T-bet Binding to Newly Identified Target Gene Promoters Is Cell Type-independent but Results in Variable Context-dependent Functional Effects**

Received for publication, December 21, 2005, and in revised form, January 17, 2006. Published, JBC Papers in Press, February 10, 2006. DOI 10.1074/jbc.M513613200

Kristin M. Beima1, Michael M. Miazgowicz1, Megan D. Lewis1, Pearly S. Yan9, Tim H-M. Huang5, and Amy S. Weinmann11

From the 1Department of Immunology, University of Washington, Seattle, Washington 98195 and the 5Ohio State University, Columbus, Ohio 43210

Recently developed target gene identification strategies based upon the chromatin immunoprecipitation assay provide a powerful method to determine the localization of transcription factor binding within mammalian genomes. However, in many cases, it is unclear if the binding capacity of a transcription factor correlates with an obligate role in gene regulation in diverse contexts. It is therefore important to carefully examine the relationship between transcription factor binding and its ability to functionally regulate gene expression. T-bet is a T-box transcription factor expressed in several hematopoietic cell types. By utilizing a chromatin immunoprecipitation assay coupled to genomic microarray technology approach, we identified numerous promoters, including CXCR3, IL2RB, and CCL3, that are bound by T-bet in B cells. Most surprisingly, the ability of T-bet to associate with the target promoters is not dependent upon the cell type background. Several of the promoters appear to be functionally regulated by T-bet. However, we could not detect a functional consequence for T-bet association with many of the identified promoters in overexpression studies or an examination of wild type and T-bet−/− primary B, CD4+, and CD8+ T cells. Thus, there is a high variability in the functional consequences, if any, that result from the association of T-bet with individual target promoters.

Upon exposure to pathogenic stimuli, an intricately coordinated immune response composed of specialized cells is required for host defense. In order for these tightly regulated cellular responses to occur, individual hematopoietic cell types must carry out very precise and coordinated alterations in their gene expression patterns to up-regulate specific functional capabilities. A great deal of research has been undertaken to better address the molecular mechanisms by which critical, lineage-restrictive transcription factors contribute to this process in the immune system, but much still remains unknown.

T-box expressed in T cells (T-bet) is a member of the T-box transcription factor family and plays a critical role in the generation of CD4+ T helper 1 (Th1) cells (1). Th1 cell responses are critical for the clearance of altered self-cells such as those infected by pathogens or cancerous cells. Overexpression of T-bet in Th2 or naive CD4+ T cells commits them to a Th1 phenotype (1). Further solidifying the critical nature of T-bet in Th1 development, T-bet−/− mice have a profound defect in generating a Th1 cell response (2). Taken together, the data strongly indicate that T-bet plays a critical role in Th1 development and cell-mediated immunity.

The importance of T-bet in generating a productive immune response is not limited to its role in Th1 development. Several studies have highlighted the role of T-bet in B, NK, NKT, DC, and CD8+ T cell development and function (3–6). For example, immunoglobulin isotype class switching is defective in T-bet−/− mice, and this defect appears to be B cell intrinsic and not solely due to the deficient Th1 response (3). In addition, NK and NKT cells intrinsically require T-bet to develop into functionally mature cells (4). In contrast to CD4+ T cells, the requirement for T-bet in CD8+ T cells is less pronounced (2). Data using dominant negative constructs suggest that the less stringent requirement for T-bet in CD8+ T cells is at least in part due to the expression of another closely related T-box family member, eomesodermin (Eomes), in these cells (7). At least in some cases, Eomes can act in a functionally redundant manner with T-bet activity. Taken together, the current data suggest that T-bet is a very important transcription factor in several immune cell types, and there may be some cellular specificity to the role it plays. However, the molecular mechanism responsible for these apparent cell type differences, including whether they result from direct or indirect effects, is poorly understood.

To begin to address the role that T-bet plays in functionally distinct cell types, it is first necessary to identify the genes that are directly regulated by T-bet in different cellular settings. Recent advances in target gene identification strategies based upon the chromatin immunoprecipitation (ChIP)2 assay coupled to genomic microarray technology (chip) now make it possible to identify, in a more global manner, the promoters that are bound by a transcription factor within the context of the normal chromatin environment of a cell. Therefore, ChIP-based techniques provide a very compelling starting point to uncover the gene expression networks with the potential to be directly regulated by a transcription factor.

One assumption that is commonly made when interpreting data from ChIP-based assays is that the detected binding of a transcription factor at a promoter results in an obligate functional role for that factor in the regulation of the associated gene. Another common assumption made when examining a large data set from ChIP-chip studies is that if a select target gene is regulated then all genomic loci bound by the transcription factor are also regulated in a similar manner. However, transcription

---

1 To whom correspondence should be addressed: Dept. of Immunology, University of Washington, Box 357650, 1959 NE Pacific St., Seattle, WA 98195. Tel.: 206-616-7235; Fax: 206-543-1013; E-mail: weinmann@u.washington.edu.

