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Gene 377 (2006) 130–139
GENE
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T-box proteins differentially activate the expression of the endogenous interferon γ gene versus transfected reporter genes in non-immune cells

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Received 21 December 2005; received in revised form 17 March 2006; accepted 25 March 2006
Available online 5 June 2006
Received by A.J. van Wijnen

Abstract

The T-box transcription factor T-bet is expressed in a number of hematopoetic cell types and plays an essential role in the lineage determination of Th1 T-helper cells. In the absence of T-bet, $CD4^+$ T-cells fail to induce IFNγ, the cytokine whose expression characterizes Th1 cells. Here we show that, surprisingly, T-bet induces the expression of endogenous IFNγ in non-immune human cells, including 293 and other cell lines. Thus T-bet can induce IFNγ expression independently of its role in T-cell lineage determination. In addition, mutations in T-bet, and chimeras of T-bet with other transcription factors including the T-box transcription factor, *TBX2*, differentially affect the ability of T-bet to activate expression of endogenous IFNγ versus a T-site regulated reporter gene. A truncated T-betVp16 fusion protein strongly activates the T-site reporter but fails to activate endogenous IFNγ. Conversely, native T-bet strongly activates endogenous IFNγ expression but only weakly activates the reporter gene. Fusion of the Vp16 activation domain to full-length T-bet greatly increases its activation of both endogenous IFNγ and transfected T-site reporter gene expression. In contrast, TBX2Vp16 potently activates the T-site reporter but has a negligible effect on endogenous IFNγ expression. Butyrate treatment of T-bet expressing cells potentiates the expression of endogenous IFNγ but weakly represses expression of the T-site reporter gene. These data indicate that induction of endogenous IFNγ can be uncoupled from differentiation into the Th1 lineage and that the expression of endogenous IFNγ versus a T-site reporter gene is differentially regulated by T-bet and other T-box proteins.
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Keywords: Transcription; HDAC; T-bet; T-cells; Differentiation; Hematopoiesis

1. Introduction

T-box genes encode a family of transcription factors that play important roles during embryonic and post-embryonic development in a variety of tissues (Naiche et al., 2005). As with many transcription factor families, the proteins encoded by these genes share a conserved DNA-binding domain, the T-box. Most T-box proteins bind the same canonical palindromic T-site in vitro, although evidence in many organisms and cell culture indicates that different T-box proteins regulate unique downstream targets in vivo (Conlon et al., 1996; Takeuchi et al., 2003; Butz et al., 2004; Plageman and Yutzey, 2004; Messenger et al., 2005; Porsch et al., 2005; Takahashi et al., 2005). Endogenous T-box binding sites and target genes have been reported for only a few T-box proteins. One of these is T-

Abbreviations: 293, human embryonic kidney 293 cells; Ap, activator protein; ATF/CREB, activating transcription factor/c-AMP response element binding protein; βgal, β-galactosidase; β2M, β2-microglobulin; CMV IEP, cytomegalovirus immediate early promoter; EcR, ecdysone receptor; EMS, electrophoretic mobility shift; HRP, horseradish peroxidase; HDAC, histone deacetylase complex; HSVtk, herpes simplex thymidine kinase promoter; IFNγ, interferon gamma; *IFNG*, interferon gamma gene; IRF-1, interferon response factor 1; luc, luciferase; murA, muristerone A; NFAT, nuclear factor activated in T-cells; NFκB, nuclear factor kappa B; NLS, nuclear localization signal; PBS, phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate; ponA, ponasterone A; QPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcription–polymerase chain reaction; STAT, signal transducer and activator of transcription; SV40 polyA, simian virus 40 late polyadenylation signal; T/2, T-half site; TBST, tris-buffered saline with Tween20; Th1, type 1 T-helper cells; Th2, type 2 T-helper cells; Txk, member of Tec family kinases; Vp16, herpes simplex virus protein 16; YY1, ying–yang 1.

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doi:10.1016/j.gene.2006.03.016

bet; a T-box protein whose expression is restricted to hematopoietic cells that express the cytokine IFNγ. Mice lacking T-bet are viable but exhibit a number of phenotypes, including asthma, associated with a skewing of T-helper cells towards a Th2 phenotype (Finotto et al., 2002; Finotto and Glimcher, 2004). Expression of T-bet in naïve or Th2 polarized T-cells or cell lines has been shown to induce IFNγ expression as well as other markers of Th1 polarization (Szabo et al., 2000, 2002).

A number of studies have suggested that the IFNγ gene is likely a direct target of activation by T-bet in $CD4^+$ T-cells (Mullen et al., 2001; Soutto et al., 2002; Cho et al., 2003; Lovett-Racke et al., 2004; Tong et al., 2005). However, since the expression of T-bet in T-cells initiates a cascade of events that lead to Th1 polarization, it has not been possible to separate out the role of T-bet in the transcriptional activation of target genes from its role in the polarization of T-cells. To do this we developed a non-hematopoietic cell system in which to study the target selectivity of T-bet and other T-box proteins and the role of domains other than the DNA-binding domain in transactivation.

By comparing the ability of several T-box proteins, including T-bet, to activate transiently transfected reporter constructs versus endogenous genes, we show that T-bet and to a much lesser extent TBX2, can induce endogenous IFNγ transcription in non-hematopoietic cell lines and that this induction is potentiated by the HDAC inhibitor, sodium butyrate. These data uncouple the induction of IFNγ expression from T-cell polarization. In addition, the differential effects of T-bet and other normal and chimeric T-box proteins on the activation of endogenous IFNγ and transient reporter genes demonstrate that there are different requirements for transactivation of endogenous versus transfected genes. This finding has important implications for the analysis of the regulation of gene expression in cultured cells.

2. Materials and methods

2.1. Cell culture and transient transfection assays

293, HeLa and JEG-3 cells were maintained in α-MEM (Invitrogen) supplemented with 10% fetal calf serum and penicillin–streptomycin at 37 °C/5%CO_2. 24 h prior to transfection, cells were seeded at 2×10^5 cells/ 35 mm dish. The CMV expression vectors used were constructed as described previously (Sinha et al., 2000) and in Fig. 1. The reporter and normalizing constructs tkGL2, 4xT/2tkGL2 and CMVβgal were described previously (Sinha et al., 2000). 293 cells were transfected with the indicated amounts of each expression vector, 1 μg 4xT/2tkGL2 reporter plasmid and 50 ng CMV-βgal or 25 ng RL-SV40 to normalize for transfection efficiency. HeLa and JEG-3 cells were transfected with 250 ng of the TBET expression vectors and the indicated reporters. Transfections were performed using lipofectamine reagent (Invitrogen) and assayed using the Single or Dual Luciferase Reporter Assay System Kits (Promega) as described previously (Sinha et al., 2000). Firefly luciferase values were normalized to renilla luciferase or β-galactosidase activity levels. Transfections were performed in duplicate in at least two independent experiments and results are expressed as the mean ± the standard error. Where indicated transfected cells were treated with 1 mM sodium butyrate (Sigma) in PBS or PBS alone for 24 h.

