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Differential target gene activation by TBX2 and TBX2VP16: evidence for activation domain-dependent modulation of gene target specificity

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Abstract

The determinants of in vivo target site selectivity by transcription factors are poorly understood. To find targets for the developmentally regulated transcription factor TBX2, we generated stable transfectants of human embryonic kidney cells (293) that express a TBX2-ecdysone receptor (EcR) chimeric protein. While constitutive expression of TBX2 is toxic to 293 cells, clones expressing TBX2EcR are viable in the absence of an EcR ligand. Using cDNA arrays and quantitative PCR, we discovered nine genes whose expression was increased, but no genes whose expression was reduced, following 24 h of induction with Ponasterone A (PonA), a ligand for EcR. Since TBX2 was reported previously to be a transcriptional repressor, we also generated cell lines expressing a TBX2VP16EcR protein which we showed was a potent conditional transcriptional activator in transient transfection assays. Treatment of these cells with PonA induced the expression of five genes, none of which were affected in TBX2EcR-expressing cells. This discordance between TBX2- and TBX2VP16-regulated genes strongly suggests that specific transactivation domains can be a major determinant of gene target site selectivity by transcription factors that possess the same DNA-binding domain.

Keywords: T-box; Transactivation; Selectivity; Transcription; Lethality

1. Introduction

TBX2 is a member of the highly conserved T-box family of transcription factors. Multiple T-box genes are present in all metazoa examined but have not been found in bacteria, yeast or plants. Members of this gene family are essential for normal embryonic development in a variety of organisms including nematodes, fruitflies, frogs, zebrafish and mammals (Smith, 1999). Mutations have been generated in nine murine T-box genes including *T* (Stott et al., 1993), *Tbx1* (Jerome and Papaioannou, 2001), *Tbx3* (Davenport et al., 2003), *Tbx4* (Naiche and Papaioannou, 2003) and others. In all cases, inactivating mutations lead to developmental abnormalities in a subset of the tissues in which the gene is normally expressed. Mutations in *TBX3*, *TBX5*, *TPIT* and *TBX22* are found in patients with ulnar mammary syndrome (Bamshad et al., 1997), Holt Oram sydrome (Li et al., 1997), adrenocorticotrophin deficiency (Pulichino et al., 2003) and cleft palate with ankyloglossia (Braybrook et al., 2001), respectively. Mutations in several T-box genes, including *Tbx1*, *TBX3* and *TBX5*, have phenotypes in the heterozygous state, suggesting that gene function is highly dose-dependent. Since T-box genes play essential roles in a variety of developmental processes, determination of the mechanism of target gene selectivity by T-box proteins is an important goal.

Abbreviations: aa, amino acids; βGal, β-galactosidase; CMV, cytomegalovirus; Cy-3, -5, cyanine-3, -5; EcR, ecdysone receptor; HA, hemagglutinin; HSV, Herpes simplex virus; LBD, ligand binding domain; Luc, luciferase; PonA, Ponasterone A; QPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species; VP16, Herpes simplex virus protein 16.

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Although it was among the first mammalian T-box genes identified, there has yet to be a report of a mouse or a human disorder associated with mutations in the *TBX2* gene. However, studies on mis-expression of *Tbx2* in developing chick limbs suggest that both *Tbx2* and the highly related *Tbx3* genes are involved in specifying digit identity (Suzuki et al., 2004). *Tbx2* is expressed in a number of other locations during murine embryonic development including the otic vesicle, lungs, heart, kidneys and mammary glands (Chapman et al., 1996). *Tbx2* is also expressed in adult mice and humans in lungs, kidneys, heart and placenta (Campbell et al., 1995) and likely plays important roles in the development of one or more of these tissues.

While all members of the T-box family of proteins possess a highly conserved T-box DNA-binding domain, the downstream target specificity of various family members likely differ. We showed previously that differences in DNA binding and transcriptional regulatory activity between single T-box proteins can be detected on a simple chimeric promoter (Sinha et al., 2000); however, the situation is likely to be more complex on endogenous promoters. To determine whether the T-box DNA-binding domain was the major determinant for gene target specificity of TBX2 in 293 cells, we asked whether two proteins with the same DNA-binding domain but different transcriptional modulation domains affected the same target genes. Surprisingly, TBX2EcR and TBX2VP16EcR activated the expression of completely distinct sets of genes in 293 cells. These data indicate that the DNA-binding domain of TBX2, while essential for transcriptional regulation, is strongly influenced by cis-acting transcriptional modulation domains in determining its specificity for target genes.

2. Methods

2.1. Vector constructs

The TBX2-EcR fusion construct was made by cloning the sequence encoding residues 321–878 of the *Drosophila* ecdysone receptor ligand binding domain (EcR LBD) in frame and downstream of the entire TBX2 coding sequence in HA-TBX2 (Sinha et al., 2000). The construct directing the expression of the TBX2VP16-EcR fusion protein was made by fusing fragments encoding residues 3–80 of the transactivation domain from the Herpes simplex virus protein 16 (VP16) and residues 321–878 of the EcR to the sequence encoding residues 1–411 of TBX2 in HA-TBX2. The R122A mutants had an alanine replacing arginine 122 of TBX2 and have no detectable DNA-binding activity (Sinha et al., 2000).