2 The abbreviations used are: ChIP, chromatin immunoprecipitation; ChIP-chip, ChIP assay coupled to genomic microarray technology; IFN, interferon; IL, interleukin; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; P/IL, phorbol 12-myristate 13-acetate and ionomycin; Eomes, eomesodermin
factor family members, such as the T-box family, often have highly homologous DNA binding domains but diverge in the domains required for functional activity. This presents the real possibility that divergent family members may have the ability to associate with similar genomic loci, but they differ in their ability to regulate gene expression. In addition, the requirements for the activity of a transcription factor in the context of complex promoter structures in distinct cellular settings or activation conditions is often unclear. Therefore, a rigorous analysis examining both the ability of a transcription factor to bind to a locus and cause a functional consequence on gene expression in diverse cellular settings is important. This will aid in the understanding of a particular transcription factor family if the ability to associate with a target sequence is in fact the key regulated event in its ability to influence gene transcription or rather that binding is more promiscuous, and other downstream events are required for more tightly controlled functional activity. In this study, we examine the correlation between binding and functional activity in several distinct cell types to begin to address this question for the T-box transcription factor family member T-bet.

Utilizing a ChIP-based target gene approach, we have identified numerous promoter regions specifically bound by T-bet in B cells. Interestingly, T-bet has the ability to bind to the same subset of promoter regions in NK and T cells, suggesting that binding, at least for these targets, is independent of the cellular setting. However, upon examination of the functional consequence for T-bet binding to these promoters, the data indicate that some targets may be differentially regulated dependent upon the cell type background. Surprisingly, a significant number of target genes are not affected by either the overexpression or absence of T-bet, regardless of the ability of T-bet to associate with their promoter. In some of these cases, it is possible that either T-bet is not the functional family member at these promoters or there is functional redundancy in the activity of T-bet with other T-box family members or regulatory events. Indeed, Brachyury, another T-box family member present in the B cell line utilized for these studies, is able to associate with the same promoter regions that are bound by T-bet. Thus, the data demonstrate that the association of T-bet with a target promoter can result in several different outcomes, including obligatory, modest context-dependent, or no detectable regulatory role. The mechanism for these variable outcomes may involve the differential requirement for T-bet at distinct steps in transcriptional activity at each promoter. In support of this possibility, histone H3K9 acetylation is dependent upon T-bet expression only at the promoters that require T-bet for gene expression.

**EXPERIMENTAL PROCEDURES**

**Chromatin Immunoprecipitation**—The chromatin immunoprecipitation procedure was performed as described previously (8, 9). Briefly, cells were cross-linked in 1% formaldehyde followed by nuclei isolation. Nuclei were sonicated and precleared with pansorbin cells (Calbiochem) prior to immunoprecipitation with specific or control antibodies overnight at 4 °C. Following wash and elution steps, cross-links were reversed at 65 °C for 4 h. For samples to be used in the microarray analysis, two consecutive immunoprecipitations were performed with the same antibody before the cross-links were reversed. The DNA in the immunoprecipitated samples was purified by proteinase K digestion followed by phenol/chloroform extraction and DNA precipitation. Samples were then either analyzed by standard PCR with promoter-specific primers or amplified by ligation-mediated PCR for microarray analysis. The T-bet and Brachyury antibodies used in these studies were from Santa Cruz Biotechnology; the histone H3 acetyl-K9 antibody was from Upstate Biotechnology, Inc., and the IgG antibody was from MP Biomedicals.

For the ChIP-chip analysis, a human promoter microarray from NimbleGen Systems, Inc., was co-hybridized with either a T-bet-precipitated and total input control or an IgG-precipitated sample with the total input control. The IgG-precipitated/total microarray was utilized to eliminate nonspecific signals because of either nonspecific precipitation or ligation-mediated PCR amplification artifacts. Putative positive promoters were those that contained multiple consecutive and positive oligonucleotide probe signals. Only promoter regions that were confirmed in a subsequent standard ChIP analysis are shown in this paper because we did not perform an exhaustive analysis with the arrays, but rather we used them as a screening tool. The standard ChIP experiments shown as validation for the targets are representative of at least three independent experiments. In all ChIP experiments, IFNγ was first examined as a positive control and IL-4 as a negative control.

**Primary Cell Isolation**—All mouse studies were performed with approval and by IACUC guidelines. CD4⁺ T cells, CD8⁺ T cells, and B cells were isolated from BALB/c wild type and T-bet-/-/- mice. CD8⁺ T cells and B cells were isolated from spleen and lymph nodes using Miltenyi microbeads (CD8α⁺ T cell isolation kit catalog number 130-090-859 or CD43 microbeads catalog number 130-049-801, respectively). CD8⁺ T cells were stimulated with plate-bound αCD28 (clone 37.51; Pharmingen) and αCD3 (clone 145-2C11; Pharmingen) in the presence of IL-2 (10 ng/ml) and IL-12 (10 ng/ml) for 3 days. B cells were either left unstimulated or stimulated with IL-12 (10 ng/ml), IL-18 (10 ng/ml), and αCD40 (10 μg/ml) for 12 h. CD4⁺ T cells were isolated from spleen and lymph nodes using the MagCollect mouse CD4⁺ T cell isolation protocol (MAGM202; R & D Systems). CD4⁺ T cells were activated with plate-bound αCD3 and αCD28 in the presence of αIL-4 (generously provided by NCI, National Institutes of Health) overnight and then cultured in Th1 conditions (IL-2 at 10 ng/ml and IL-12 at 5 ng/ml) for 3 days. Cells were split 3 days after cytokine addition to a density of 2 × 10⁶ cells/well and cultured for another 3 days in Th1 conditions before harvest. In all experiments, cell purity was monitored following purification by flow cytometry.

**RT-PCR**—RNA from primary CD4⁺ T cells, CD8⁺ T cells, or B cells was isolated using the QIAgen RNA purification protocol with the optional DNase step included. RT-PCR primers were designed to span at least one intronic region. RT-PCR was carried out using the EZ TaqT polymerase kit (Applied Biosystems).