2.2. Stable transfection

A TbetEcR vector was constructed and used to generate a stable TbetEcR expressing 293 cell line with the same strategy used previously to produce a Tbx2EcR expressing cell line (Butz

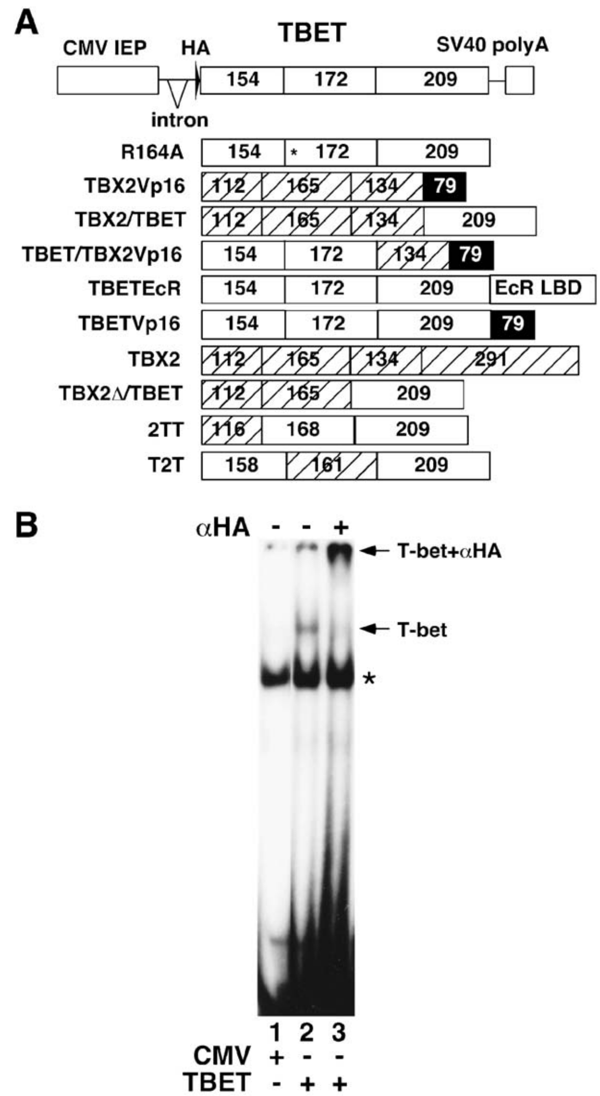

Fig. 1. T-box expression constructs and T-bet DNA-binding activity. *A. Expression constructs.* All constructs were expressed from the same CMV vector containing a CMV IEP, intron, HA-tagged T-box protein and SV40 polyA site described previously (Sinha et al., 2000). Constructs TBET, R16A and 2TT contain two additional residues (PS) at the C-terminus of the protein. Numbers within the boxes indicate the number of residues present in the N-terminal, DNA-binding, C-terminal, and Vp16 domains. *B. T-bet DNA-binding activity.* EMSA assay using T oligo and extracts from 293 cells transfected with 500 ng of the following plasmids. *Lane 1*, CMV; *lanes 2 and 3*, TBET. In *lane 3* 1 μl of αHA antibody (12CA5, Boehringer Mannheim) was added to the EMSA reaction. The asterisk marks a non-specific band and arrows indicate specific bands.

et al., 2004). 24 h prior to transfection cells were seeded at a density of 2×10^5 cells/ 35 mm dish. Cells were co-transfected with a puromycin-selectable plasmid and the TbetEcR expression vector using lipofectamine reagent (Invitrogen). Two days following the DNA transfection, each dish was split into two 100 mm dishes and puromycin (1 μg/ml) was added to the medium the following day. Stable clones were isolated after 10–14 days and maintained in medium containing puromycin. Where indicated, cells were treated with 15 μM PonA (Invitrogen) in EtOH, 10 μM MurA (Invitrogen) in EtOH, EtOH alone or 1 μg/ml CHX (Sigma) for the times given in the figure legends.

2.3. RNA extraction, cDNA synthesis and QPCR

Cells were harvested in Trizol (Invitrogen) and total RNA isolated according to the manufacturer's instructions. 5 μg total RNA was reverse-transcribed at 42 °C for 50 min in the presence of random hexamers and SuperScript II Reverse Transcriptase (Invitrogen). *IFNG* mRNA expression levels were measured by Quantitative Real-Time PCR (QPCR) using the primers: 5′-GTGTGGAGACCATCAAGGAAGA C-3′ and 5′-AGTTCAG CCATCACTTGGATGAG-3′. Transfected TBET mRNA expression levels were measured using primers: 5′-AACCAGAA AGTTAACTGGCCTG-3′ and 5′-GTAATCTGGAACATCG TATGGG-3′. The latter primers do not amplify transfected DNA. QPCR was carried out with the SYBR Green Supermix (Bio-Rad) using a Real-Time PCR instrument (Bio-Rad) according to the manufacturer's instructions. In each reaction *IFNG* expression was assayed in duplicate or triplicate and normalized to the level of β2-microglobulin as described previously (Butz et al., 2004). Data are expressed as the mean ± the standard error. Non quantitative PCR reactions were performed using the same primers and either 32 (β2-microglobulin) or 35 (IFNγ) cycles of amplification.

2.4. Western blotting

Cell extracts were resolved on 10% SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore). After blocking overnight, membranes were probed with a 1:400 dilution of αHA monoclonal antibodies (clone 12CA5, Boehringer Mannheim) for 3 h to assess protein expression. Western blots were washed in TBST and incubated with a 1:1000 dilution of HRP anti-mouse antibodies (Amersham) for 1 h. Proteins were detected using ECL Plus Western blotting detection reagents (Amersham) according to the manufacturer's instructions and exposed to X-ray films (Kodak).

2.5. Indirect assay for IFNγ protein expression

293 or JEG-3 cells were transiently transfected as described above and media removed 48 h post transfection. The media was filtered through 0.2 μ filters to remove cells and 1 ml was added to test 293 cells for 1 h. Cell extracts were prepared and assayed for STAT binding activity using an Electrophoretic mobility shift assay.

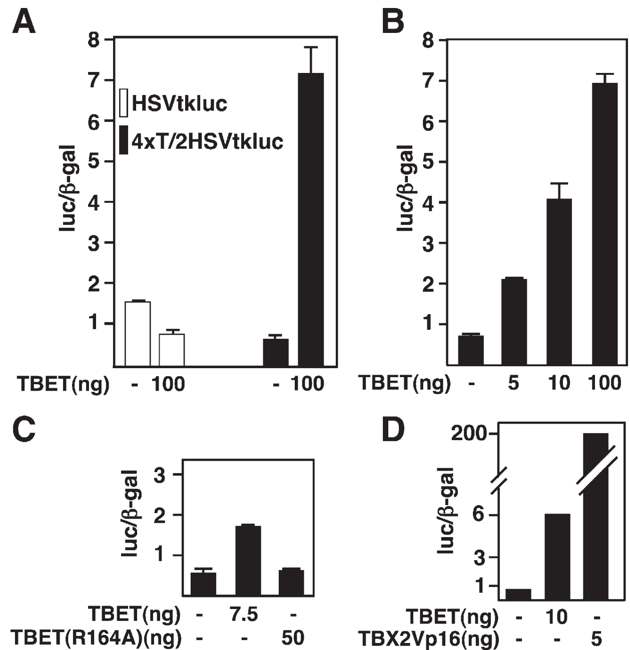

Fig. 2. Activation of reporter construct expression by TBET, R164A and TBX2Vp16. 293 cells were transfected and assayed for luciferase and β-galactosidase activity as described in Materials and methods. Bars show mean ± S.E. for duplicate extracts. *A. T-bet activates expression of reporter construct containing T-box DNA-binding sites.* Cells were transfected with HSVtkluc (open bars) or 4xT/2HSVtkluc (filled bars) plus empty CMV vector (−) or TBET (+). *B. Activation by T-bet is DNA concentration dependent.* Cells were transfected with 4xT/2HSVtkluc and indicated amounts of TBET vector. *C. A missense mutation in the DNA-binding (T-box) domain destroys activation by T-bet.* Cells were transfected with 4xT/2HSVtkluc and indicated amounts of TBET or R164A vectors. *D. T-bet is a weaker activator of the reporter construct than TBX2Vp16.* Cells were transfected with 4xT/2HSVtkluc and indicated amounts of TBET or TBX2Vp16 vectors.