2.2. Cell culture and stable transfection assay

293 cells were maintained in α-Minimum Essential Medium (Invitrogen) containing 10% fetal calf serum and antibiotics (penicillin–streptomycin) at 37 °C/5% CO_2. These cells were chosen for analysis because TBX2 is expressed in embryonic kidney and preliminary data showed low levels of TBX2 in 293 cells by DNA-binding assays and supershifting with antiTbx2 antibodies (not shown). The role of Tbx2 in kidney is unknown. Twenty-four hours prior to transfection, cells were seeded in duplicate in 35-mm dishes at a density of 2×10^5 cells per dish. Cells were co-transfected with a puromycin-selectable plasmid and the TBX2EcR or TBX2VP16EcR expression vectors using lipofectamine reagent (Invitrogen). Two days following the DNA transfection, each 35-mm 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. Based on Western blots, approximately 50% of the clones expressed proteins of the expected size. The levels of TBX2EcR and TBX2EcRVP16 expressed in the transfectants was ~10 times the levels of endogenous TBX2 in 293 cells and was equal to that seen in some breast cancer cell lines including MCF7 (not shown). Where indicated, cells were treated with PonA (Invitrogen) in EtOH (final concentration, 15 μM) or EtOH alone for the times given in the figure legends.

2.3. Transient transfection assay

Twenty-four hours before transfection, cells were seeded in duplicate in 35-mm dishes at a density 2×10^5 cells per dish. To assess the transcriptional modulatory properties of TBX2EcR, cells were transfected using lipofectamine (Invitrogen) with the indicated amounts of a vector expressing TBX2EcR, 1 μg of p14ARF-642Luc reporter plasmid (gift from Dr. D. Holzschu) and 50 ng CMVβGal (MacGregor and Caskey, 1989) to normalize for transfection efficiency. To test the function of stably expressed TBX2VP16EcR, cells were transiently transfected with 1 μg 4xT/2HSVtkLuc reporter (Sinha et al., 2000) and 50 ng CMVβGal. Transfected cells were treated with PonA or EtOH for 24 h and extracts were prepared and assayed using the Luciferase Assay system Kit (Promega) as instructed by the manufacturer. Luciferase values were normalized to β-galactosidase activity levels. Transfections were performed in duplicate in at least two independent experiments.

2.4. Long-term growth inhibition assay

Cells were seeded in 6 well dishes at a density 2×10^4 cells/well and grown for 20 days (split 1:20 on day 10). PonA (or EtOH) was added on days 1, 4, 7 and 10 to a final concentration 10 μM. On day 20, the plates were stained with Coomassie blue to visualize the differences in growth.

2.5. Microarray studies

Total RNA was isolated 24 h after PonA (or EtOH) addition. In initial studies, probes were labeled with Cy-3 and Cy-5 fluorescent dyes and hybridized to microarrays containing oligonucleotides for ~21,000 human mRNAs (U. Cincinnati Array Facility). In later studies, labeled probes were hybridized to cDNA microarrays representing ~2500 human genes and ESTs (Roswell Park Cancer Institute). Hybridizations were performed in triplicate with RNA from three independent experiments using two different clones of each stable cell line.

2.6. RNA extraction, cDNA synthesis and QPCR

Cells were harvested in Trizol (Invitrogen) at specific times after PonA addition and total RNA was isolated according to the manufacturer's instructions. Total RNA (5 μg) was reverse-transcribed at 42 °C for 50 min in the presence of random hexamers and SuperScript II Reverse Transcriptase (Invitrogen). mRNA expression levels were measured by quantitative real-time PCR (QPCR). All genes were amplified using the appropriate specific primers (sequences available upon request). QPCR was carried out with the SYBR Green QPCR Kit (Applied Biosystems) using the Real-Time PCR instrument (Bio-Rad) according to the manufacturer's instructions. In each reaction, gene expression levels were assayed in triplicate and normalized to the level of β2-microglobulin. All analyses were performed multiple times using RNA from at least two independent experiments and two independent clones.

2.7. Statistical analysis

Values representing fold change in transcript levels and luciferase activity/β-galactosidase activity were analyzed by calculation of the mean and standard deviation. Statistical significance was evaluated using one-tailed, one-sample *t*-test.

3. Results

3.1. TBX2 expression is lethal to 293 cells

To search for genes whose expression is regulated by TBX2, we attempted to stably express full-length TBX2 protein in the human 293 cells. Deletion mutants TBX2Δ283–702 which lacks the nuclear localization signal and transcriptional regulatory domain of TBX2, and TBX2Δ407–702 which lacks the transcriptional regulatory domain alone, were used as controls (Sinha et al., 2000). Cells were co-transfected with a puromycin-selectable plasmid and colonies were picked and expanded prior to extracting RNA and protein. Although the colony numbers were similar (data not shown), colonies isolated from TBX2-transfected cells grew more slowly and a higher proportion of them were lost during the selection. While >50% of the clones transfected with any of the TBX2 constructs expressed RNA or protein, only in the cases of TBX2Δ283–702 or TBX2Δ407–702 were proteins of the expected size detected. None of seven TBX2 stable transfectants expressed full-length protein as assessed by Western blotting (Table 1, lines 2 and 3 versus 1). These data indicate that full-length TBX2 expression is toxic to 293 cells.

To test whether this toxicity required the binding of TBX2 to DNA, we transfected 293 cells with a vector expressing a missense mutant of TBX2 (TBX2R122A) that localizes to the nucleus but has no DNA binding activity (Sinha et al., 2000). Stable transfectants expressed primarily full-length protein (Table 1, line 4). The amount of mutant protein expressed in TBX2R122A, TBX2Δ283–702 and TBX2Δ407–702 transfectants was also similar (data not shown). These results suggest that TBX2 expression in 293 cells is incompatible with long-term growth in culture and that toxicity requires the localization of TBX2 to the nucleus, binding to DNA and the transcriptional regulatory domain of the protein.