**Transient Transfection**—Transient transfection analysis of EL4 cells was performed using the AMAXA nucleofection system. 4 million cells per transfection were resuspended in nucleofection solution V. Setting O-17 was used for nucleofection with the standard protocol. The T-bet pcDNA3 expression construct was provided by Christopher Wilson.

**Western Blot Analysis**—Whole cell lysates from equal cell numbers of the human 721 B cell line, YT NK cell line, and Jurkat T cell line were loaded. The human 721 B cell line was kindly provided by Bill Sugden, and the YT NK cell line was kindly provided by Paul Sondel. Membranes were hybridized with the T-bet-specific antibody (Santa Cruz Biotechnology) before being stripped and reprobed with an antibody against GAPDH (Santa Cruz Biotechnology) as a loading control.

**RESULTS**

**T-bet Associates with the IFNγ Promoter in B Cells**—T-bet is expressed in B cells in response to several stimuli (1, 10), but very little is known about the genes that are directly regulated by T-bet in this cellular setting. To begin to address the molecular role that T-bet plays in B cell responses, it is necessary to identify the genes that are direct T-bet targets in B cells.
Variable Consequences for T-bet Association with Target Genes

We utilized the human 721 B cell line for our studies. Low levels of T-bet are constitutively expressed in either unstimulated or IFN-γ-stimulated 721 B cells (Fig. 1A). To identify T-bet targets, we utilized a ChIP-chip approach to provide a screening method to identify the DNA regions that are bound by T-bet in the context of the cellular environment of IFN-γ-stimulated B cells.

To ensure the feasibility of a ChIP-based approach for this study, a preliminary standard ChIP experiment utilizing a T-bet-specific antibody before being stripped and reprobed with an antibody against GAPDH as a loading control. β, the chromatin immunoprecipitation experiment was performed with a T-bet-specific antibody (lane 1) or an IgG control (lane 3) in the human 721 B cell line. A standardized aliquot of the total input chromatin is also shown (lane 2). Primers specific to the IFN-γ or IL-4 promoter regions were used for PCR amplification of the ChIP samples as indicated to the left of the gel image.

We performed a control. Following reversal of cross-links, the precipitated DNA was purified and amplified by ligation-mediated PCR. To identify the promoter regions that are specifically associated with T-bet, the T-bet-purified sample was co-hybridized with a total input control on a human promoter microarray. In parallel, the IgG-precipitated sample was subjected to the same analysis to subtract false positives because of nonspecific precipitation of DNA fragments. This analysis was performed as an initial screen only, and promoters were confirmed in standard ChIP assays utilizing promoter-specific primers to ensure that all targets presented in this study have been validated in this nonexhaustive screen.

The data in Fig. 2 show the validation of numerous promoter regions that are specifically bound by T-bet in B cells. Also shown is one of the false positives from this screening, $CCR1$, which is not enriched in the T-bet-purified chromatin in comparison to the IgG control. The $STAT1$ promoter, which has been shown previously to be bound by T-bet (13), was identified in our screen, which suggests that we are in fact identifying true target promoters. In addition, XCXR3, which is functionally regulated by T-bet in Th1 cells (14), was uncovered in our analysis by virtue of the association of T-bet with its promoter. $IL2RB$, which is important in activated and memory T cell responses, was also identified and validated.

Interestingly, several of the promoter regions that are bound by T-bet in B cells, such as $XCXR3$ and $PSGL1$, are differentially expressed in Th1 relative to Th2 cells (15). Although these promoter regions may have been predicted because of the well characterized role for T-bet in Th1 development, it was somewhat surprising that T-bet was able to bind to these promoters in B cells. Several other validated target genes, such as $RAD51$, $CALM2$, and $MAPK1$, are expressed in a more ubiquitous pattern, and therefore, one may not have predicted that T-bet associates with these genes in B cells. In addition, the promoters for $CCL3$ and $CCL3L1$ are both bound by T-bet (Fig. 2). $CCL3$ and $CCL3L1$ are important proinflammatory chemokines that are known to be suppressive for human immunodeficiency virus infection (16, 17). This locus has been the site of gene duplication events throughout evolution, and a recent study suggested that the copy number of $CCL3L1$ impacts the susceptibility to human immunodeficiency virus infection (16). We also detected T-bet association with another chemokine that resides in this region (Fig. 2B).

**FIGURE 1.** Examination of T-bet expression levels in human cell lines and association of T-bet with the IFN-γ promoter in B cells. A, Western analysis of T-bet protein levels in the human YT NK cell line (lane 1), Jurkat T cell line (lanes 2 and 3), and the 721 B cell line (lanes 4–6). Cells were either left unstimulated (lanes 1, 2, and 4) or stimulated (Stim) with IFN-γ (lane 5) or phorbol 12-myristate 13-acetate and ionomycin (P/Ι) (lanes 3 and 6) as indicated above the image. The membrane was probed with a T-bet-specific antibody before being stripped and reprobed with an antibody against GAPDH as a loading control. B, the chromatin immunoprecipitation experiment was performed with a T-bet-specific antibody (lane 1) or an IgG control (lane 3) in the human 721 B cell line. A standardized aliquot of the total input chromatin is also shown (lane 2). Primers specific to the IFN-γ or IL-4 promoter regions were used for PCR amplification of the ChIP samples as indicated to the left of the gel image.

