, and retroviral gene transduction was performed at day 9 rather than day 2 of culture. In these effector Th2 cells, control GFP-transduced cells were IL-4 and IL-5 producers (77% and 59%) with barely detectable IFN $\gamma$  producer cells present (<1%) (Figure 7A). Remarkably, introduction of T-bet into effector Th2 cells redirected them into Th1 cells as evidenced both by the induction of IFN $\gamma$  expression and the loss of IL-4 and IL-5 expression. Fully 83% of T-bet-transduced Th2 cells now produced IFN $\gamma$  while the percentage of cells producing IL-4 and IL-5 was reduced to 26% and 4%, respectively. Additionally, there was a dramatic reduction in the amount and number of IL-2-producing cells from 41% to 5%. These T-bet-transduced cells are therefore not Th0 cells that produce IL-2, IFN $\gamma$ , and IL-4. We conclude that T-bet has not simply induced IFN $\gamma$  expression in Th2 cells but appears to be reprogramming Th2 cells into the opposing Th1 subset.



**Figure 7. T-bet Converts Effector Th2 into Th1 Cells Independently of IFN $\gamma$  and Redirects Tc2 Cells into the Tc1 Pathway**  
 FACS-purified wild-type 129 CD4<sup>+</sup>, *IFN $\gamma$ R*<sup>-/-</sup> CD4<sup>+</sup>, or BALB/c CD8<sup>+</sup> T cells were activated with anti-CD3/CD28 in the presence of rIL-4, anti-IFN $\gamma$ , and IL-12 mAbs to promote Th2 or Tc2 differentiation. Retroviral infection with either GRP RV or T-bet/GFP RV was performed on day 9 of culture, and cells were expanded with IL-2 and maintained under Th2/Tc2 culture conditions. On day 14, GFP-positive cells were sorted and analyzed on day 15 by intracellular cytokine analysis. (A) Effector Th2 cells, (B) effector *IFN $\gamma$ R*<sup>-/-</sup> Th2 cells, or (C) effector Tc2 cells.

**T-bet Represses IL-4 and IL-5 Production in Th2 Cells through an IFN $\gamma$ -Independent Pathway**

T-bet clearly induces endogenous IFN $\gamma$  production in a number of different cell types and represses IL-4 production in developing and effector Th2 cells. However, T-bet did not repress IL-4 promoter transactivation in EL4 cells. One explanation could be that the IFN $\gamma$  pro-

duced by T-bet-transduced Th2 cells inhibits IL-4 production. To address this issue, we examined T-bet's ability to repress IL-4 production in Th2 cells that were insensitive to IFN $\gamma$  signaling due to genetic deficiency of the IFN $\gamma$  receptor (*IFN $\gamma$ R*<sup>-/-</sup>) (Huang et al., 1993). *IFN $\gamma$ R*<sup>-/-</sup> CD4<sup>+</sup> T cells were activated under Th2-polarizing conditions, and retroviral transduction was per-



formed on day 9. Intracellular cytokine analysis showed Th2 development in the *IFN $\gamma$ R<sup>-/-</sup>* CD4<sup>+</sup> T cells occurred similarly to the control wild-type cells as evidenced by the similar number of IL-4 and IL-5 producers (82% and 81%) and the presence of few IFN $\gamma$ -producing cells (<1%). When T-bet was introduced into these *IFN $\gamma$ R<sup>-/-</sup>* Th2 cells, a dramatic reduction in IL-4 and IL-5 expression (21% and 5%, respectively), a marked increase in the number of IFN $\gamma$ -producing cells (73%), and a decrease in the number of IL-2-producing cells (43% to 8%) was observed. Thus, T-bet's ability to repress IL-4 and IL-5 is not an indirect effect of IFN $\gamma$  overproduction on the T cells.