3.2. TBX2EcR represses cell growth and transcription in a manner similar to TBX2

Since constitutive expression of TBX2 is lethal to 293 cells, we isolated stable transfectants expressing a conditionally active TBX2 protein, consisting of HA-tagged TBX2 fused to the *Drosophila* EcR LBD. Previous studies have shown that the EcR LBD can suppress the activity of proteins to which it is fused and this suppression can be overcome by treatment with ecdysone (Christopherson et al., 1992; No et al., 1996). Clones were screened for expression of TBX2EcR protein by Western blot and DNA binding assays (data not shown). Cytotoxic/cytostatic activity of the chimeric protein in the

Table 1
293 stable transfectants expressing wild-type or mutant TBX2

No.	Construct	Total number of colonies	Number of colonies expressing TBX2	Number of colonies expressing protein of predicted size
1	TBX2	15	7	0
2	TBX2Δ283–702	9	5	5
3	TBX2Δ407–702	12	8	7
4	TBX2R122A	23	14	11

293 cells were transfected with TBX2 (line 1), TBX2Δ283–702 (line 2), TBX2Δ407–702 (line 3) or TBX2R122A (line 4). Cell extracts were resolved on a 10% SDS-PAGE gel, and analyzed by Western blot using αHA antibodies. Table shows the number of clones expressing TBX2 protein of predicted size as determined by Western blot analysis.

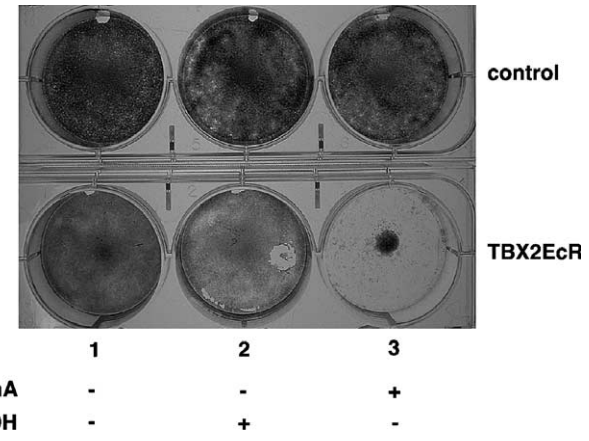

Fig. 1. TBX2EcR induces cell lethality in a manner similar to TBX2. Cells stably expressing TBX2EcR were grown for 20 days as described inSection 2.4. PonA (line 3) or EtOH (line 2) were added in final concentration 10 μM. Plates were stained with Coomassie blue to determine the extent in growth. A clone isolated from the puromycin selection but not expressing TBX2EcR was used as a control.

absence and presence of the ecdysone analog PonA was assessed by a long-term growth inhibition assay. PonA treatment of TBX2EcR-expressing clones resulted in greatly reduced cell number compared to clones treated with EtOH or non-treated (Fig. 1, column 3 versus columns 2 and 1). No such differences were detected in control cells which do not express TBX2EcR. These data indicate that TBX2EcR likely induces cell lethality in a manner similar to TBX2.

It has been reported previously that TBX2 represses the transcription from the cell cycle inhibitor *p19/p14ARF* promoter (Jacobs et al., 2000). We therefore used a p14ARF-responsive reporter plasmid (p14ARF-642Luc) to assess the transcriptional modulatory properties of TBX2EcR. As expected, TBX2 represses p14ARF-642Luc expression (Fig. 2A, bar 2 versus 1) while the DNA-binding deficient mutant TBX2R122A represses to a much lesser extent (Fig. 2A bar 3 versus 2). TBX2EcR also represses p14ARF-642Luc in the presence of PonA, although more weakly than TBX2 (Fig. 2B, bar 2 versus 1; bar 4 versus 3; bar 6 versus 5). This somewhat weaker repression is consistent with results we have obtained using other T-box/EcR fusion proteins, where the addition of the EcR LBD reduces to some degree the transcriptional modulatory activity of the protein (unpublished data). The slight repression by TBX2EcR in the presence of EtOH (Fig. 2B, bars 1, 3 and 5) may be due to proteolysis of TBX2EcR generating low levels of TBX2 activity. As expected, the TBX2EcR protein containing a mutation that abolishes DNA binding caused little or no repression of p14ARF expression (Fig. 2B, bars 7–12). These findings indicate that TBX2EcR in the presence of PonA has similar biological activities as native TBX2 and could be used for the identification of genes whose expression is regulated by TBX2.

3.3. Identification of TBX2 downstream target genes

To identify downstream target genes responsible for the lethality seen in TBX2-expressing cells, we analyzed global gene expression using long oligonucleotide and cDNA microarrays. To distinguish early TBX2-induced