**FIGURE 2.** Confirmation analysis for direct T-bet target genes identified in B cells. Shown is a representative ChIP experiment performed in IFN-γ-stimulated 721 B cells. Chromatin was precipitated with a T-bet-specific antibody (lanes 1 and 4) or an IgG control antibody (lanes 3 and 6). A standardized aliquot of the input chromatin (lanes 2 and 5) is also shown. Primers specific to the promoter regions for putative T-bet targets that were identified in the ChIP-chip screen were used for PCR analysis as indicated to the left of the gel images. IFN-γ and IL-4 were examined as a positive and negative control, respectively.

**TABLE 1.** Validation of T-bet binding to the IFN-γ promoter.

| Promoter | ChIP | Total | IgG |
|----------|------|-------|-----|
| IFN-γ    | 3    | 1     | 2   |
| IL-4     | 2    | 1     | 3   |
| STAT1    | 6    | 5     | 6   |
| CCL3     | 3    | 2     | 3   |
| CCL3L1   | 4    | 3     | 6   |
Variable Consequences for T-bet Association with Target Genes

T-bet Binding Is Independent of Cell Type Background—It is interesting to note that several of the T-bet target promoters that we identified in B cells are either regulated by T-bet in other cell types or differentially expressed in Th1 versus Th2 development. Therefore, one may hypothesize that T-bet will be able to bind to a portion of these promoters in other cell types. To address whether there is cell type specificity to the association of T-bet with these newly identified target promoters, we examined T-bet binding to these promoters in T and NK cells.

We first examined the human Jurkat T cell line. In resting cells, T-bet protein expression is absent; however, it is highly induced by phorbol 12-myristate 13-acetate and ionomycin (P/I) stimulation (Fig. 1A). Before testing whether T-bet could associate with the target promoters in the P/I-stimulated Jurkat T cells, we first performed an additional negative control to demonstrate that the enrichment of the novel target promoters in the T-bet-precipitated chromatin sample is in fact dependent upon T-bet expression. We performed a ChIP experiment in the absence of T-bet expression in resting Jurkat T cells (Fig. 1A and Fig. 3A). Importantly, the targets presented here are not enriched in the T-bet-precipitated chromatin in the absence of T-bet protein expression, in either resting Jurkat T cells (Fig. 3A) or HeLa cells (data not shown). These data suggest that the signal observed indeed is dependent upon T-bet expression.

It is worth noting that several false positives were uncovered during the analysis of the resting Jurkat T cells. For these regions, enrichment of a strong signal was detected in the T-bet-precipitated chromatin in the absence of T-bet protein expression in either unstimulated Jurkat cells or HeLa cells (data not shown). These signals may have been due to cross-reactivity of the T-bet antibody with another protein. The identification of these false positives makes it clear that potential targets identified in a ChIP-chip experiment, a procedure that is highly dependent upon antibody specificity, should be screened using the same antibody in a cell type that does not express that protein. This added control will help to eliminate false positives that are because of antibody cross-reactivity or impurities, which are common with most antibody preparations.

We next examined the targets in P/I-stimulated Jurkat T cells to determine whether T-bet can associate with the same set of promoter regions in a T cell background. Most interestingly, all of the targets identified in the B cells are also bound by T-bet in the Jurkat T cells (Fig. 3B). This observation was somewhat surprising because we hypothesized that the chromatin environment generated in different cellular backgrounds may provide at least some specificity for the ability of T-bet to associate with a subset of the target promoters. To further address this question in another cellular background, we next performed a ChIP experiment in the human YT NK cell line. The YT NK cell line has a high level of constitutive T-bet expression (Fig. 1A) and was previously utilized to identify RUNX1 as a functionally regulated T-bet target gene (4). Similar to the results in the T cells, T-bet associates with the newly identified B cell target genes in the NK cell line as well (Fig. 3C). These results suggest that the cell type background does not play a large role in restricting the inherent ability of T-bet to bind to the promoters.

T-bet Levels or Stimulation Conditions Can Influence Binding—Because of the lack of cell type-restricted binding for the T-bet targets identified in B cells, we next examined the cell type binding specificity for two T-bet targets, RUNX1 and CDK6, that were originally identified in a ChIP-chip screen in the YT NK cell line using a CpG island microarray (4) (Fig. 4A). As we observed with the T-bet targets that were originally identified in B cells, T-bet is able to associate with the RUNX1 and CDK6 promoters in the T cell background as well (Fig. 4B). Once again, binding specificity does not appear to be restricted by the cell type background.

We next performed a ChIP experiment in the 721 B cell line to determine whether T-bet is able to associate with the CDK6 and RUNX1 promoters in a B cell background. Interestingly, neither of these promoters appeared to be bound by T-bet in the IFNγ-stimulated 721 B cells as indicated by the lack of enrichment in the T-bet-precipitated chromatin in comparison to the nonspecific antibody control (Fig. 4C). In some experiments, we did detect a low level enrichment, but this signal was inconsistent (data not shown). Interestingly, when we examined RUNX1 and CDK6 in P/I-stimulated 721 B cells, both promoters were consistently enriched in the T-bet-precipitated chromatin, indicating that they are bound by T-bet in this setting (Fig. 4C).