#### T-bet Redirects Effector Tc2 Cells into the Tc1 Pathway

Cytotoxic CD8<sup>+</sup> T cells may also be divided into IFN $\gamma$ -producing (Tc1) and IL-4-producing (Tc2) subsets (Croft et al., 1994). We therefore asked whether introduction of T-bet into effector Tc2 cells could redirect them to the Tc1 phenotype. Purified BALB/c CD8<sup>+</sup> T cells were activated with anti-CD3/CD28 in the presence of Tc2-polarizing conditions, and retroviral infection was performed on day 9. Figure 7C demonstrates that T-bet transduction into Tc2 cells induced a striking increase in IFN $\gamma$ -producing cells (14% versus 86%) and dramatically repressed the production of IL-2 (16% versus 2%), IL-4 (34% versus 4%), and IL-5 (45% versus 2%). We conclude that T-bet expression is able to convert differentiated CD8<sup>+</sup> Tc2 cells to Tc1 cells.

#### T-bet Converts Polarized Th2 Cells into Th1 Cells

Relatively little is known about the biological mechanisms through which T box transcription factors exert their functions. It is possible that T-bet may be involved in cell cycle control and could give a selective growth advantage to infected cells. It was also possible that retroviral infection was occurring in a small population of uncommitted Thp cells that then predominated over the course of the experiment. To address these possibilities, naive CD4<sup>+</sup> DO11.10 T cells were activated under Th2-polarizing conditions for 15 days, and retroviral transduction was performed. On day 21, GFP<sup>+</sup> and GFP<sup>-</sup> cells were sorted from both the GFP RV and T-bet/GFP RV-transduced cultures, and proliferation assays demonstrated that the growth rates between all populations were similar (data not shown). Intracellular cytokine staining of the GFP<sup>+</sup> populations demonstrates that provision of T-bet/GFP to polarized Th2 cells converts them into Th1 cells as evidenced by the increased number of cells producing IFN $\gamma$  (<1% to 71%) and TNF $\alpha$ , another cytokine preferentially produced by Th1 cells (26% to 46%). The effect of T-bet not only initiated a Th1 program but also inhibited the Th2 program as evidenced by the reduced number of T-bet/GFP-transduced cells producing IL-4 (68% to 42%) and IL-5 (48% to 2%).

#### T-bet Converts Fully Polarized Th2 Cells into Th1 Cells by a Mechanism Directly Dependent on T-bet Expression

Reversibility of Th1 and Th2 populations is largely abrogated after 1 week in culture. However, stable commitment to a Th phenotype occurs after 3 weeks of stimulation under Th-polarizing conditions (Murphy et al., 1996).