2.6. Electrophoretic mobility shift assays

Electrophoretic mobility shift (EMS) assays were carried out as described previously (Sinha et al., 2000) using either a ^{32}P-labelled T oligonucleotide (5′-CTAGATTTCACACCTAGGT GTGAAATCTAG-3′) or a STAT oligonucleotide (5′-TCGAG CCTGATTTCCCCGAAATGACGGC-3′). The latter sequence was derived from the IRF-1 promoter and was the gift of S. Haque, Lerner Research Institute, Cleveland OH.

3. Results

3.1. T-bet DNA binding and activation of reporter gene expression

Based on the published murine T-bet sequence (Szabo et al., 2000), we cloned T-bet from cDNA prepared from PMA/ionomycin activated peripheral human T-cells and expressed it from the strong CMV promoter (Fig. 1A). To assess the DNA-binding activity of human T-bet, the protein was expressed in human 293 cells, extracts were prepared and binding of T-bet to a canonical T-site was measured in an Electrophoretic Mobility Shift assay (Fig. 1B). T-bet bound to the T-site (Fig. 1B, lanes 2

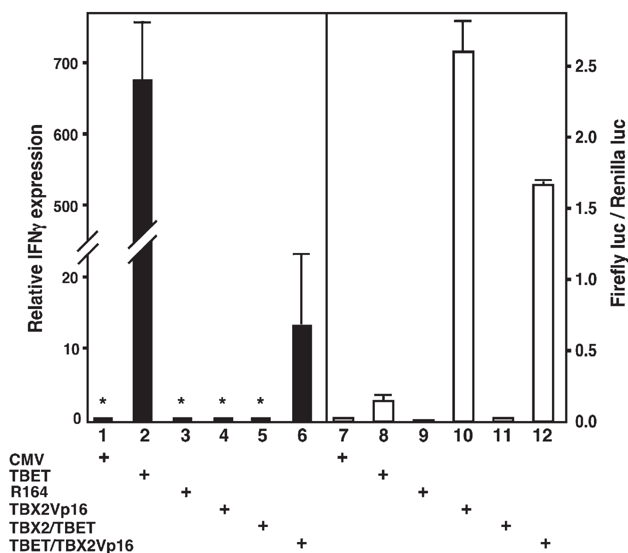

Fig. 3. Effect of the C-terminus of TBET on endogenous IFNγ and transfected T-site reporter gene induction. 293 cells were transfected with the following plasmids: *lanes 1 and 7*, CMV; *lanes 2 and 8*, TBET; *lanes 3 and 9*, R164A; *lanes 4 and 10*, TBX2Vp16; *lanes 5 and 11*, TBX2/TBET; *lanes 6 and 12*, TBET/TBX2Vp16. *Lanes 1–6 (IFNγ induction)*: RNA was prepared from transfected cells and QPCR was performed as described in Materials and methods. Here and in Figs. 6–8 *asterisks* indicate samples for which IFNγ signals were below the sensitivity of the assay (40 cycles). IFNγ transcript levels in those samples were set to 40 cycles and an expression level of 1 and were used to calculate relative levels of IFNγ expression. *Lanes 7–12 (Reporter gene expression)*: 293 cells were transfected with 1 μg of 4xT/2HSVtkluc, 25 ng control RL-SV40 vector and 50 ng of the indicated vectors and cell extracts were prepared and assayed for firefly and renilla luciferase as described in Materials and methods.

and 3), the same DNA sequence that we showed previously is bound by TBX2 (Sinha et al., 2000) and which has been shown to be recognized by several other T-box proteins [reviewed in Naiche et al. (2005)]. Transcriptional activation by T-bet was assessed by expressing T-bet with a luciferase reporter gene driven by a 4 half-T-site (T/2) containing minimal tk promoter that was previously used to assess activation and repression of transcription by TBX2 and other T-box proteins (Sinha et al., 2000). T-bet activated expression of the T/2-site reporter vector ~8 fold, while no activation was seen of the same promoter lacking the T/2-sites (Fig. 2A, black versus white bars). Promoter activity increased with increasing amounts of the T-bet expression vector (Fig. 2B). In addition, an arginine to alanine mutation in the T-bet DNA-binding domain that is homologous to an inactivating mutation described previously in the TBX2 DNA-binding domain (Sinha et al., 2000) abolished promoter activation (Fig. 2C, R164 versus T-bet). To assess the relative potency of T-bet as a transcriptional activator, we compared the activity of the T/2-site reporter in the presence of T-bet and TBX2Vp16, previously shown to be a strong activator of T-site dependent transcription (Sinha et al., 2000). While T-bet activated the T-site reporter expression ~6 fold, TBX2Vp16 activated it ~200 fold (Fig. 2D) at 1/2 the amount of expression vector. Thus TBX2Vp16 is a much stronger activator than T-bet of the transiently transfected T/2-site reporter gene.

3.2. T-bet activation of IFNγ expression in 293 cells

Since T-bet had been reported to activate IFNγ expression in T-cells, we measured IFNγ mRNA in 293 cells transfected with vectors expressing T-bet or T-box containing proteins (Fig. 3 black bars). T-bet increased the level of IFNγ transcripts by over 600 fold as detected by QPCR (Fig. 3, bar 2 versus bars 3–5). This strong activity of T-bet for IFNγ induction is in contrast to its relatively weak activity on the transient T/2-site reporter gene