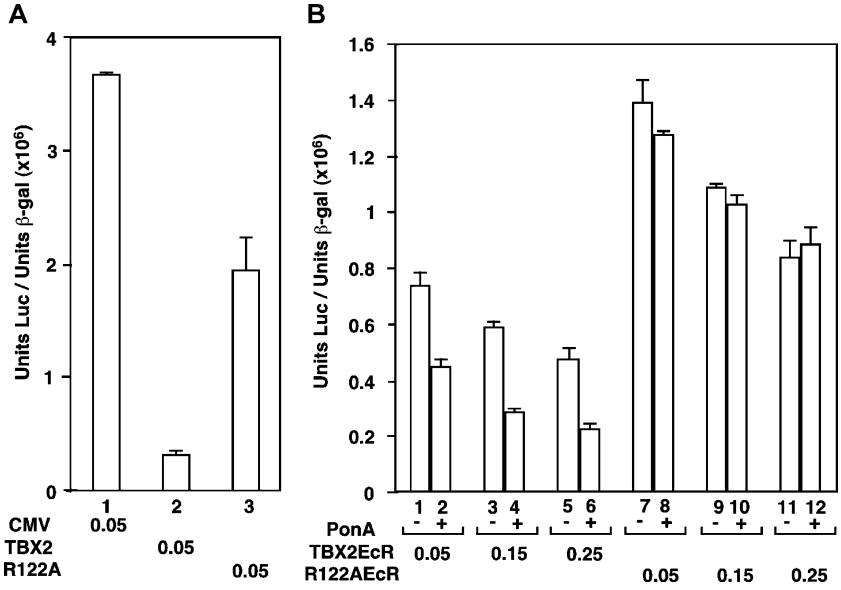

Fig. 2. TBX2EcR represses transcription from p14ARF promoter in a manner similar to TBX2. (A, B) 293 cells were transfected and analyzed for luciferase (Luc) activity as described in Section 2.3. The figure shows the luciferase activity for p14ARF-642Luc (1 μg) relative to control co-transfected β-galactosidase activity (CMVβgal, 50 ng) in the presence of the indicated amounts (in micrograms) of vectors expressing no TBX2 (A, bar 1), TBX2 (A, bar 2), TBX2R122A (A, bar 3), TBX2EcR (B, bars 1–6) or TBX2R122AEcR (B, bars 7–12). TBX2EcR and TBX2R122AEcR transfectants were treated with 15 μM PonA or EtOH for 24 h. Each construct was tested in duplicate. Values are the mean±S.D of the luciferase activity divided by the β-galactosidase activity.

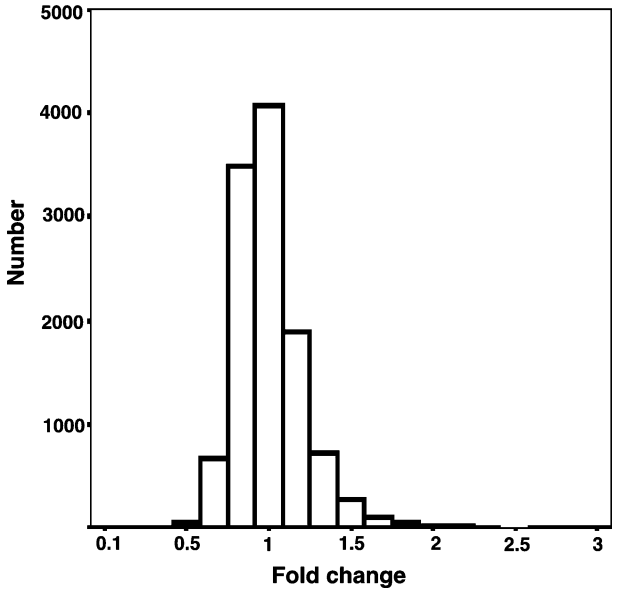

Fig. 3. Histogram is derived from microarray results and shows the range of gene expression changes in TBX2EcR-expressing cells treated with 15 μM PonA for 24 h versus cells treated with EtOH (control).

changes from later alterations due to cell death, we assessed gene expression changes in TBX2EcR cells after 24 h of PonA treatment. This time was chosen because trypan blue exclusion studies indicated that little or no cell death was occurring after 24 h of PonA treatment (data not shown).

Our analysis showed that TBX2 does not have large (5- to 10-fold) effects on transcript levels over the time period analyzed. Only relatively small changes (≤2–3 fold) in gene expression were observed in TBX2EcR-expressing cells treated with PonA (Fig. 3). To confirm the microarray data, we assessed the gene transcript levels in PonA-treated TBX2EcR cells using QPCR. Sixty-five percent of the genes identified as up-regulated in PonA-treated cells on the arrays showed reproducible induction (9 of 14), whereas QPCR failed to confirm any TBX2EcR-repressed genes (0 of 6 tested). We identified nine genes, whose expression levels are induced 2- to 3-fold in TBX2EcR cells treated with PonA compared to EtOH-treated cells (Fig. 4, black bars versus line). Results were confirmed using multiple RNA preparations from two independently isolated TBX2EcR-expressing clones.

Two other controls were used to confirm the specificity of induction of gene expression in PonA-treated TBX2EcR cells. There was no increase in expression levels of the identified genes in non-transfected 293 cells treated with PonA (Fig. 4, diagonal bars versus line), nor in 293 cells stably expressing a TBX20EcR fusion construct (Fig. 4, dotted bars versus line). Unlike TBX2, TBX20 expression is not toxic to 293 cells although both proteins act as weak repressors in transient transfection assays with a reporter construct (data not shown).