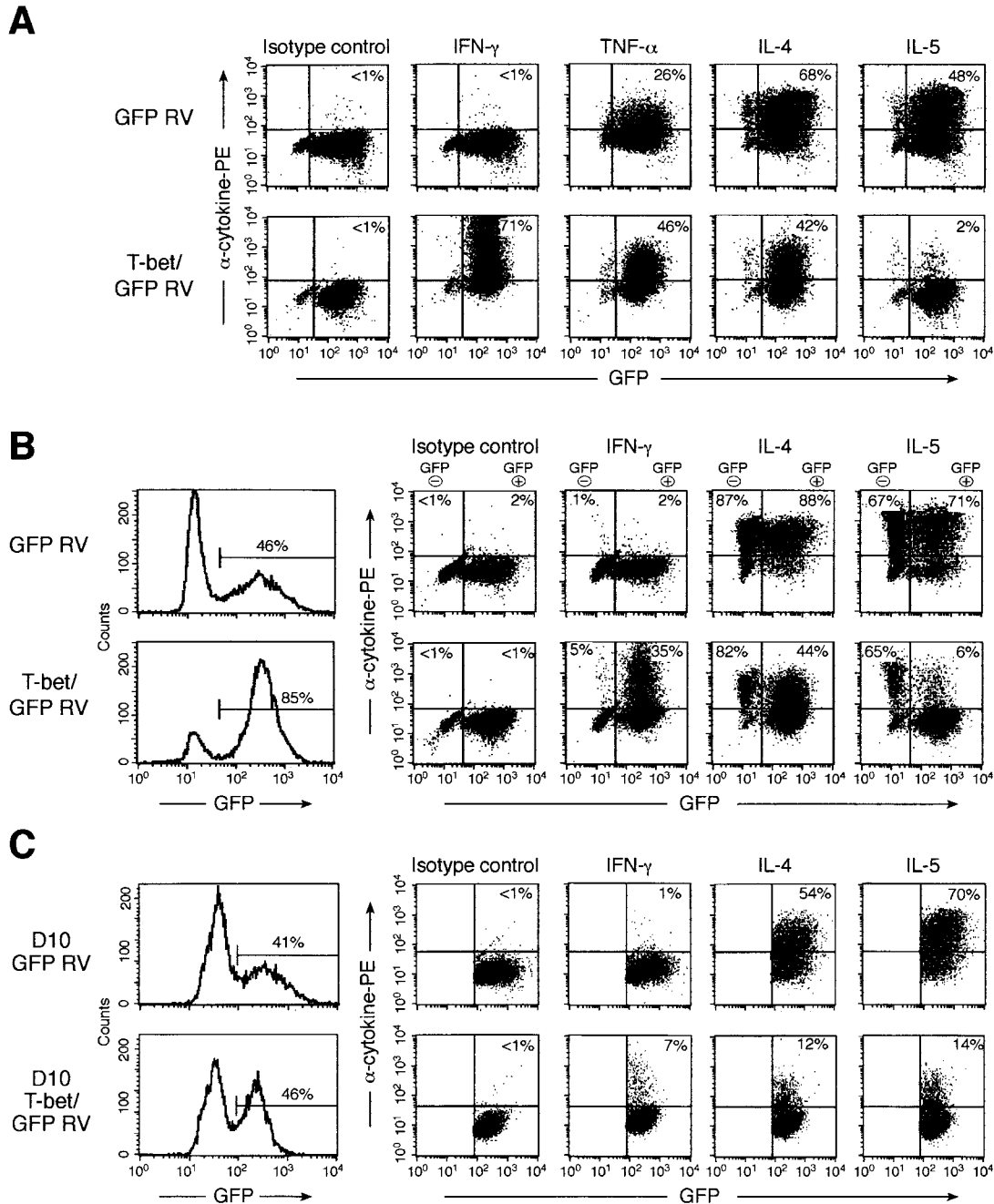
To test whether T-bet could reverse the Th2 differentiation program in a population of stably committed Th2 cells, CD4<sup>+</sup> DO11.10 T cells were stimulated under Th2-polarizing conditions for 22 days (Figure 8B), and retroviral transduction was then performed. The histograms in Figure 8B indicate the retroviral transduction efficiencies between the GFP RV (46%) and T-bet GFP RV (86%) populations. On day 28, intracellular cytokine staining was performed on the unfractionated populations. Indicated in the upper left panel of each FACS plot is the percent cytokine-positive cells of the nontransduced cell population (GFP<sup>-</sup>), while the percent cytokine-positive cells of the retrovirally transduced cell population (GFP<sup>+</sup>) is indicated in the upper right panel. In the GFP<sup>+</sup> populations, provision of T-bet/GFP to stably committed Th2 cells converts them into Th1 cells as evidenced by the increased number of cells producing IFN $\gamma$  (2% to 35%) and by the reduced number of T-bet/GFP-transduced cells producing IL-4 (88% to 44%) and IL-5 (71% to 6%). Comparison with the control GFP<sup>-</sup> population shows that the cytokine profiles of the nontransduced populations are nearly identical in the GFP RV and T-bet/GFP RV-treated cultures, with respect to IL-4 (87% versus 82%), IL-5 (67% versus 65%), and IFN $\gamma$  (1% versus 5%). Thus, T-bet's ability to repress IL-4 and IL-5 is not due to soluble factors but is due to T-bet expression within the cell.

#### T-bet Represses IL-4 and IL-5 Production in Th2 Clones

Lastly, T-bet's ability to repress IL-4 and IL-5 production was assayed in a homogeneous population of Th2 cells. For these experiments, the D10 Th2 clone (Figure 8C) and two additional Th2 clones, HAE4A6 and 1A8, were used (data not shown). D10 cells were stimulated and retroviral infection performed on day 1, and cells were expanded with IL-2. On day 7, GFP-positive cells were enriched, restimulated, and expanded, and were analyzed on day 14 by intracellular cytokine analysis. Retroviral transduction efficiency is shown as a histogram, and the FACS plots reflect events gated on GFP<sup>+</sup> expression. In D10 cells, T-bet inhibited the Th2 genetic program as evidenced by the reduced number of T-bet/GFP-transduced cells producing IL-4 (54% to 12%) and IL-5 (70% to 14%). In this cell line, T-bet minimally induced IFN $\gamma$  production (1% to 7%). Similar results were obtained with the two additional Th2 clones, HAE4A6 and 1A8 (data not shown). These results demonstrate that T-bet's ability to suppress Th2 development extends even to long-term, established Th2 clones.