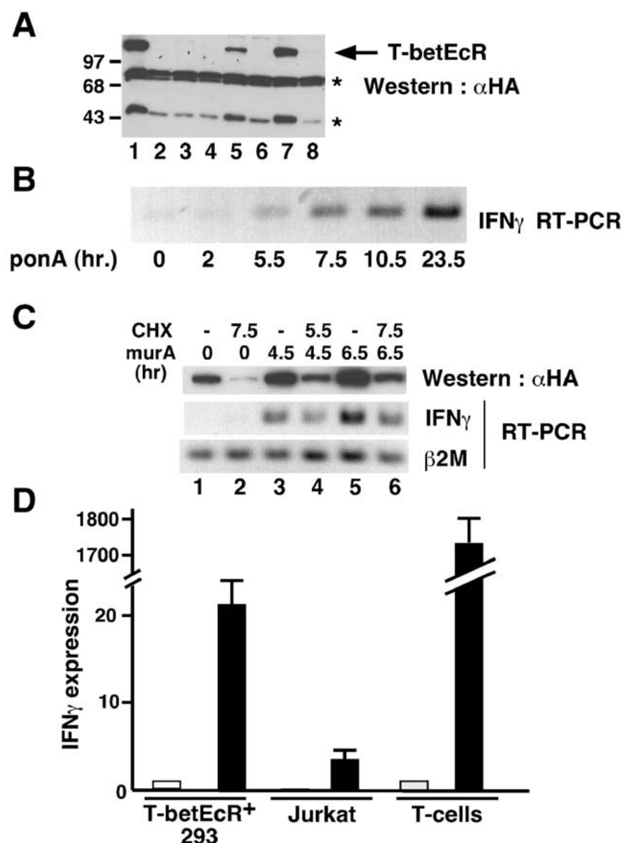

Fig. 4. Cells stably expressing T-betEcR make IFNγ in response to ligand. 293 cells were stably transfected with TBETEcR vector and pGKpuro as described in Materials and methods and clones were isolated. *A. Western blot of transient and stable transfectants for the presence of HA-tagged T-betEcR protein. Lane 1*, 293 cells transiently transfected with TBETEcR (included as a positive control); *lanes 2–8*, individual 293 clones. *Arrow* indicates position of full-length protein at predicted size of ~120 kD in *lanes 1, 5 and 7*. *Asterisks* indicate non-specific bands. *B. IFNγ induction in T-betEcR expressing cells.* Cells from the clone shown in *lane 7* of *panel A* (TBETEcR+293) were exposed to ponasterone A (15 μM) for the times indicated, harvested for RNA and analyzed by RT-PCR for IFNγ induction. *C. Cycloheximide effect on T-betEcR and IFNγ expression*. TBETEcR+293 cells were treated for times indicated with cyclohexamide (1 μg/ml) and muristerone A (10 μM) and were analyzed by Western blot for the presence of T-betEcR (*upper panel*) and by RT-PCR for IFNγ and β2-microglobulin (*middle and lower panels*, respectively). *D. IFNγ induction in TBETEcR+ 293, Jurkat and human T-cells.* QPCR was used to determine the relative IFNγ transcript levels in primary human T-cells (a gift of J. Finke, Lerner Research Institute, Cleveland Clinic Foundation) and Jurkat cells with or without activation (5 h PMA (10 ng/ml) and ionomycin (0.75 μg/ml)) and TBETEcR+293 cells with or without activation (4.5 h 10 μM muristerone A). IFNγ levels in non-activated and activated cells are shown in open and filled bars, respectively. All values are normalized to β2M levels in the same cDNA samples and expressed relative to IFNγ transcript levels in non-activated T-cells set to 1.

(Fig. 3, bar 8). The reporter gene was strongly activated by TBX2Vp16 (Fig. 3, bar 10) which failed to activate IFNγ (Fig. 3, bar 5). As expected, the DNA-binding domain mutant R164A failed to activate either IFNγ or the T/2-site reporter (Fig. 3, bars 3 and 9).

3.3. Requirement for T-bet C-terminus in IFNγ but not transient reporter induction

To ask whether the T-bet C-terminus was necessary to confer IFNγ inducibility on a chimeric T-box protein, we fused a region of the TBX2Vp16 protein (containing the NLS of TBX2 and the Vp16 transactivation domain) to the N-terminal 326 residues of T-bet (see Fig. 1A). When this protein was expressed in 293 cells, it strongly activated T/2-site reporter gene expression (Fig. 3, bar 12) but only weakly activated endogenous IFNγ expression (Fig. 3, bar 6, note break in scale on left). These data indicate that swapping the C-terminus of T-bet for another transactivation domain significantly impaired the ability of the chimeric protein to activate endogenous IFNγ transcription and strongly suggests that the C-terminus of T-bet is involved in endogenous target gene selection.

3.4. IFNγ induction by stably expressed T-betEcR

To ask whether IFNγ induction in 293 cells is a direct effect of T-bet or an indirect effect mediated through induction of another protein, we made a vector expressing a chimeric T-bet-ecdysone receptor ligand binding domain protein (Fig. 1A) and stably introduced this vector into 293 cells (Fig. 4A). We showed previously that the ecdysone receptor ligand binding domain (EcR-LBD) could repress the function of another T-box protein, TBX2, and render its transcriptional activity inducible by ecdysone analogues (Butz et al., 2004). In 293 cells expressing T-betEcR, treatment with ecdysone analogue ponasterone A caused a time-dependent increase in IFNγ transcript levels (Fig. 4B). Tbx2EcR expressing 293 cells (Butz et al., 2004) showed no such increase (data not shown), indicating that IFNγ induction was not a non-specific effect of ponasterone A.

To determine whether this increase in IFNγ expression required de novo protein synthesis, we compared T-betEcR levels (Fig. 4C, upper panel) and IFNγ induction (Fig. 4C, middle panel) in the absence and presence of cycloheximide. In the absence of an ecdysone analogue, treatment with cycloheximide reduced T-betEcR levels and no expression of IFNγ was detected (Fig. 4C, lane 2 versus lane 1). In the presence of the ecdysone analogue muristerone A for 4.5 or 6.5 h, T-betEcR levels were slightly increased over those seen in uninduced cells and strong IFNγ expression is seen (Fig. 4C top and middle panels, lanes 3 and 5). Addition of cycloheximide 1 h prior to induction with muristerone A prevented the increase in T-betEcR protein levels but only slightly reduced the increase in IFNγ transcripts (Fig. 4C, lanes 4 and 6). These data suggest that T-betEcR protein is stabilized by ecdysone analogues and that ongoing protein synthesis is not needed for the T-bet induction of IFNγ transcripts. Thus it appears that T-bet activity directly increases IFNγ transcript levels with no requirement for ongoing protein synthesis.