The lack of binding in the IFNγ-stimulated B cells but the detected association in YT NK cells and P/I-stimulated 721 B and Jurkat T cells suggest that there are in fact some context-dependent differences in the ability of T-bet to associate with at least some promoters. One possibility for these apparent differences is the level of T-bet expressed in the various cell lines and stimulation conditions. It is possible that T-bet is able to associate with high affinity binding sites at low expression levels, whereas low affinity binding sites require high T-bet expression levels to be occupied. Our initial ChIP-chip screen was performed in IFNγ-stimulated 721 B cells, which express a low level of T-bet (Fig. 1A). Thus, it is possible that we selected for targets that have a high affinity for T-bet at the low expression levels, therefore resulting in the identification of targets that are more likely to be ubiquitously bound by T-bet. It is also possible that the stimulation conditions up-regulate cofactors that aid in the recruitment of T-bet to target promoters and result in some specificity for target gene selection. Taken together, the data suggest, at least for a subset of targets, that T-bet expression levels or possibly other
cooperative factors up-regulated in specific stimulation conditions may be more important for selecting specific genomic regions where T-bet binds than the inherent cell type background where it is expressed.

T-bet Overexpression Up-regulates a Subset of Target Genes—The data generated in the above experiments address the ability of T-bet to associate with specific genomic regions. However, these binding studies do not address what role T-bet plays, if any, in the regulation of these genes. It is possible that there may be a degree of cellular specificity in the functional capability of T-bet at a given promoter that is independent of its ability to bind. Mechanistically, this would suggest that functional specificity is downstream of DNA binding capacity. Cooperation with other lineage-restricted factors may play a role in modulating activity of T-bet in different cellular settings. Another possibility is that T-bet may not be required for the functional promoter activity for some of the identified genes. Other T-box family members, such as Eomes, may be the relevant family member. Therefore, although T-bet can associate with the promoters identified here, its role in the regulation of these genes needs to be carefully addressed.

To begin to address the functional role of T-bet in the regulation of these targets, we performed a series of studies to examine the mRNA expression levels for the identified targets in the presence and absence of T-bet. We first performed overexpression experiments in EL4 T cells. T-bet transcripts are barely detectable in resting EL4 T cells (Fig. 5). We transfected the cells with a T-bet overexpression construct, and we examined its effect on the mRNA expression levels for the endogenous target genes. Upon T-bet overexpression, the transcripts for several target genes are significantly increased. Cxcr3, Ccl3, Ifnγ, Stat1, and Il2Rβ are all up-regulated upon T-bet overexpression, suggesting that T-bet has the ability to activate these genes. However, many target genes were not altered upon T-bet overexpression. This may imply that T-bet alone does not have the ability to alter the levels of these targets. It is possible that a cooperative factor, perhaps activation or cell-type restricted, is required for T-bet to function at specific promoters. Alternatively, T-bet may not play a significant role in their regulation.

Examination of Primary Wild Type and T-bet<sup>−/−</sup> Cells Uncovers Distinct Differences in the Role of T-bet at Novel Promoters—To address these questions and further analyze the targets that are regulated by T-bet in the overexpression system, we examined target gene levels in several cell types isolated from wild type and T-bet<sup>−/−</sup> mice. We isolated primary naive B cells, CD8<sup>+</sup> T cells, and CD4<sup>+</sup> T cells from wild type and T-bet<sup>−/−</sup> mice. To induce T-bet expression, primary B cells were stimulated with αCD40, IL-12, and IL-18, whereas the CD8<sup>+</sup> T cells were stimulated with plate-bound αCD3 and αCD28 in the presence of IL-2 and IL-12. The CD4<sup>+</sup> T cells were stimulated with plate-bound αCD3 and αCD28 and were subjected to Th1 skewing conditions. We isolated RNA from the primary wild type and T-bet<sup>−/−</sup> cells and performed an RT-PCR analysis to examine the expression levels for the newly identified T-bet target genes in each cellular setting (Fig. 6, A–C).

We detected a dramatic decrease in Cxcr3 mRNA expression levels in T-bet<sup>−/−</sup> CD4<sup>+</sup> T cells relative to wild type cells (Fig. 6B). This is consistent with a study published previously that showed a decrease in Cxcr3 mRNA and protein expression levels in T-bet<sup>−/−</sup> Th1 cells (14). These data, in conjunction with our ChIP binding results, suggest that T-bet is directly required for Cxcr3 gene expression in Th1 cells. We next examined Cxcr3 expression in B cells and CD8<sup>+</sup> T cells. Interestingly, Cxcr3 levels decrease in T-bet<sup>−/−</sup> B cells but are not affected by the loss of T-bet in the CD8<sup>+</sup> T cells (Fig. 6, A and C). It is worth noting that Cxcr3 expression was not up-regulated to a large extent in the CD8<sup>+</sup> T cells in these in vitro stimulation conditions so further studies will be informative in these cells.

We also examined the other novel targets that are bound by T-bet in each of these primary cell populations. We observed a small decrease in Rad51 and Ccl3 expression levels in T-bet<sup>−/−</sup> CD8<sup>+</sup> T cells.
Variable Consequences for T-bet Association with Target Genes

(FIG. 6C). In contrast, T-bet−/− B cells had a modest increase in the expression of Rad51 and Ccl3 relative to wild type cells (FIG. 6A). Rad51 was not altered in T-bet−/− CD4+ T cells, although subtle differences in the low level of Ccl3 expression were more variable in this cell type (FIG. 6B and data not shown). We observed a minor decrease in Kdr expression in T-bet−/− CD4+ T cells, but it was not affected by T-bet loss in CD8+ T cells or B cells (FIG. 6, A–C). Collectively, these data suggest that the identified target genes may be differentially regulated by T-bet in a context-dependent manner. It also suggests that for the majority of regulated targets, with the exception of Cxcr3 and Ifnγ, T-bet is not required for expression but rather modestly modulates gene expression levels dependent upon the context in which it is expressed.