#### Discussion

CD4<sup>+</sup> T helper cells have been classified into two subsets based on their distinct patterns of cytokine secretion. The hallmark cytokine produced by the Th1 subset is IFN $\gamma$ , and the corresponding cytokine of the Th2 subset is IL-4. In contrast to the substantial progress that has been made in identifying the factors that direct Th2-specific IL-4 expression, no information is available on the corresponding Th1-specific factors that control IFN $\gamma$  expression. Here, we provide strong evidence that the selective expression of T-bet, a novel T box family member, accounts for the Th1-specific expression of IFN $\gamma$ .



**Figure 8. T-bet Converts Fully Polarized Th2 Cells into Th1 Cells and Represses IL-4 and IL-5 Production in Th2 Clones**  
 (A and B) FACS-purified naive DO11.10 TCR transgenic CD4<sup>+</sup> T cells were activated with 0.3  $\mu$ M OVA peptide and irradiated BALB/c splenocytes in the presence of Th2-inducing conditions. Retroviral infection was performed on day 15 or day 22 of culture, and cells were expanded with IL-2 and maintained under Th2 culture conditions until day 21 or 28.  
 (A) On day 21, GFP-positive cells were sorted, and they were analyzed on day 22 by intracellular cytokine analysis.  
 (B) On day 28, retroviral transduction efficiency was determined by FACS and plotted as a histogram. Nontransduced (GFP<sup>-</sup>) and retrovirally transduced (GFP<sup>+</sup>) cells were analyzed by intracellular cytokine analysis. The percent cytokine-positive cells of the total nontransduced cell population (GFP<sup>-</sup>) is indicated in the upper left panel of each FACS plot and the percent cytokine-positive cells of the total retrovirally transduced cell population (GFP<sup>+</sup>) is indicated in the upper right panel.  
 (C) D10 Th2 clone was activated with 0.1 mg/ml conalbumin presented by irradiated AKR splenocytes. Retroviral infection was performed on day 1 of culture, and cells were expanded with IL-2. On day 7, GFP-positive cells were enriched, restimulated, expanded, and analyzed on day 14 by intracellular cytokine analysis. Retroviral transduction efficiency was determined by FACS and plotted as a histogram. The FACS plots reflect events gated on GFP<sup>+</sup> expression.

T-bet is expressed only in Th1 and not in Th2 cells and is induced in the former upon transmission of signals through the TCR. The expression of T-bet correlates

with IFN $\gamma$  expression in Th1 cells, NK cells, and B cells, and T-bet is a potent transactivator of the IFN $\gamma$  gene. Most convincingly, retroviral-mediated transduction of

T-bet into either Thp, Th2, or Tc2 cells results in a marked induction of IFN $\gamma$  expression. What is most exciting is that the function of T-bet appears to extend beyond the simple control of IFN $\gamma$  gene transcription. Thus, T-bet converts both effector Th2 cells and effector Tc2 cells into the opposing Th1 and Tc1 subsets, respectively. Moreover, T-bet can reprogram a stably committed population of fully polarized Th2 cells into the Th1 phenotype and can even repress IL-4 and IL-5 production in Th2 clones. Taken together, these data lead us to conclude that T-bet is responsible for the genetic program that initiates Th1 lineage development from naive Thp cells and acts both by initiating Th1 genetic programs and by repressing the opposing Th2 programs.

From this study, T-bet clearly regulates IFN $\gamma$  production in a number of cell types: EL4, primary T cells, developing Th2 cells, effector Th2 and Tc2 cells, and fully polarized Th2 cells. However, in Th2 clones, while T-bet was capable of repressing IL-4 and IL-5 production it was unable to induce substantial IFN $\gamma$  production. It may be that there is a point in the life span of a Th2 cell where T-bet is incapable of inducing IFN $\gamma$  secretion because the chromatin configuration at the relevant loci is no longer accessible or because the array of factors necessary for IFN $\gamma$  gene expression is no longer present. Much remains to be learned about the exact mechanism by which T-bet regulates IFN $\gamma$  production. For instance, although we have found regions in the IFN $\gamma$  gene that resemble T box binding sites, we have not yet determined that T-bet binds to these sites. A thorough dissection of all of the upstream, downstream, and intronic regions of the IFN $\gamma$  gene will be necessary to address this issue.