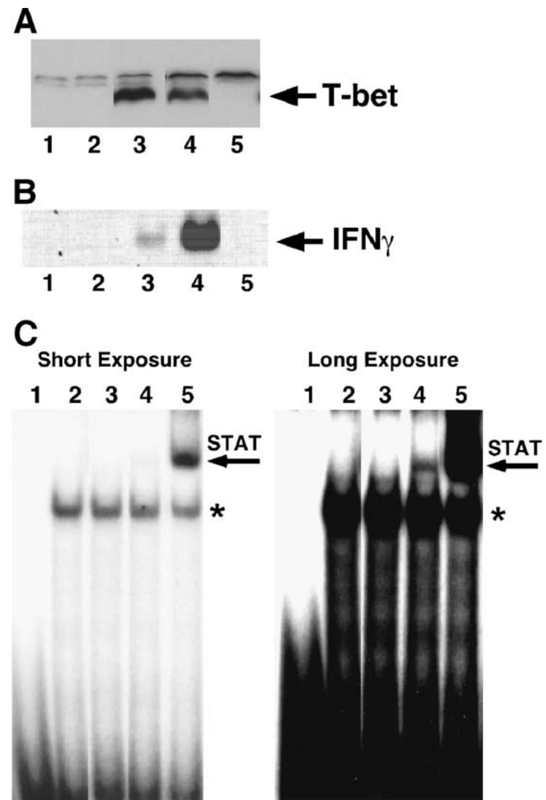

Fig. 5. TBET activates endogenous IFNγ RNA and protein expression in JEG-3 and 293 cells. JEG-3 or 293 cells were transfected and protein, RNA or conditioned medium was harvested for assay. *A. Western blot of cell extracts using αHA to detect HA-tagged T-bet. Lane 1*, mock transfected JEG-3; *lane 2*, JEG-3 transfected with 250 ng TBET plasmid; *lane 3*, JEG-3 transfected with 1.25 μg TBET plasmid; *lane 4*, 293 transfected with 250 ng TBET plasmid; *lane 5*, mock transfected 293. *B. IFNγ transcript induction in T-bet-expressing 293 and JEG-3 cells.* RT-PCR using IFNγ primers and RNA from transfected cells. *Lane 1*, mock transfected JEG-3; *lane 2*, JEG-3 transfected with 250 ng TBET plasmid; *lane 3*, JEG-3 transfected with 1.25 μg TBET plasmid; *lane 4*, 293 transfected with 250 ng TBET plasmid; *lane 5*, mock transfected 293. *C. IFNγ protein expression in T-bet expressing 293 and JEG-3 cells.* EMSA assay using a STAT probe and extracts from test 293 cells treated with conditioned medium from transfected cells shown in B. *Lane 1*, no 293 cell extract; *lanes 2-5*, extracts of 293 cells incubated with medium from: *lane 2*, mock transfected 293 cells; *lane 3*, JEG-3 transfected with 250 ng TBET plasmid; *lane 4*, JEG-3 transfected with 1.25 μg TBET plasmid; *lane 5*, 293 cells transfected with 250 ng TBET plasmid. The *asterisks* indicates a non-specific band and the *arrows* indicate STAT binding activity. Short (*left*) and long (*right*) exposures of the X-ray film are shown.

3.5. Level of IFNγ transcripts induced by T-bet in immune and non-immune cells

To assess the relative levels of IFNγ transcripts that are produced in non-immune T-bet expressing cells, we compared the levels of IFNγ transcripts in the uninduced and muristerone-induced T-betEcR+ cells with those from unstimulated and PMA/ionomycin stimulated Jurkat and primary human T-cells (Fig. 4D). Levels of IFNγ transcripts are at similar very low levels in all 3 cell types before induction (Fig. 4D, open bars). After 4.5 h of muristerone treatment (which is submaximal for induction of IFNγ, see Fig. 4C) IFNγ expression in T-betEcR+ cells is ~6 fold higher than the levels found in the PMA/

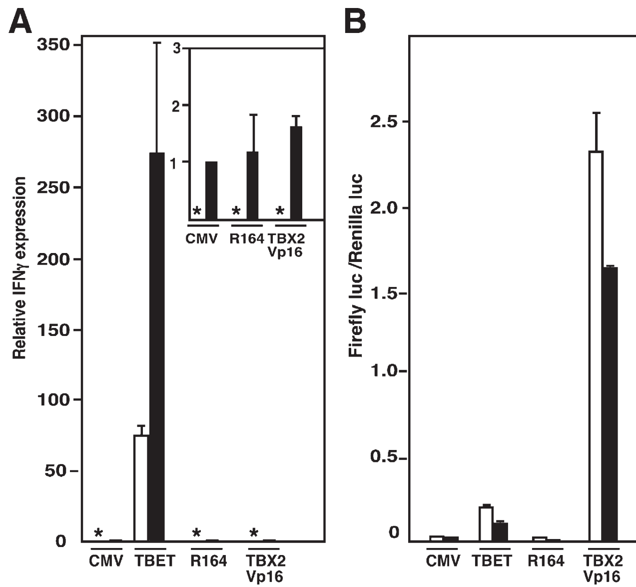

Fig. 6. Sodium butyrate potentiates endogenous IFNγ but not reporter gene expression in the presence or absence of TBET. 293 cells were transfected with the indicated T-box constructs and assayed for the ability of the protein to activate expression of (*A*) endogenous IFNγ or (*B*) a transfected T-site reporter construct in the absence (*open bars*) or presence (*filled bars*) of 1mM sodium butyrate. *A. Expression of endogenous IFNγ* . IFNγ was assayed by QPCR and expression levels were normalized to β2M. The inset shows an expanded scale without the TBET samples. *B. Expression of transfected T-site reporter gene.* Cells were transfected with the indicated constructs and data are expressed as firefly luciferase/renilla luciferase.

ionomycin-induced Jurkat T-cell line (Fig. 4D). This level of induction was ~80 fold lower than the very high levels seen in PMA/ionomycin-treated human T-cells (Fig. 4D). Since Jurkat cells have been frequently used as a model system for T-cells, these data indicate that the levels of IFNγ transcripts induced by T-betEcR in non-immune human 293 cells are greater than those seen in a cell culture model of T-cell function.

3.6. Induction of IFNγ expression by T-bet in other cell lines

To ask whether 293 cells are unique as non-immune cells in their competence for induction of IFNγ by T-bet, we asked whether JEG-3, a human choriocarcinoma cell line, could express IFNγ transcripts in the presence of T-bet. Transient transfection of JEG-3 cells with 1250 ng of vector expressing T-bet induced low levels of IFNγ transcripts as assessed by RT-PCR (Fig. 5B, lane 3). The levels of IFNγ transcripts are lower in JEG-3 cells than in 293 cells transfected with 1/5 as much as the TBET vector (Fig. 5B, lane 3 versus 4) although the T-bet protein levels achieved are higher (Fig. 5A, lane 3 versus 4). Some of this difference may reflect a lower proportion of JEG-3 versus 293 cells expressing T-bet. As PCR is a very sensitive technique for measuring gene expression we asked whether the levels of IFNγ transcription we observed were sufficient to lead to the production of functional IFNγ protein. To do this, we used an indirect assay for IFNγ which assesses the ability of conditioned medium from transfected cells to induce STAT DNA-binding activity in non-transfected 293 cells. Induction of STAT activity is a well characterized function of IFNγ (Schindler and Darnell, 1995). Conditioned medium collected from 293 or JEG-3 cells transiently expressing T-bet induced STAT DNA-binding activity in test 293 cells (Fig. 5C, lanes 5 and 4, respectively). Conditioned media from T-bet expressing 293 cells (Fig. 5C, lane 5) induced more STAT DNA-binding activity than media from T-bet expressing JEG-3 cells (Fig. 5C, lane 4), consistent with the higher levels of IFNγ transcripts in 293 versus JEG-3 cells (Fig. 5B, lanes 4 and 3, respectively). When less T-bet