We next used QPCR to determine the time course of TBX2EcR-mediated gene activation. The nine genes up-regulated upon TBX2 activation fall into two groups with different expression patterns. Genes of the first set (*PIG3, TP53INP1, p21, ACTA2* and *MMP2*) exhibit maximal expression at 24 h of PonA treatment with a subsequent decrease (Fig. 5A). In contrast, *CTGF, IER3, EGR1* and *DUSP6* are expressed more highly at 48 h than at 24 h (Fig. 5B). Interestingly, *CTGF* expression drastically increases at 48 h of TBX2 activation and maintains almost the same level at 96 h. *IER3* and *MMP2* also maintain high levels of induction at 96 h. While *IER3, EGR1* and *CTGF* expression levels begin to increase by 17 h after PonA addition, none of the genes are induced at 6 h. Such

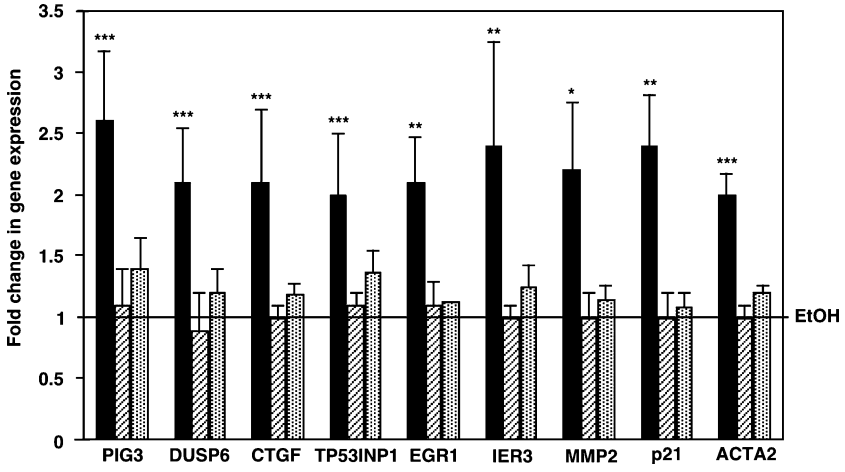

Fig. 4. Genes up-regulated in TBX2EcR-expressing cells. Data represent fold changes in expression levels of indicated genes in TBX2EcR cells (black bars), non-transfected 293 cells (diagonal bars) and TBX20EcR cells (dotted bars) treated with 15 μM PonA compared to EtOH-treated cells. Expression levels in EtOH-treated cells were set to 1 (line). At 24 h after PonA addition, RNA was collected, reverse-transcribed and subjected to QPCR for mRNA quantification as described in Section 2.6. All reactions were performed multiple times using RNA from at least two independent experiments. Values are the mean of multiple (n=2–17) QPCRs±S.D. ***p<0.0005, **p<0.005, *p<0.025.

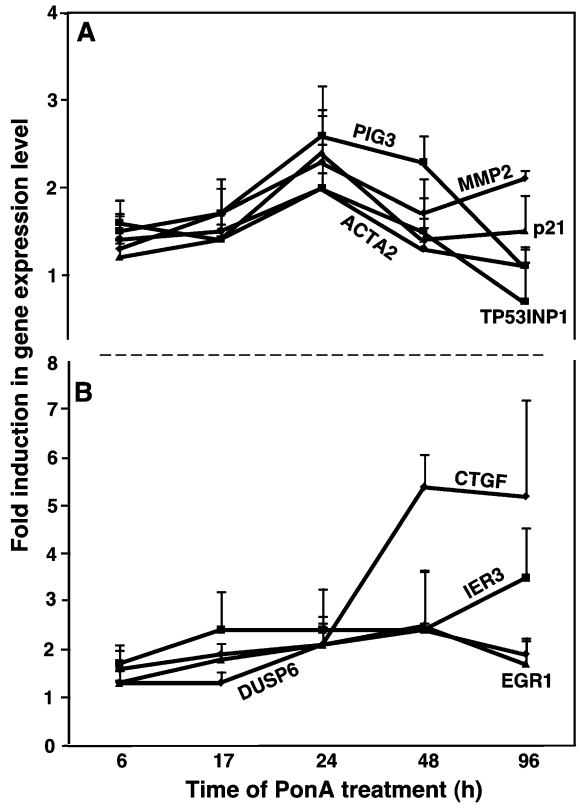

Fig. 5. Time-dependent expression profiles of TBX2EcR-activated genes. (A, B) Data represent fold induction in transcript levels in TBX2EcR-expressing cells treated with PonA (15 μM) versus EtOH at different time points. RNA was harvested at indicated time points of PonA treatment and analyzed as in Fig. 4. Values are the mean±S.D.

a slow time course of induction suggests that some or all of the genes may be indirect rather than direct targets of TBX2 (see Discussion).

3.4. Transcriptional modulatory domain affects the selectivity of the target genes regulated by the TBX2 DNA-binding domain

Since TBX2 had previously been shown to function as a transcriptional repressor, it was somewhat surprising that we did not identify any TBX2-repressed genes. In an effort to generate a more potent modulator of TBX2-responsive genes and to test whether TBX2 target gene specificity resides solely in the DNA-binding domain, we made stable cell lines expressing a chimeric TBX2 protein where the C-terminus of TBX2 was replaced with the VP16 transactivation domain, followed by the EcR LBD.

We assessed the activity of this chimeric protein in vivo using the previously characterized TBX2-responsive reporter construct 4xT/2HSVtkLuc (Sinha et al., 2000). Native TBX2 represses the expression of this reporter gene (Sinha et al., 2000). PonA treatment of cells expressing TBX2VP16EcR resulted in an ~10-fold increase of luciferase activity from 4xT/2HSVtkLuc compared to EtOH-treated cells (Fig. 6, bar 4 versus 3). No such increase was seen in cells not expressing TBX2VP16EcR protein (Fig. 6, bar 2 versus 1). These data indicate that TBXVP16EcR can effectively activate the same TBX2-responsive promoter that is repressed by TBX2.