How does T-bet simultaneously transactivate the IFN $\gamma$  gene and repress IL-2 transcription? The consensus T box binding site was defined by target site selection *in vitro*. Inspection of the murine IL-2 promoter reveals an excellent T box site at -240 to -220. Presumably, the binding of T-bet to the IL-2 promoter explains its isolation in our yeast one hybrid screen where the readout depended simply on binding of T-bet to drive a reporter gene. Additionally, we have found three T box sites in the IFN $\gamma$  gene locus, two sites at positions -2300 to -2291 and -1957 to -1948 and one in the third intron at position +4655 to +4665. While these T box sites in the IFN $\gamma$  and IL-2 genes appear similar in transient transfection assays, T-bet induced IFN $\gamma$  promoter activity and repressed IL-2 promoter activity. An attractive model for a transcription factor acting as a context-specific repressor or activator is the *Drosophila* transcription factor dorsal. Dorsal typically acts as a transcriptional activator, yet it becomes a context-specific repressor when dorsal binding sites are adjacent to VRE sites that bind the HMG1/2 protein DSP1 (Ip, 1995).

We do not yet understand why repression of IL-2 appears strongest in effector Th2 cells. Naive Thp cells, early after stimulation, produce large amounts of IL-2, which is then gradually replaced in polarized Th cells by the effector cytokines IFN $\gamma$  and IL-4 (Seder and Paul, 1994). As Th1 cells continue to polarize toward the mature Th1 phenotype, IL-2 expression is progressively turned off. In contrast, Th2 cells are thought to turn off IL-2 production after the first week of differentiation. We find, however, substantial numbers of IL-2-producing

cells present in Th2 cultures. This discrepancy may reflect the increased sensitivity of the intracellular cytokine assay for IL-2. Thus, we believe that Th2 cells produce IL-2 albeit at lower levels than Th1 cells; however, we cannot rule out that there also exists a population of IL-2-producing uncommitted cells within our Th2 cultures. Regardless, the repression of IL-2 by T-bet as seen in this study is consistent with a function for T-bet in driving lineage commitment from a naive precursor cell into a fully polarized Th1 effector cell. In contrast to the striking results obtained using Th2 and Tc2 cells, only modest reduction of IL-2-producing cells was observed in developing Thp cells transduced with T-bet. These results may be explained by the presence of a developmentally regulated corepressor (Fisher and Caudy, 1998), or T-bet may synergize with ZEB, a negative regulator of IL-2 transcription, which binds the NRE site of the IL-2 promoter and is constitutively expressed in Th2 clones (Yasui et al., 1998).

How does T-bet repress the Th2 program in Thp and Th2 cells? It is unlikely that it is a direct effect since T-bet did not repress IL-4 promoter transactivation. Since the observed repression also occurred in the absence of IFN $\gamma$  receptor signaling, it cannot be explained by IFN $\gamma$  acting directly on the T cell. An intriguing possibility is that T-bet directly inhibits the activity of the GATA-3 gene. The very pronounced effect of T-bet on IL-5, a cytokine gene directly regulated by GATA-3, is consistent with this explanation. In this scenario, the somewhat lesser effect of T-bet on inhibition of IL-4 gene expression could be explained by the predominant role of c-maf in controlling this gene. The effect of T-bet in repressing the Th2 program while simultaneously enhancing the Th1 program is reminiscent of GATA-3, which indirectly represses IFN $\gamma$  expression through influencing expression of the IL-12 receptor  $\beta$ 2 chain while simultaneously inducing Th2 differentiation (Ouyang et al., 1998). Perhaps GATA-3 suppresses T-bet expression during Th2 development. Thus, the predominance of T-bet or GATA-3 may ultimately determine the fate of a developing Th cell toward the Th1 or Th2 lineage, respectively.