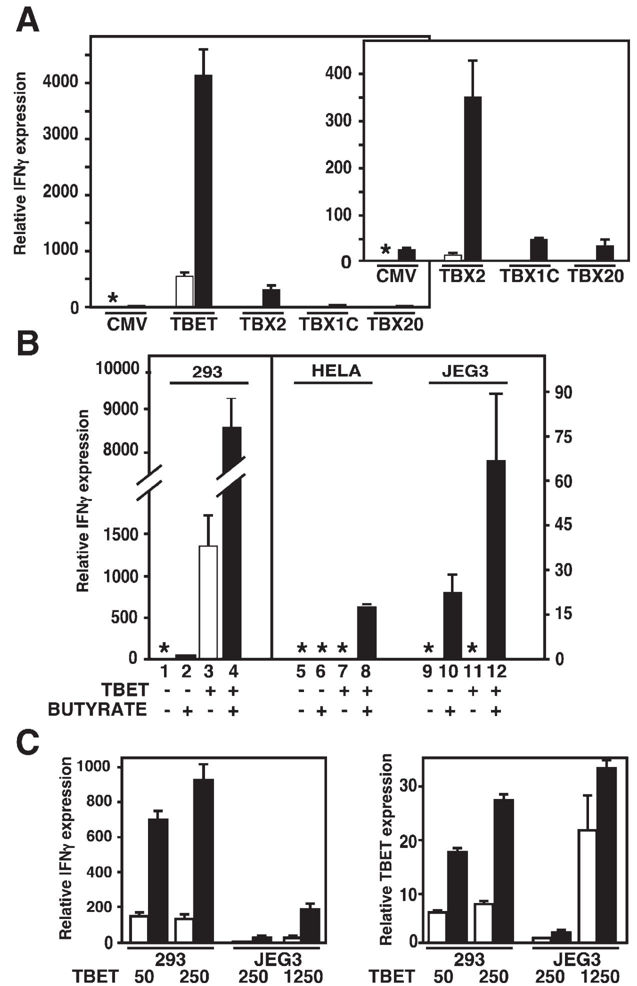

Fig. 7. Effect of sodium butyrate on T-box protein induction of IFNγ in 293, JEG-3 and HeLa cells. RNA was prepared from cells transfected with the indicated plasmids and treated with sodium butyrate as described in Materials and methods. Relative levels of IFNγ or TBET expression were determined using QPCR normalized to β2M and the lowest expression level in each panel was set equal to 1. *A. Induction of IFNγ by various T-box proteins.* All transfections were in 293 cells using 250 ng of the indicated plasmid. In the inset on the right the data for TBET has been excluded and the scale expanded. *B. T-bet and sodium butyrate induction of IFNγ in three cell lines.* 293 (*left panel*), JEG-3 and HeLa cells (*right panel*) were transfected with 250 ng CMV (*lanes 1, 2, 5, 6, 9 and 10*) or TBET (*lanes 3, 4 ,7, 8, 11 and 12*) and incubated in media without or with sodium butyrate (− and +, respectively). Transcript levels were expressed relative to levels in the absence of TBET and sodium butyrate. Note the different scales on the left and right. *C. IFNγ and T-bet expression in 293 and JEG-3 cells.* RNA was prepared from 293 and JEG-3 transfected with the indicated amounts of TBET vector in ng and assayed for levels of IFNγ (*left panel*) or TBET (*right panel*) by QPCR.

vector was transfected into JEG-3 cells, neither T-bet expression, IFNγ transcripts, nor STAT activity was induced (Fig. 4A and B, lane 2 and C, lane 3, respectively). No induction of STAT activity was seen with conditioned medium from control CMV-transfected 293 cells (Fig. 5C, lane 2). These data demonstrate that T-bet can induce IFNγ expression in both 293 cells and JEG-3 cells and that active IFNγ protein is also produced in these cells.

3.7. Potentiation by sodium butyrate of IFNγ expression but not transient reporter expression

Previous studies had suggested that activation of IFNγ expression by T-bet in T-cells was accompanied by chromatin remodeling (Mullen et al., 2001; Avni et al., 2002; Tong et al., 2005). To test for a role of protein acetylation in T-bet activation of endogenous IFNγ in our cell system, we compared IFNγ transcript levels and luciferase activity in transfected 293 cells treated with the known inhibitor of protein deacetylases, sodium butyrate (Fig. 6, filled bars) versus untreated cells (Fig. 6, open bars). Sodium butyrate increased the levels of IFNγ transcripts in T-bet expressing cells (Fig. 6A, lanes labeled TBET) by >3fold and caused a small but measurable increase in IFNγ transcripts in control cells or cells expressing the R164A T-bet or TBX2Vp16 proteins (Fig. 6A, inset). In contrast, butyrate inhibited activation of expression of the luciferase reporter gene by both T-bet and TBX2Vp16 (Fig. 6B). Thus butyrate potentiated the activation of endogenous IFNγ by T-box proteins but inhibited the expression of the transiently transfected reporter gene. It is of interest that butyrate-treated 293 cells had very low but detectable levels of IFNγ transcripts in this and some other experiments. While these levels are near the limits of detection of QPCR, they suggest that some expression from the IFNγ locus can occur in butyrate-treated 293 cells even in the absence of T-bet.

Since butyrate potentiated the T-bet mediated increase in IFNγ transcript levels, we determined whether butyrate affected IFNγ expression in the presence of three other T-box proteins that we have shown previously also bind T- and T/2-sites, TBX2, TBX1C and TBX20 (Sinha et al., 2000; Butz et al., 2004). No induction of IFNγ was seen by either TBX1C or TBX20 in the absence or presence of butyrate (Fig. 7A, inset). Surprisingly, butyrate mediated a significant increase in IFNγ transcript levels in cells expressing TBX2, although transcript levels were less than 1/10th those seen in the presence of T-bet (Fig. 7A, inset versus main panel). These data indicate that TBX2 is a much less potent activator of IFNγ than T-bet and that the other T-box proteins tested do not increase IFNγ expression even in the presence of butyrate. We then asked whether T-bet and butyrate could increase IFNγ expression in two other non-immune cell lines HeLa (cervical epithelial) and JEG-3 (choriocarcinoma). Although expression levels were ~100 fold lower than in 293 cells, T-bet induced IFNγ transcript levels in both HeLa and JEG-3 cells in the presence of sodium butyrate (Fig. 7B, lanes 8 and 12 versus 6 and 10). We showed in Fig. 5 that increasing the amount of TBET DNA transfected into JEG-3 cells from 250 ng to 1250 ng increased the expression of T-bet and IFNγ. We therefore decided to quantify those differences and ask whether they were maintained in cells treated with sodium butyrate. At the high concentration of TBET DNA (Fig. 7C, left panel), weak IFNγ expression could now be detected in JEG-3 cells not treated with sodium butyrate (Fig. 7C, left panel, open bars marked JEG-3 250 or 1250 ng). In butyrate-treated T-bet expressing JEG-3 cells, IFNγ transcript levels were ~1/6 of those seen in 293 cells