Given the strong transcriptional activation by TBX2VP16EcR, we examined the expression levels of the previously determined TBX2EcR target genes in PonA-treated TBX2VP16EcR-expressing cells by QPCR. Surprisingly, genes activated in TBX2EcR cells treated with PonA show little, if any, effect upon PonA treatment of TBX2VP16EcR-expressing cells (Table 2). Of the nine genes, only *PIG3* expression level was reduced in TBX2VP16EcR cells upon PonA treatment. Thus the expression of most of the TBX2EcR-activated genes was unaffected in PonA-treated TBX2VP16EcR cells.

To identify TBX2VP16EcR-responsive genes, we initially assessed gene expression changes using cDNA microarrays and confirmed putative targets using QPCR. Use of QPCR confirmed 2- to 3-fold activation of five genes in TBX2VP16EcR cells treated with PonA versus EtOH (Fig. 7, black bars versus line), whereas no differences were detected in non-transfected PonA-treated 293 cells (Fig. 7, diagonal bars versus line). As was seen above, the expression of these TBX2VP16EcR-activated genes was not altered in PonA-treated TBX2EcR cells (Table 3). Since different genes are regulated by TBX2EcR and TBX2VP16EcR, these data strongly suggest that the transcriptional modulatory domain associated with a T-box DNA-binding domain may influence target gene specificity

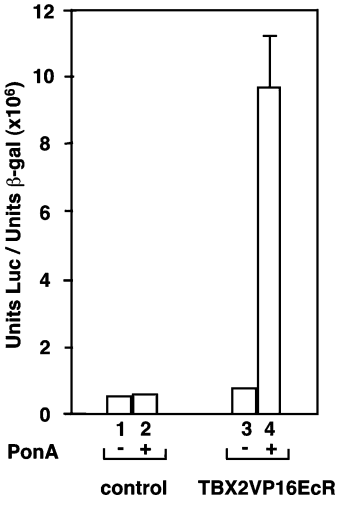

Fig. 6. TBX2VP16EcR activates TBX2-responsive reporter gene. Cells stably expressing TBX2VP16EcR (bars 3–4) were transfected with 1 μg of 4xT/2HSVtkLuc reporter plasmid and 50 ng CMVβgal, treated with 15 μM PonA or EtOH for 24 h and analyzed for luciferase and β-galactosidase activity as described in Section 2.3. A matched stable transfectant not expressing TBX2VP16EcR protein was used as a negative control (bars 1–2). Transfections were performed in duplicate in two independent experiments. Values are the mean±S.D of the luciferase activity divided by the β-galactosidase activity.

Table 2
Gene expression changes in TBX2EcR and TBX2VP16EcR cells

Gene symbol	Gene name	Fold change TBX2EcR	Fold change TBX2VP16EcR
PIG3	Quinone oxidoreductase homolog	2.6±0.6**** n=17	0.6±0.1* n=3
DUSP6	Dual specificity phosphatase 6	2.1±0.5**** n=14	0.9±0.2 n=4
CTGF	Connective tissue growth factor	2.1±0.6**** n=11	0.8±0.3 n=3
TP53INP1	Tumor protein p53 inducible nuclear protein 1	2.0±0.5**** n=9	0.7±0.1 n=2
EGR1	Early growth response 1	2.1±0.4*** n=4	0.8±0.4 n=5
IER3	Immediate early response 3	2.4±0.8*** n=7	0.8±0.3 n=2
MMP2	Matrix metalloproteinase 2	2.3±0.6** n=4	0.8±0.2 n=3
p21	Cyclin-dependent kinase inhibitor 1A	2.4±0.4*** n=4	0.9±0.3 n=4
ACTA 2	Actin, alpha 2	2.0±0.2**** n=5	0.7±0.2 n=2

QPCR results representing fold changes in expression levels of indicated genes in TBX2EcR- and TBX2VP16EcR-expressing cells treated with 15 μM PonA for 24 h compared to cells treated with EtOH. Values are the mean of multiple (n=2–17) QPCRs±S.D.
* p<0.05.
** p<0.025.
*** p<0.005.
**** p<0.0005.

(see Discussion). Consistent with this hypothesis, we found that TBX2VP16 neither activates nor represses transcription of the p14ARF reporter construct, although it appears to bind the promoter as indicated by its ability to relieve TBX2-induced repression (data not shown).

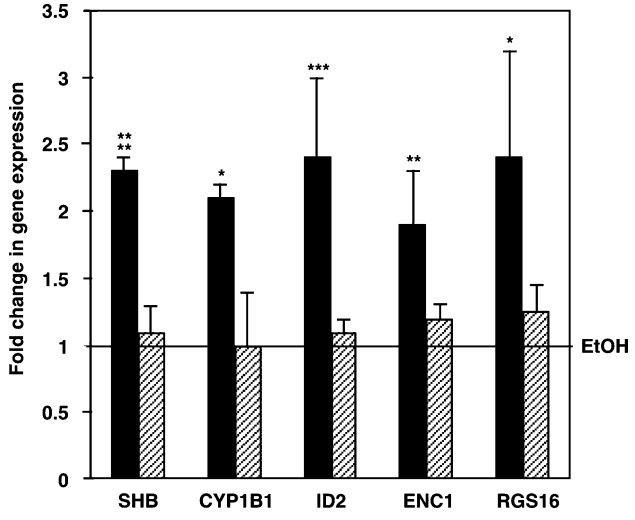

Fig. 7. Genes up-regulated in TBX2VP16EcR-expressing cells. Data represent fold changes in expression levels of indicated genes in TBX2VP16EcR cells (black bars) and in non-transfected 293 cells (diagonal bars) treated with 15 μM PonA compared to cells treated with EtOH. Expression levels in cells treated with EtOH were set to 1 (line). At 24 h after PonA addition, RNA was collected and analyzed as in Fig. 4. Values are the mean of multiple (n=2–4) QPCRs±S.D. ****p<0.005, ***p<0.01, **p<0.025, *p<0.05.