Surprisingly, the overexpression or loss of T-bet did not impact gene expression levels for a significant number of the newly identified targets in any of the cell types tested (FIGS. 5 and 6, A–C). It is possible that their activity may be regulated in another T-bet expressing cell type, such as NK or dendritic cells, or that alternative stimulation conditions that induce required cofactors may result in a more pronounced role for T-bet. In addition, redundancy with other T-box family members, such as Eomes, may mask the role for T-bet at a subset of these targets.

Indeed, we detected mRNA for Eomes and another T-box family member, Tbx6, in these cell populations (data not shown). However, it is also possible that some of these targets are not truly functionally regulated by T-bet.

Brachury Associates with T-bet Target Promoters—Because of the lack of cell type specificity in T-bet binding patterns and our inability to demonstrate a functional role for T-bet in the regulation of a significant number of genes, we hypothesized that it is possible that we have identified the target genes for the broader T-box family and that a portion of these may not in fact be functionally regulated by T-bet. The T-box DNA binding domain is highly conserved in this family and is able to interact with similar DNA sequences in vitro (18). The functional specificity for various family members is thought to be derived in part from the differential expression patterns for these proteins during development as well as from the N- and C-terminal domains that are not as highly conserved between family members (18).

To begin to address this broad question, we wanted to examine if other T-box proteins could interact with the T-bet target genes. Unfortunately, we were unable to examine Eomes binding in these studies because of the lack of an available antibody that works in the ChIP assay. Instead, we examined the ability of the original T-box family member, Brachury, to bind to these newly identified target promoters. Brachury is expressed in the 721 B cell line but not the YT NK cell line (data not shown). We performed a ChIP analysis to determine whether Brachury can associate with these T-bet target genes. Interestingly, Brachury can bind to the T-bet target promoters in the 721 B cells (FIG. 7A). As a control to demonstrate that this interaction is specific, and not because of cross-reactivity of the antibody with T-bet, we performed the same experiment in the YT NK cell line that does not express Brachury but does express high levels of T-bet. In YT NK cells, we no longer detect any enrichment of the target promoters in the Brachury-precipitated chromatin (FIG. 7B). These results suggest that Brachury can bind to at least a subset of T-bet target promoters. Unlike Eomes, Brachury does not share significant homology with T-bet outside of the DNA binding domain. Therefore, the data suggest that diverse members of the T-box family can interact with a similar set of genes, and one may hypothesize that their ability to regulate these genes is possibly derived from the less conserved activation or repression domains found in each protein.
Variable Consequences for T-bet Association with Target Genes

T-bet Is Required for Acetylation at the Cxcr3 and Ifnγ Promoters—To start to address the mechanism by which T-bet regulates gene expression, we examined the chromatin structure at the T-bet target gene promoters. Previous studies have shown that there is a loss in acetylation at the Ifnγ promoter in T-bet−/− Th1 cells (19). Therefore, we examined the histone H3K9 acetylation status at the T-bet target promoters in primary wild type and T-bet−/− CD4+ Th1 cells. Interestingly, H3K9 acetylation was completely dependent upon T-bet expression at the Cxcr3 and Ifnγ promoters, which require T-bet for expression (Fig. 8). In contrast, the absence of T-bet did not affect H3K9 acetylation at targets such as Il-2Rβ, Calm2, and Jmjd1A, where T-bet plays either a modest or no detectable role in expression (Fig. 8). These data suggest that the association of T-bet with target promoters results in distinct mechanistic events that are dependent upon the context of the promoter to which it binds. At least for the subset of targets examined in this study, the absolute requirement for T-bet in target gene regulation coincides with a critical role in H3K9 acetylation, whereas T-bet association with other promoters does not impact this event.

DISCUSSION

In this study, we have identified several genomic loci that are specifically bound by T-bet in B, NK, and T cells. Although little binding specificity is detected in relationship to the cell type, the ability of T-bet to associate with two of the promoters appears to be dependent upon T-bet expression levels or stimulation conditions. The functional role for T-bet in the regulation of target gene expression is highly variable and appears to have a component of cell type specificity. T-bet is absolutely required for Cxcr3 expression in Th1 cells and significantly required in B cells; however, we could detect no required role in CD8+ T cells. Surprisingly, many of the genes whose promoters are bound by T-bet are not significantly altered in either an overexpression system or in its absence. It is possible that a subset of these represent target genes that have a functional redundancy with or required role for other T-box family members. Collectively, the data suggest that there is additional complexity in the functional regulation of the target genes that is independent of DNA binding.

The recently developed ChIP-chip technique (also termed genome-wide location analysis) is a powerful approach to identify the genomic loci specifically bound by a transcription factor. Numerous studies have been performed to provide valuable information about the binding patterns for diverse transcription factors (9, 20–22). In most cases, it has been assumed that demonstrating binding is equivalent with a functional role. However, most studies have not examined this question with more than a handful of select target genes. In some ChIP-chip studies that have proceeded to examine gene expression consequences with more than a few select targets, it has been noted that no significant role could be detected for the association of the transcription factor with many target genes (22, 23). If the regulated step for a transcription factor to activate or repress gene activity is at the level of DNA binding, the ChIP-chip approach will uncover the genes that are the functional targets for that factor in a specific cellular setting. However, if the ability of a transcription factor to regulate gene expression is triggered by an event downstream of DNA binding (e.g. functional interactions dependent upon cellular setting or stimulation conditions), then a genome location analysis may not be representative in all cases of functional activity. Indeed, our studies show that transcription factor binding in many circumstances does not modulate the transcriptional activity of an individual gene, suggesting that transcription factors may be able to access their binding sites in many circumstances when all of the complements for activity are not present. Alternatively, binding may be more promiscuous than is generally believed and is not always productive. Taken together, these results all highlight the need to be careful in the interpretation of ChIP-based studies and that the binding of an individual transcription factor may not always result in transcriptional regulatory activity.