It will be of interest to understand the basis for the tissue-specific expression of T-bet itself since our preliminary data demonstrate that T-bet is regulated not only by signals stemming from the TCR but also by signal transduction pathways downstream of the IL-12 receptor. Thus, IL-12 induces an increase in levels of T-bet RNA and protein, and T cells derived from mice lacking Stat4 have decreased expression of T-bet (S. J. S., unpublished data). These data fit nicely with the role of IL-12 and Stat4 in driving the development of the Th1 lineage (Gately et al., 1998). While a critical role for IL-12/Stat4 in driving the differentiation of naive Thp cells has been established, evidence has emerged that effector Th1 cells can produce IFN $\gamma$  through TCR stimulation only. While IL-12 may augment IFN $\gamma$  production in these cells, it is clearly not required. This is especially true for CD8<sup>+</sup> cells where a Stat4-independent pathway for IFN- $\gamma$  production has been demonstrated (Carter and Murphy, 1999). Analysis of T-bet gene transcription itself in different cell types should prove informative.

*Brachyury* is the founding member of a family of transcription factors that share a 200–amino acid DNA-binding domain designated the T box (Papaioannou, 1997; Smith, 1997). The isolation of the *Brachyury* gene (Herrmann et al., 1990) led to the identification of a family of related genes in various species. Each T box gene has a distinct and developmentally regulated pattern of expression. Throughout evolution, T box family genes have been conserved in sequence and expression pattern in species including *Xenopus*, zebrafish, chick, and humans, as well as more distant species such as amphioxus, ascidians, echinoderms, *Caenorhabditis elegans*, and *Drosophila* (Papaioannou, 1997; Smith, 1997). The essential role of T box family members in developmental processes has been exemplified in several systems. The *Brachyury* mutation was first described in heterozygous mutant animals with a short tail. Homozygote mice were more severely affected as evidenced by defective mesoderm formation, regression of the notochord, and embryonic death from failure of the allantois to connect with the placenta (Papaioannou, 1997; Smith, 1997). Recent studies determined that ectopic expression of *Tbx4* in chick limbs repatterns limb identity from forelimb to hindlimb (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). Mutations in the human T box genes *TBX5* and *TBX3* are responsible for the autosomal dominant genetic diseases Holt-Oram syndrome and ulnar-mammary syndrome, respectively (Bamshad et al., 1997; Basson et al., 1997). Both syndromes are characterized by developmental defects reflected by the pattern of *Tbx5* and *Tbx3* expression. Holt-Oram syndrome affects the heart and upper limbs while ulnar-mammary syndrome affects limb, apocrine gland, tooth, and genital development. The mutations in these patients usually involve a point mutation in one allele of the T box gene, and thus it has been postulated that haploinsufficiency of *Tbx5* and *Tbx3* causes these two diseases. These discoveries emphasize the critical importance of this family in vertebrate development.

The recent prominence of the T box gene family arises from its clear importance in diverse developmental processes as exemplified most dramatically by the T box mutations in human disease. The generation of mature T cells from stem cells and the differentiation of Th cells from naive Th precursors can also be viewed as tightly regulated developmental processes. Our discovery that T-bet is responsible for the development of the Th1 lineage demonstrates an important role for this newest T box family member in the lymphoid system. A systematic structure-function analysis, coupled with approaches to determine T-bet function *in vivo*, should provide further insight into molecular mechanisms that direct lineage commitment in the immune system.

#### Experimental Procedures

##### Mice, Cell Lines, Cytokines, Antibodies, and Plasmids

BALB/c mice were obtained from Jackson Laboratories and DO11.10 TCR-transgenic mice from K. Murphy (Hsieh et al., 1993), MBP TCR-transgenic mice from C. Janeway (Lafaille et al., 1994), and *IFN $\gamma$ R<sup>-/-</sup>* mice from R. Schreiber (Huang et al., 1993). Cell lines and primary cells were maintained in complete medium (CM) containing RPMI 1640 supplemented with 10% fetal calf serum (HyClone Laboratories), glutamine (2 mM), penicillin (50 units/ml), streptomycin (50  $\mu$ g/ml), HEPES (100 mM), nonessential amino acids (1 $\times$ ,