Table 3
Gene expression changes in TBX2VP16EcR and TBX2EcR

Gene symbol	Gene name	Fold change TBX2VP16EcR	Fold change TBX2EcR
SHB	(Src homology 2 domain containing) adaptor protein B	2.3±0.1**** n=3	1.0±0.2 n=3
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	2.1±0.1* n=2	0.8±0.1 n=2
ID2	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	2.4±0.6*** n=4	0.9±0.0 n=2
ENC1	Ectodermal-neural cortex (with BTB-like domain)	1.9±0.4** n=4	1.3±0.4 n=4
RGS16	Regulator of G-protein signalling 16	2.4±0.8* n=3	1.1±0.3 n=2

QPCR results representing fold changes in expression levels of indicated genes in TBX2VP16EcR- and in TBX2EcR-expressing cells treated with 15 μM PonA for 24 h versus cells treated with EtOH. Values are the mean of multiple (n=2–4) QPCRs±S.D.
* p<0.05.
** p<0.025.
*** p<0.01.
**** p<0.005.

4. Discussion

We have shown that constitutive expression of TBX2 is lethal to 293 cells and that toxicity requires the localization of TBX2 in the nucleus, binding to DNA and the transcriptional regulatory activity of the protein (Fig. 1; Table 1). Since TBX2 expression appears toxic to 293 cells, to search for genes regulated by TBX2 we generated TBX2EcR, which is toxic only in the presence of the ligand PonA. Using a time point (24 h) at which the cells are still ~95% viable, we identified nine genes, induced upon PonA treatment in TBX2EcR cells but not in TBX2VP16EcR cells (Table 2), and five genes induced in TBX2VP16EcR cells but not in TBX2EcR cells (Table 3). This discordance in gene expression changes in the two cell types suggests that the VP16 activation domain may strongly influence TBX2 target selectivity.

The genes up-regulated in PonA-treated TBX2EcR-expressing cells are associated with stress induction and are involved in the regulation of cell viability, growth, proliferation and cytoskeleton remodeling. For example, the ability of *EGR1* to stimulate the generation of the reactive oxygen species (ROS) (Bek et al., 2003), allows us to group this gene with *PIG3*, known to generate ROS which damage mitochondrial components and induce apoptosis (Polyak et al., 1997). The *P21* gene up-regulated in PonA-treated

TBX2EcR-expressing cells can be induced by ROS (Russo et al., 1995) as well as by EGR1 (Ragione et al., 2003). The overexpression of *IER3* is known to either induce or suppress apoptosis depending on cellular context (Wu, 2003). The IER3 anti-apoptotic effect is dependent on the presence of active extracellular signal-regulated kinases (ERK) (Garcia et al., 2002). In turn, dephosphorylation of ERK1/2 by DUSP6 has been shown to induce apoptosis via Bcl-2 proteolysis (Rossig et al., 2002). In light of these data, overexpression of *DUSP6* in TBX2EcR-activated cells may negatively regulate a number of anti-apoptotic pathways.

The ability of DUSP6 to inactivate ERKs1/2, which are responsible for the regulation of several signal transduction pathways, links *DUSP6* to another group of genes up-regulated following TBX2 activation. This subset of genes, involved in extracellular matrix formation and remodeling, includes *CTGF*, *MMP2* and *ACTA2*. CTGF is reported to negatively affect the anti-apoptotic program in cells by reducing the levels of Bcl2 (Hishikawa et al., 1999). CTGF has also been implicated in the induction of *MMP2* expression (Fan and Karnovsky, 2002). This metalloproteinase has been shown to induce apoptosis via extracellular matrix degradation (Wu and Huang, 2003). Thus our data suggest that TBX2 mediates an increase in the RNA levels of several genes which may contribute to cell lethality by affecting cellular apoptotic pathways. It will be important in future studies to determine whether one or all of these TBXEcR-induced genes plays an essential role in the TBX2-mediated toxicity seen in 293 cells.

We compared our results to previously reported microarray analysis of genes differentially expressed in Tbx2-overexpressing mouse NIH3T3 fibroblasts and rat osteosarcoma cells (Chen et al., 2001). Of the six genes previously confirmed to be directly or indirectly up-regulated by Tbx2, none were found to be targets in our work. These differences may be related to the different species, cell lines and experimental systems used, or to the long-term toxicity of TBX2 to 293 cells but not NIH3T3 or osteosarcoma cells. A few genes have been shown previously to be directly or indirectly repressed by Tbx2 in other cell lines and tissues including *TRP-1* (Carreira et al., 1998), *p19/p14ARF* (Jacobs et al., 2000), *ANF* (Habets et al., 2002) and *Cx43* (Borke et al., 2003); however, none of these endogenous TBX2 targets were affected in our 293 cell system (data not shown). In addition, Tbx2 has been shown recently to directly repress *p21* expression in B16 and MCF-7 cells by siRNA-mediated down-regulation of endogenous *Tbx2* (Prince et al., 2004). Since we saw an increase in *p21* transcript level in PonA-treated TBX2EcR-expressing 293 cells (Table 2), these data suggest: (1) that TBX2 can either activate or repress *p21* expression depending on the cell type used, (2) TBX2EcR activity differs from that of Tbx2, or (3) activation of *p21*, and the other TBX2 targets seen here may be indirect rather than direct.