In this study we began to address the mechanisms by which T-bet is able to regulate genes in diverse cell types. A summary of the characteristics of the newly identified T-bet target genes is provided in Table 1. It was somewhat surprising to us that T-bet has the ability to associate with the same set of promoter regions in human B, NK, and T cell lines. We had hypothesized that because the chromatin structure at individual genes may vary in different cellular backgrounds, this would limit accessibility to a subset of binding sites in a cell type-specific manner and provide a means to establish cell specific transcriptional control of target genes. However, at least for the targets identified here, this does not appear to be the case.

It is interesting to note that the Ifnγ promoter is not highly acetylated in the IFNγ-stimulated 721B cells.3 Despite this inactive chromatin state, T-bet can bind to the Ifnγ promoter in this setting. These data imply that T-bet has the ability to be recruited to regions of the genome that are not in a fully active chromatin state and may place T-bet at the early stages of gene activation. This may help to explain the lack of cell type restricted binding observed in this study. In further support of the possibility that T-bet acts during the early stages of promoter accessibility, T-bet expression is required for H3K9 acetylation at the Cxcr3 and Ifnγ promoters. Collectively, these data suggest that T-bet has

3 M. Lewis and A. Weinmann, unpublished data.
the ability to access its binding site prior to the acetylation of the promoter and is critical, either directly or indirectly, in recruiting histone acetylase activity to the promoters. It is worth noting that this mechanism for T-bet activity is observed only at the promoters that require T-bet for expression but not those that are more modestly regulated. Thus, this appears to be one critical aspect by which T-bet regulates transcription, but other modes for influencing transcriptional activity are likely to occur at steps subsequent to chromatin remodeling events.

It should be noted that this analysis was not intended to be a comprehensive identification of T-bet target genes in all cellular settings. By performing our initial analysis in a cell type with very low T-bet expression levels in each unique cellular setting to determine whether these targets are also ubiquitously bound by T-bet. It is perhaps likely that a subset of target promoters will in fact have a cell type-restricted binding pattern when more targets are uncovered. If cell type-restricted binding targets are discovered in future studies, it will be important to determine the differences in promoter structure that account for the restriction of some targets but not others, such as the targets identified in this study. It is also worth noting that we did not address possible cell type-dependent differences in the affinity of T-bet for various targets, but rather we focused on the intrinsic ability of T-bet to associate with individual target promoters. It will perhaps be informative in future experiments to perform a careful titration of T-bet expression levels in each unique cellular setting to determine whether there are cell type-dependent affinity differences.

Despite the ubiquitous binding pattern for T-bet, we did encounter some differential target gene regulation dependent upon the cellular background. Three distinct classes of targets were encountered (Table 1). The first class of targets, Cxcr3 and Ifi30, require T-bet for gene expression in at least one cell type. In the second class of targets, the association of T-bet with the target promoter results in a modest augmentation or repression of gene expression, but this role is context-dependent (despite the ubiquitous nature of binding). Finally, a third class of targets is bound by T-bet, but no functional role in gene expression was detected in any of the contexts examined to date. It is possible that some of these targets may be modulated by T-bet in other cell types. For instance, Runx1, although not affected in the contexts examined in this study, is modestly regulated by T-bet in NK cells (4). Nevertheless, it is clear that binding does not always result in functional effects in all contexts and that other signaling events likely contribute to the activity of T-bet independently of DNA binding capacity.

It is likely that some of the targets that we have identified are functionally redundant targets with Eomes. Previous studies have shown that IFNγ mRNA and protein expression levels are dramatically decreased in T-bet−/− Th1 cells but are much less affected in CD8+ T cells (2). It appears that functional redundancy between T-bet and Eomes explains this differential requirement for T-bet (7). It is possible that the expression patterns for T-bet and Eomes also play a role in the differential surface expression level of IL2Rβ in NK and NKT cells isolated from wild type and T-bet−/− mice. NK cells express both T-bet and Eomes, whereas NKT cells only express T-bet (4). Interestingly, IL2Rβ surface expression is normal in T-bet−/− NK cells but dramatically diminished in T-bet−/− NKT cells (4). Therefore, it is possible that IL2Rβ may be influenced by both of these factors. Indeed, a recent study does provide evidence that T-bet and Eomes do in fact play a functionally redundant role in IL2Rβ expression in CD8+ T cells (24). Taken in conjunction with our binding data demonstrating that T-bet can bind to the IL2Rβ promoter, the data collectively suggest T-bet has the ability to regulate this promoter. A more complete understanding of the members of this family in the immune system is required to address the overlapping and exclusive functions for this protein family during the immune response.

One of the most surprising results of our ChIP-chip studies is that T-bet can bind to a significant number of targets where we can demonstrate no functional role despite examining numerous different cellular settings. It is known that the different T-box family members can frequently bind to very similar sequences in vitro (18). It is possible that T-bet does not play a tangible role at some of the identified targets and instead other T-box family members regulate their expression. Our studies highlight the need for a functional analysis of the target genes that are identified in ChiP experiments. With large transcription factor families, one must be careful to equate the binding of a transcription factor to a promoter with an obligate role in gene function.