sodium pyruvate (1 mM), and  $\beta$ -ME (50  $\mu$ M). Jurkat is a human T cell lymphoma; EL4 a mouse thymoma; M12 a B cell lymphoma; NK3.3 a human NK cell line obtained from H. Young and J. Kornbluth (Kornbluth et al., 1982); YT a human NK cell line obtained from Dr. J. Yodoi (Yodoi et al., 1985); PL17 a mouse Th1 clone obtained from Dr. P. Allen; AE7 and D1.1 mouse Th1 clones; and D10 and CDC35 mouse Th2 clones. All Th1 and Th2 clones were passaged as described by Ho et al. (1996). Recombinant IL-4 was the gift of R. Coffman and A. O'Garra (DNAX), human rIL-2 the gift of Chiron Corporation, rIL-12 the gift of Dr. M. Gately (Hoffman LaRoche), and rIL-18 purchased from Peprotech. Monoclonal anti-IL-12, monoclonal anti-IFN $\gamma$  (R4/GA2), monoclonal anti-IL-4 (11B11), and P-selectin-Ig were purchased from Pharmingen. The CCR5 rabbit polyclonal antibody was the gift of P. Ponath (Leukocyte). Both the T-bet polyclonal antisera, produced in rabbits, and mAbs were raised against full-length recombinant bacterially produced T-bet protein. The 4B10 mAb was produced by fusion of mouse spleen cells to the SP2/O-Ag14 myeloma and is of the IgG1 subtype.

##### Th1 cDNA Library Construction and Transformation

Total RNA was purified from the Th1 clone OF6 (gift of S. Smiley) activated with anti-CD3 and anti-CD28 for 3 and 6 hr. Poly A<sup>+</sup> RNA was isolated on oligo dT magnetic beads (Dyna), double stranded cDNA synthesized by SuperScript Choices System (Gibco/BRL), EcoRI adaptors ligated, cDNA fragments size fractionated on a Siesep400 spun column (Pharmacia), and cloned in the EcoRI site of pJG4-5. The ligated product was transformed into DH5a (Gibco/BRL).

The murine IL-2 promoter (−400 to −40) was PCR amplified and cloned into the XbaI/SacI sites of pHISi-1 (Clontech), linearized with XhoI, and stably integrated into the EGY48 yeast genome (pIL2/HIS/EGY48). The OF6 Th1 cDNA library was transformed into pIL2/HIS/EGY48 using the Yeastmaker yeast transformation system kit following the manufacturer's instructions (Clontech). The transformed yeast were grown in yeast media containing 2% glucose, 26.7 g/L DOB (drop out base) (Bio101), 0.72 g/L CSM (complete supplement mixture) -HIS -TRP, (Bio101) for 2 hr at 30°C, washed twice in dH<sub>2</sub>O, and grown for 16 hr in yeast medium containing 2% galactose, 2% raffinose, 26.7 g/L DOB, and 0.72 g/L CSM -HIS -TRP, and then plated onto agar plates containing the same media supplemented with 145 mM aminotriazole (Sigma).

##### Cell Purification and In Vitro Cultures

Naive CD4<sup>+</sup> T cells, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and B220<sup>+</sup> B cells were purified by FACS (Mo Flo, Becton Dickinson) using anti-Mel14 (CD62L), CD4, CD8, or B220 (Pharmingen) to 98%–99% purity. For *in vitro* activation, 2  $\times$  10<sup>6</sup>/ml T cells were resuspended in CM and activated with plate-bound 2  $\mu$ g/ml anti-CD3 (2C11) and 2  $\mu$ g/ml anti-CD28 (Pharmingen) for 3 days. For *in vitro* activation of DO11.10 TCR T cells, 0.5  $\times$  10<sup>6</sup>/ml were stimulated with 0.3  $\mu$ M OVA peptide (323–339) with irradiated BALB/c splenocytes (10  $\times$  10<sup>6</sup>/ml, 2000 rads) for 3 days. Both types of cultures were then split 1:4 in CM with 200 U/ml IL2 and cultured for 4 days. In Figures 3 and 5, DO11.10 or MBP TCR transgenic spleen cells were fractionated on histopaque gradients, washed, and resuspended at 3  $\times$  10<sup>6</sup> cells/ml with 0.3  $\mu$ M OVA or with 6  $\mu$ M MBP peptide (Ac1–11), respectively. Cells were split 1:4 on day 3 with CM with 200 U/ml IL-2. To induce Th1 and Th2 differentiation as indicated, the above cultures included 5 ng/ml rIL-12 and 10  $\mu$ g/ml anti-IL4 for Th1 cell development or 10 ng/ml rIL4, 10  $\mu$ g/ml anti-IFN $\gamma$ , and 10  $\mu$ g/ml anti-IL-12 for Th2 cell development. For secondary and tertiary Th2 cultures, cells were harvested on day 7 and day 14 after primary stimulation, washed, and restimulated at 1  $\times$  10<sup>6</sup> cells/ml with 2  $\mu$ g/ml plate-bound anti-CD3 and 2  $\mu$ g/ml anti-CD28 in Th2-inducing culture conditions as indicated above.