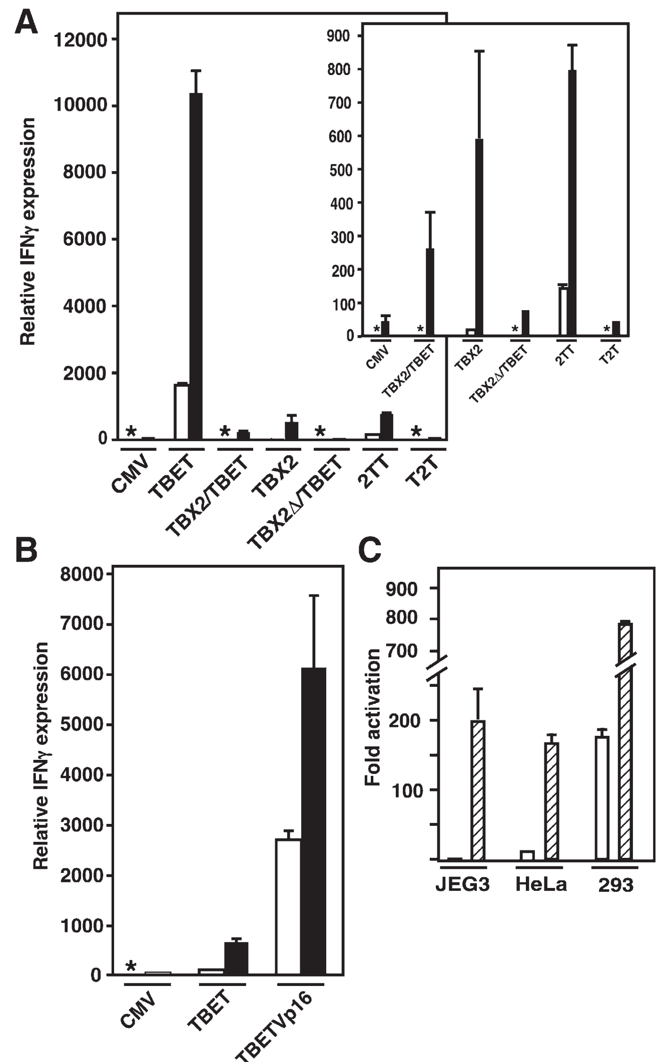

Fig. 8. Regions of TBET required for IFNγ induction and the effect of a Vp16 transactivation domain. *A. IFNγ induction by chimeric T-box proteins.* A series of chimeric T-box constructs (see Fig. 1) were transfected into 293 cells and their ability to induce IFNγ transcript levels in the absence (*open bars*) or presence (*filled bars*) of 1 mM sodium butyrate was assessed by QPCR. Inset shows an expanded scale without the TBET samples. *B. Effect of sodium butyrate on IFNγ induction by T-bet and T-betVp16.* 293 cells were transfected with CMV, TBET or TBETVp16 and treated without (*open bars*) or with (*filled bars*) 1 mM sodium butyrate as described in Materials and methods. Data are expressed as fold increase in IFNγ transcript levels relative to untreated CMV-transfected cells. *C. Effect of sodium butyrate on IFNγ induction by T-bet and T-betVp16 in three cell lines.* JEG-3, HeLa and 293 cells were transfected with CMV, TBET (*open bars*) or TBETVp16 (*hatched bars*) and treated with 1 mM sodium butyrate as described in Materials and methods. Data are expressed as fold increase in IFNγ transcript levels relative to butyrate-treated CMV-transfected cells.

transfected with 1/5 as much DNA (Fig. 7C, left panel, 293 closed bar 250 versus JEG-3 closed bar 1250). In contrast, T-bet transcript levels in JEG-3 cells transfected with 1250 ng of the T-bet expressing plasmid were slightly higher than in 293 cells transfected with 250 ng of the plasmid (Fig. 7C, right panel, 293 closed bar 250 versus JEG-3 closed bar 1250). These data again indicate that JEG-3 cells express less IFNγ in response to T-bet than do 293 cells, even at similar levels of T-bet expression. Lastly, treatment with butyrate increased expression of T-bet in both 293 and JEG-3 cells, but in all cases this increase was less than the fold increase in IFNγ expression seen (for example Fig. 7C, JEG-3 open and closed bars labeled 1250 in the right versus left panels).

3.8. IFNγ induction by chimeric T-bet proteins

We showed in Figs. 2 and 3 that T-bet potently activates endogenous IFNγ expression but only weakly activates a transient T-site reporter gene, while TBX2Vp16 and TBET/TBX2Vp16 strongly activate the reporter gene with minimal or no effect on endogenous IFNγ expression. These data suggested that the C-terminus of T-bet is essential for maximal activation of IFNγ expression. To identify other region(s) of T-bet needed for activation of IFNγ expression and determine the effect of the Vp16 domain on this process we generated a number of deletion and chimeric T-bet proteins (Fig. 1A). While intact T-bet activated IFNγ expression more than 1000 fold, replacement of the N-terminal 154 residues of T-bet with the N-terminal 116 residues of TBX2 abolished activation (Fig. 8A, T-bet versus 2TT). The latter protein was also much less active than T-bet in inducing transcription of the T/2-site reporter construct (not shown). Similarly, replacement of the 172 residue T-box DNA-binding domain of T-bet with the 161 residue DNA-binding domain of TBX2 also abolished activation of both IFNγ (Fig. 8A, T2T) and the reporter construct (not shown). Together with the data from Fig. 3, these results indicate that all 3 major regions of T-bet; the N-terminal, the T-box DNA-binding, and the C-terminal domains, are essential for maximal activation of IFNγ expression. To determine whether the Vp16 activation domain could contribute to activation of IFNγ expression we generated an expression vector in which this domain was fused in frame to full-length T-bet. The resulting T-betVp16 protein had a greatly enhanced ability to activate IFNγ expression, showing an ∼7 fold increase over T-bet in IFNγ expression in the presence of butyrate and an ∼30 fold increase over T-bet in the absence of butyrate (Fig. 8B, closed and open bars marked TBETVp16 versus TBET, respectively). It is of interest that while butyrate stimulated the T-bet mediated expression of IFNγ by ∼10 fold, it stimulated the T-betVp16-mediated activation by only ∼2 fold. These data suggest that the greater activity of T-betVp16 may be at least in part due to overcoming the effects of acetylation of histones or other proteins (see Discussion).

Since T-betVp16 activated IFNγ expression more potently than T-bet, we examined IFNγ expression levels in butyrate-treated JEG-3 and HeLa cells expressing either T-bet or T-betVp16 (Fig. 8C). In both JEG-3 and HeLa cells T-betVp16 activated IFNγ expression much more potently than did T-bet (∼150 and <10 fold, respectively, hatched bars versus open bars). In this experiment T-betVp16 was ∼5 fold more potent than T-bet in inducing IFNγ expression in butyrate-treated 293 cells. Thus the addition of the Vp16 activation domain to T-bet greatly increased its ability to activate IFNγ expression in all 3 cell types, although the effect of theVp16 domain was greater in JEG-3 and HeLa than in 293 cells.

4. Discussion

T-bet has previously been characterized as a transcription factor that mediates the polarization of $CD4^+$ T-cells into Th1-type cells. A hallmark of this cell fate is the expression of IFNγ. Here we show that T-bet can induce IFNγ in 293 and other non-immune cells (Figs. 2–8), showing that the induction of IFNγ is independent of the more general induction of T-cell differentiation. In addition, we have shown that IFNγ induction in this non-immune system can occur in the presence of a protein synthesis inhibitor (Fig. 4), and that chimeras with other T-box proteins fail to significantly induce IFNγ expression (Figs. 3, 6–8). Butyrate treatment of cells potentiates the induction of IFNγ suggesting a role for protein acetylation in the maintenance of a repressed state at the IFNγ locus (Figs. 6–8). Some fraction of this potentiation by butyrate may be due to increased expression of T-bet in the transfected cells (Fig. 7C). Finally, fusion of the HSV-Vp16 activation domain to T-bet increases the levels of IFNγ transcripts in cells, indicating that T-bet alone cannot maximally activate IFNγ expression in non-hematopoetic cells (Fig. 8). Together, these data suggest that T-bet is a direct transcriptional activator of IFNγ and that this activation can be independent of the induction of T-cell differentiation.