In comparison with the TBX2EcR-induced genes described above, the TBX2VP16EcR-induced genes comprise a distinct group with few obvious linkages. SHB is an SH2 containing adaptor protein involved in cell signaling (Welsh et al., 1994), CYP1B1 is a cytochrome *P*450 enzyme (Sutter et al., 1994), ID2 is a dimerization partner and inhibitor of basic helix-loop-helix transcription factors (Hacker et al., 2003), ENC1 is a potential beta-catenin target proposed to play a role in colorectal carcinogenesis (Fujita et al., 2001), whereas RGS16 is a regulator of G-protein signaling (Snow et al., 1998). While having no clear biochemical connections, these genes may affect a number of cell signaling pathways that could influence cell proliferation. Perhaps the most interesting feature of this set of genes is their lack of overlap with the TBX2EcR-regulated genes identified above. It is the potential mechanistic connection between these two sets of genes that we discuss below.

The finding that largely non-overlapping sets of genes are affected in TBX2EcR- versus TBX2VP16EcR-expressing cells has important implications for the mechanisms of target gene selectivity by T-box proteins. Studies of transcription factor activation of promoters in transiently transfected cells have in general focused on the role of specific cis-acting elements in directing transcription factor binding, and on the role of defined transcriptional activation domains on the recruitment of and/or modification of basal transcription, co-activator/repressor, or chromatin remodeling factors. Much less is known about the mechanisms that influence the recognition and activation of specific target genes in their in vivo chromosomal context. For example, while it is recognized that different transcriptional activation domains can affect different aspects of the transcription process (e.g. initiation versus elongation (Blau et al., 1996; Brown et al., 1998), little is known about the potential role of transcriptional activation domains on the selectivity of target genes in vivo.

There are multiple mechanisms by which transcriptional activation and repression domains could influence target gene selectivity and modulation in vivo: (1) direct modification of the DNA-binding specificity or affinity of the cognate DNA-binding domain, (2) formation of a complex with other site-specific transcription factors and subsequent modification of DNA-binding specificity or affinity of the complex, (3) the ability to function synergistically with other transcription factors bound independently to a given target gene, (4) recruitment of co-activators or co-repressors that differ in their abilities to activate or repress specific target genes, and others. Some of these mechanisms have been identified as likely occurring on specific promoters in transiently transfected cells, but their role in general target gene selectivity in vivo is still unclear. A major goal of our future studies is to determine whether these or other mechanisms are mediating the differential activation of gene expression seen in TBX2EcR- and TBX2VP16EcR-expressing cells.

One important consideration in identifying the mechanism of differential gene activation by TBX2EcR and TBX2VP16EcR is whether the genes identified here are direct or indirect targets of these chimeric transcription factors. If the genes are direct targets of TBX2-binding, then it should be possible to distinguish between differential occupancy (mechanisms 1 and 2) and differential activation (mechanisms 3 and 4) of the target genes in vivo using chromatin immunoprecipitation. However, if these genes are indirect targets of the TBX2 and are regulated by intermediates that are themselves direct targets, then it will be essential to identify these direct targets in order to distinguish between the mechanisms. The PonA-inducible system used here has advantages over other inducible systems since activation of the transcription factor does not require ongoing protein synthesis, and we attempted to assess whether the genes are direct targets by using cycloheximide to block protein synthesis upon induction with PonA. Unfortunately, the TBX2EcR proteins used here appear relatively unstable and in the absence of protein synthesis are rapidly degraded, precluding a simple comparison of induction in the absence and presence of cycloheximide (data not shown). Reengineering the proteins for increased stability should overcome these issues.

In addition, it is possible that the different apparent sets of targets are due to the induction of different cell fates by the two forms of TBX2 used here. However, both forms of TBX2 appear lethal to 293 cells (not shown) and thus if they are inducing different cell fates, then both fates end in the same process, cell death. We are currently investigating whether any of the targets identified contribute directly to 293 cell death.

Finally, in an effort to assess whether the genes identified are direct or indirect targets of TBX2, we searched for TBX2-binding sites (full consensus and partial matches) in the 3 kb region upstream of the start site of transcription and in the 5′ UTRs of the putative target genes and several control genes that were unchanged in the transfectants. Putative TBX2-binding sites were found at many locations in both classes of genes, but no increase in the frequency of TBX2-binding sites was detected in the proposed targets. Since important control regions of genes can sometimes extend for hundreds of kilobases around the promoter, it will likely be necessary to first determine whether the putative target genes are direct targets, and then perform a more detailed promoter analysis of these confirmed target genes using transfection assays and DNA-binding assays.

Irrespective of whether the genes identified here are direct or indirect targets of TBX2, their lack of overlap and the differential regulation of their expression within 24 h of PonA treatment suggest that TBX2EcR and TBX2VP16EcR regulate distinct sets of genes in 293 cells. This differential activation of gene expression from proteins containing the same DNA-binding domain suggests that transcription factor activation and repression domains, together with influencing the overall levels of gene expression on specific promoters, likely affect the selectivity of target genes. Determining the mechanism of this apparent modulation of target gene selection remains an important goal.

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