##### Northern and Western Blot Analysis

Total RNA was isolated using TRIZOL reagent (Gibco/BRL), and 10  $\mu$ g of each sample was separated on 1.2% agarose 6% formaldehyde gels, transferred onto GeneScreen membrane (NEN) in 20 $\times$  SSC overnight, and covalently bound using a UV Stratalink (Stratagene). Northern blots were hybridized using radiolabeled T-bet,



HPRT, or GAPDH cDNA probes. Whole cell extracts or nuclear extracts were prepared as described (Szabo et al., 1995; Gouilleux et al., 1994; respectively). Extracts (30  $\mu$ g) were separated by 10% PAGE followed by electrotransfer to nitrocellulose membranes and probed with polyclonal antisera specific for T-bet followed by horseradish peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

#### Transient Transfection Assays

EL4 cells were transfected using a Bio Rad electroporator (280V, 975  $\mu$ F) using  $5 \times 10^6$  cells in 0.4 ml RPMI supplemented with 20% FCS per transfection with 10  $\mu$ g reporter plasmid and 10  $\mu$ g expression plasmid. Luciferase assays were performed as described (Szabo et al., 1993). The IFN $\gamma$  reporter-luciferase construct (IFN $\gamma$ .luc) was the gift of T. Hoey and is derived from the plasmid pB9, which contains the entire human (Gray and Goeddel, 1982). The pGL2 luciferase gene was inserted into the first exon of the IFN $\gamma$  gene. The luciferase reporter constructs (IL-2.luc and IL-4.luc) were the gift of K. Murphy (Szabo et al., 1993).

#### Retroviral Constructs and Transduction

The GFP-RV bicistronic vector was obtained from K. Murphy (Ouyang et al., 1998) and the Phoenix-Eco packaging cell line from G. Nolan (Hofmann et al., 1996). The GFP-RV vector was constructed by inserting the encephalomyocarditis virus internal ribosomal entry sequence (IRES) and the GFP allele into the MSCV2.2 retroviral vector as described (Ouyang et al., 1998). The MMLV vector, pGCIREs, will be described in detail elsewhere (G. L. C. and C. G. F., unpublished data). Both vectors express two cDNAs, T-bet and the cDNA encoding GFP, simultaneously using an IRES to initiate translation of each mRNA separately. Transfection of the packaging cell line and retroviral transductions of primary T cells were performed as described (Ouyang et al., 1998).

#### Intracellular Cytokine Staining and FACS Analysis

Intracellular cytokine staining was performed as described (Ouyang et al., 1998). Retrovirally transduced T cells were stimulated with PMA (50 ng/ml) and ionomycin (1  $\mu$ M) for 4 hr with the addition of 3  $\mu$ M monensin for the last 2 hr of stimulation. Cells were harvested, washed twice in PBS, fixed in 4% paraformaldehyde for 10 min, washed twice in PBS 1% FCS, resuspended in 0.1% saponin/1% FCS/PBS, and stained using either PE-conjugated anti-cytokine antibodies (Pharmingen) or control-Ig-PE for 30 min. Cells were washed twice in 0.1% saponin/1% FCS/PBS, resuspended in PBS, and analyzed using a FACS Calibur.

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#### GenBank Accession Numbers

The human and mouse t-bet sequences described in this paper are entered in GenBank under accession numbers AF241242 and AF241243, respectively.