Acknowledgments—We thank Steve Smale, Chris Wilson, and Lynn Hajjar for critical reading of the manuscript. We also thank Alexander Rudensky and Mark Bix for helpful suggestions and Kiea Arispe for animal husbandry.

REFERENCES
1. Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G., and Glimcher, L. H. (2000) Cell 100, 655–669
2. Szabo, S. J., Sullivan, B. M., Stemmann, C., Satoskar, A. R., Sleckman, B. P., and Glimcher, L. H. (2002) Science 295, 338–342
3. Peng, S. L., Szabo, S. J., and Glimcher, L. H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5545–5550
4. Townsend, M. J., Weinmann, A. S., Matsuda, J., Salaman, R., Farnham, P. J., Biron, C. A., GAPIN, L., and Glimcher, L. H. (2004) Immunity 20, 477–494
5. Lugo-Villarino, G., Maldonado-Lopez, R., Possemato, R., Penaranda, C., and Glimcher, L. H. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 7749–7754
6. Sullivan, B. M., Juedes, A., Szabo, S. J., von Herrath, M., and Glimcher, L. H. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 15818–15823
Variable Consequences for T-bet Association with Target Genes

7. Pearce, E. L., Mullen, A. C., Martins, G. A., Krawczyk, C. M., Hutchins, A. S., Zedia, V. P., Banica, M., DiCioccio, C. B., Gross, D. A., Mao, C. A., Shen, H., Cereb, N., Yang, S. Y., Lindsten, T., Rossant, J., Hunter, C. A., and Reiner, S. L. (2003) Science 302, 1041–1043
8. Weinmann, A. S., and Farnham, P. J. (2002) Methods 26, 37–47
9. Weinmann, A. S., Yan, P. S., Oberley, M. J., Huang, T. H.-M., and Farnham, P. J. (2002) Genes Dev. 16, 235–244
10. Liu, N., Ohnishi, N., Ni, L., Akira, S., and Bacon, K. B. (2003) Nat. Immun. 4, 687–693
11. Cho, J. Y., Grigura, V., Murphy, T. L., and Murphy, K. (2003) Int. Immunol. 15, 1149–1160
12. Shnyreva, M., Weaver, W. M., Blanchette, M., Taylor, S. L., Tompa, M., Fitzpatrick, D. R., and Wilson, C. B. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 12622–12627
13. Lovett-Racke, A. E., Rocchini, A. E., Choy, J., Northrop, S. C., Hussain, R. Z., Ratts, R. B., Sikler, D., and Racke, M. K. (2004) Immunity 21, 719–731
14. Lord, G., Rao, R. M., Choe, H., Sullivan, B. M., Lichtman, A. H., Luscsinskas, F. W., and Glimcher, L. H. (2005) Blood 106, 3432–3439
15. Rogge, L., Bianchi, E., Biffi, M., Bono, E., Chang, S. Y., Alexander, H., Santini, C., Ferrari, G., Sinigaglia, L., Seiler, M., Neeb, J., Sinigaglia, F., and Certa, U. (2000) Nat. Genet. 25, 96–103
16. Gonzalez, E., Kulkarni, H., Bolivar, H., Mangano, A., Sanchez, R., Catano, G., Nibbs, R. J., Freedman, B. I., Quinones, M. P., Bamshad, M. J., Murthy, K. K., Rovin, B. H., Bradley, W., Clark, R. A., Anderson, S. A., O'Connell, R. J., Agan, B. K., Ahuja, S. S., Bologna, R., Sen, L., Dolan, M. J., and Ahuja, S. K. (2005) Science 307, 1434–1440
17. Verani, A., Scarlatti, G., Comar, M., Tresoldi, E., Polo, S., Giacca, M., Lusso, P., Siccardi, A. G., and Vercelli, D. (1997) J. Exp. Med. 185, 805–816
18. Tada, M., and Smith, J. C. (2001) Dev. Growth Differ. 43, 1–11
19. Avni, O., Lee, D., Macian, F., Szabo, S. J., Glimcher, L. H., and Rao, A. (2002) Nat. Immun. 3, 643–651
20. Ren, B., Cam, H., Takahashi, Y., Volkert, T., Terragni, J., Young, R. A., and Dynlacht, B. D. (2002) Genes Dev. 16, 245–256
21. Odom, D. T., Zizlberger, N., Gordon, D. B., Bell, G. W., Rinaldi, N. J., Murray, H. L., Volkert, T. L., Schreiber, J., Rolfe, P. A., Gifford, D. K., Fraenkel, E., Bell, G. I., and Young, R. A. (2004) Science 303, 1378–1381
22. Martone, M., Euskirchen, G., Bertone, P., Hartman, S., Royce, T. E., Luscombe, N. M., Rinn, J. L., Nelson, F. K., Miller, P., Gerstein, M., Weissman, S., and Snyder, M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 12247–12252
23. Phillip, P. L., Friedman, J. R., Schug, J., Brestelli, J. E., Parker, J. B., Bochkis, I. M., and Kaestner, K. H. (2005) PLoS Genet. 1, e16
24. Intlekofer, A. M., Takemoto, N., Wherry, E. J., Longsworth, S. A., Northrup, J. T., Panalivel, V. R., Mullen, A. C., Gasink, C. R., Kaech, S. M., Miller, J. D., Gapin, L., Ryan, K., Russ, A. P., Lindsten, T., Orange, J. S., Goldrath, A. W., Ahamed, R., and Reiner, S. L. (2005) Nat. Immun. 6, 1236–1244