Previous studies on T-bet focused on its ability to induce differentiation of naïve $CD4^+$ T-cells into the Th1 cell lineage. T-bet was shown to be both necessary and sufficient to induce differentiation of naïve T-cells into the Th1 cell fate (Szabo et al., 2000, 2002). We show with a series of chimeric protein constructs that the DNA-binding, as well as the N- and C-terminal domains of T-bet are essential for induction of IFNγ in non-immune 293 cells (Figs. 1, 3, 6–8). Two constructs, R164A (containing a missense mutation in the DNA-binding domain) and T2T (in which the DNA-binding domain of T-bet was replaced with that of Tbx2) were incapable of altering transcription of either the chimeric reporter construct or the endogenous IFNγ gene. The remaining constructs either activated, or in some cases repressed, expression of the luciferase reporter construct. Curiously, there was no correlation between activation or repression of the T-site reporter construct and the ability to activate transcription of endogenous IFNγ as Tbx2 weakly repressed luciferase activity (reported in Sinha et al. (2000)) but weakly activated IFNγ in both the absence and presence of sodium butyrate. Conversely, Tbx2Vp16 strongly activates the reporter construct but did not affect IFNγ expression. TbetTbx2Vp16 which contains the same C terminus as the former construct but the N terminus and DNA-binding domain of T-bet has similar levels of activity as Tbx2Vp16 on the reporter construct but is able to only weakly but reproducibly activate IFNγ. These results demonstrate that both a T-box DNA-binding activity and

a strong transcriptional activation domain are still insufficient to maximally activate IFNγ transcription.

Replacing the TBX2 NLS containing and Vp16 domains of TBX2Vp16 with the C-terminus of T-bet converted a strong transcriptional activator of luciferase into a weak transcriptional repressor of transient reporter genes while leaving unaltered the inability of the protein to activate endogenous IFNγ. Replacing the N-terminus of T-bet with the N-terminus of Tbx2 (construct 2TT) resulted in a protein that could still weakly activate both luciferase and IFNγ. These data suggest that the IFNγ induction activity of T-bet requires both the N- and C-terminal domains. This would be consistent with previous studies indicating that deletions in the T-box, N- or C-terminal domains of T-bet all impaired the ability of the protein to transactivate an IFNγ-luciferase reporter construct (Cho et al., 2003). However, the addition of a Vp16 transactivation domain to a full-length T-bet greatly enhanced transactivation of both the transient reporter gene and endogenous IFNγ demonstrating that while induction of IFNγ transcription requires N- and C-terminal T-bet sequences, the Vp16 transactivation domain can act in concert with the T-bet transactivation domain.

IFNγ is a highly regulated gene whose expression is primarily, although not exclusively, restricted to hematopoetic cells of NK and T lineages (Szabo et al., 2000). As the induction of IFNγ expression in these cells is part of a complex cascade of events occurring in response to cytokine signaling, identifying the cis- and transacting elements required solely for IFNγ induction has not been simple. Cis-acting elements are clearly present in both promoter proximal and distal regions of the gene (Aune et al., 1997; Bream et al., 2004). Among the transacting factors identified as binding to the IFNγ promoter or intronic regions (primarily using exogenously transfected reporter constructs), have been STAT4, STAT3, Ap-1, Ap-4, YY1, NFAT, NFκB, ATF/CREB, Txk (Cippitelli et al., 1995, 1996; Kojima et al., 1999; Rengarajan et al., 2000; Kaminuma et al., 2002; Takeba et al., 2002; Robertson et al., 2005) and T-bet (Szabo et al., 2000). T-bet has been suggested to be involved in both chromatin remodeling of the IFNγ gene and transcriptional activation based on the observation that exogenously expressed T-bet can induce Dnase I hypersensitivity (Mullen et al., 2001). Our observation that butyrate treatment potentiates IFNγ expression (Figs. 6–8) supports a model where T-bet activates IFNγ by influencing chromatin remodeling, possibly through affecting the acetylation of histones or other proteins.

We had shown previously that different endogenous genes were induced by TBX2 and TBX2Vp16 chimeric proteins regulated by the EcR-ligand binding domain (Butz et al., 2004). Here we demonstrate that transactivation of the endogenous IFNγ gene has different requirements than the activation of a transient reporter gene. Although both genes use the same T-bet DNA-binding domain for targeting of the transcription factor to the promoter, IFNγ expression appears highly dependent upon the presence of the C-terminal domain of T-bet. Such an effect of a transactivation domain on the apparent in vivo target specificity of transcription factors had been suggested in other studies. For example, the E2F family of transcription factors possess similar DNA-binding activity in vitro but appear to populate different target sites in vivo (Zhu et al., 2004). E2F2 and E2F4 stimulate apoptosis when overexpressed in cardiomyocytes, while E2F1 and E2F3 do not (Ebelt et al., 2005). This difference is likely due to differential effects on gene expression by the different proteins in vivo, even though they show similar DNA-binding activity in vitro. Some of this differential binding is produced by cooperative interactions with other transcription factors, including NF-Y, at promoter sites (Schlisio et al., 2002). Given the observed differences in gene activation by T-bet and other T-box family members in vivo, it will be of interest to determine which of the several known steps in transcription (initiation complex formation, promoter clearance, elongation, etc. (Blau et al., 1996) are activated by T-bet.

We propose 4 specific models for how the T-bet C-terminal domain may mediate potent IFNγ induction with little effect on transient reporter genes (Fig. 9): 1) Specific cooperative binding with other site-specific transcription factors that function at the IFNγ control regions, 2) synergistic activation with, but independent binding with other transcription factors at the IFNγ gene, 3) modification of the in vivo DNA-binding specificity of the T-bet DNA-binding domain by the C-terminus and 4) recruitment of specific co-activators that differ in their ability to activate expression from endogenous genes and transient reporter constructs. To distinguish between these models and other possible mechanisms for increasing IFNγ transcript levels it will be necessary to identify the other transcription factors functioning at the control elements of the endogenous IFNγ gene and to assess their binding and activity in the presence and absence of T-bet. It will also be of interest to determine whether the T-bet C-terminus specifically influences the acetylation of histones or other proteins at the IFNγ gene. In addition, it will be important to determine whether the increases in IFNγ transcript levels are due to an increased transcription rate, or to potential post-transcriptional changes in transcript processing or stability mediated by T-bet.

Finally, we note that not only T-bet, whose expression is generally restricted to cells of hematopoetic lineage, but at least

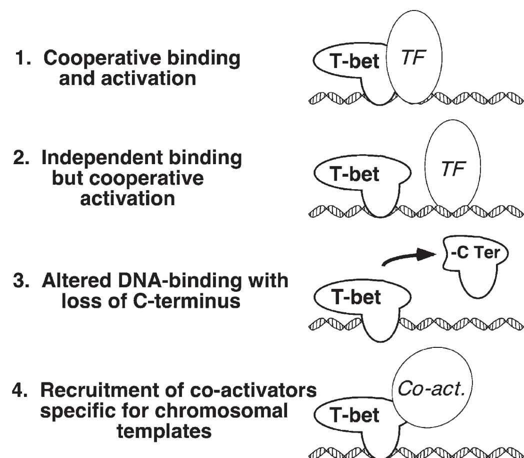

Fig. 9. Models for T-bet activation of endogenous versus transfected reporter genes. The models illustrate possible mechanisms for the differential activation of the endogenous IFNγ gene versus the transfected T-site reporter gene by T-bet and chimeric T-box proteins.

one other T-box gene, Tbx2, is capable of inducing low levels of IFNγ in non-hematopoetic cell lines. If this also occurs in vivo, it could provide a previously unrecognized source of this powerful signaling cytokine.

Acknowledgements

We thank Dr. J. Finke for providing human T-cells and Dr. S. J. Haque for the STAT oligonucleotide. This work was supported by NIH grant DK48796.

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