Tbx2 Directly Represses the Expression of the p21WAF1 Cyclin-Dependent Kinase Inhibitor

Sharon Prince,1,2 Suzanne Carreira,1 Keith W. Vance,1 Amaal Abrahams,2 and Colin R. Goding1
1Signalling and Development Laboratory, Marie Curie Research Institute, Oxted, Surrey, United Kingdom, and 2Division of Medical Biochemistry, Institute for Infectious Disease and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa

ABSTRACT

T-box factors play a crucial role in the development of many tissues, and mutations in T-box factor genes have been implicated in multiple human disorders. Some T-box factors have been implicated in cancer; for example, Tbx2 and Tbx3 can suppress replicative senescence, whereas Tbx3 can cooperate with Myc and Ras in cellular transformation. The p21WAF1 cyclin-dependent kinase inhibitor plays a key role in senescence and in cell cycle arrest after DNA damage. Here, using a combination of in vitro DNA-binding, transfection, and chromatin immunoprecipitation assays, we show that Tbx2 can bind and repress the p21 promoter in vitro and in vivo. Moreover, small interfering RNA-mediated down-regulation of Tbx2 expression results in a robust activation of p21 expression. Taken together, these results implicate Tbx2 as a novel direct regulator of p21 expression and have implications for our understanding of the role of T-box factors in the regulation of senescence and oncogenesis, as well as in development.

INTRODUCTION

Recent work has contributed to an increasing body of evidence implicating the T-box transcription factor family in the maintenance of cell identity during development (reviewed in Refs. 1–3). For example, Tbx1 mutations have been implicated in DiGeorge syndrome (4–6), Holt-Oram syndrome correlates with mutations in Tbx5 (7), Tbx4 and Tbx5 play crucial roles in limb bud outgrowth and specification of limb identity (8–11), and T-Pit determines cell fate in the pituitary (12).

In addition to their key function in development, evidence suggesting a role for T-box factors in breast cancer is accumulating. Tbx3 is required for normal breast development (13), with mutations in the Tbx3 gene linked to uInammar syndrome (14). Moreover, Tbx3 can cooperate with Ras and Myc to induce cellular transformation and suppress apoptosis (15). Importantly, both Tbx3 and the highly related factor Tbx2 are transcriptional repressors (16–19) and can suppress senescence through a mechanism involving repression of the expression of the p19ARF (p14) gene (20–22). Tbx2, like Tbx3, is also expressed in the developing breast and has been implicated in breast cancer where the Tbx2 gene is preferentially amplified in BRCA1 and BRCA2 mutants (23) and Tbx2 is overexpressed in breast cancer cell lines (21). These results suggest that T-box factors may contribute to cell cycle control and oncogenesis but it is not known whether their effect on proliferation extends beyond regulation of p19ARF.

Proliferation of mammalian cells is regulated by signals acting to control the activity and expression of components of the cell cycle machinery. A combination of cyclins, cyclin-dependent kinases (cdks), and cdk inhibitors act together to control proliferation, cell cycle arrest, apoptosis, and senescence, and deregulation of components of these key cell cycle regulators can contribute to cancer. The p21WAF1/CIP1/SDI1 cdk inhibitor (referred to as p21) is induced in differentiating cells and in response to a wide variety of cellular stresses including DNA damage, and contributes to stress-induced growth arrest (24, 25). The promoter of the p21 gene can be induced via p53 (26), and p21 expression is necessary for p53-mediated growth arrest (27–30). In contrast to the p16 cdk inhibitor, mutation of the p21 gene in human cancers occurs infrequently, and mice lacking p21 appear to undergo normal development, with no increase in the frequency of spontaneous tumor formation (27, 28). Nevertheless, some studies on the role of p21 in Ras transformation have suggested that p21 status is important. For example, in a mouse breast cancer model, the onset of Ras-induced tumors was accelerated in mice deficient in p21 (31). Any effects on the onset of tumor formation may reflect a role for p21 in replicative senescence. In senescent human fibroblasts, p21 levels are strongly elevated (32–34) and although mouse fibroblasts lacking p21 undergo normal senescence and are resistant to transformation by moderate levels of expression of oncogenic Ras (35), the cell cycle arrest associated with a high-intensity Ras/Raf signal is bypassed in p21-deficient fibroblasts (36). Additionally, a clone of human diploid fibroblasts in which the p21 gene had been disrupted exhibited a senescence bypass phenotype (37), whereas p21 expression can induce senescence in a p53-independent fashion (38, 39). Given the complex role of p21, identifying the controls regulating p21 expression is essential for understanding fully the balance between proliferation, differentiation, and senescence.

Here, we used a combination of in vitro DNA binding, transfection, and chromatin immunoprecipitation assays together with small interfering RNA (siRNA)-mediated down-regulation of Tbx2 to identify Tbx2 as a novel negative regulator of p21 expression. These results have implications for our understanding of the role of T-box factors in the regulation of senescence and oncogenesis, as well as in development.

MATERIALS AND METHODS

siRNA. Suppression of Tbx2 cellular expression was achieved using siRNA that specifically targets Tbx2 mRNA. The siRNA oligonucleotide sense sequence with two uracil residues at the 3′end was 5′-GCCGGAGCGG-GAUGGCGCUUU-3′ corresponding to amino acids 302–309 of murine Tbx2 or 5′-GGAGCGUGGGAGCCACGCUU-3′ corresponding to amino acids 102–108 of human Tbx2. B16 cells or MCF-7 cells were transfected with anti-Tbx2 siRNA or a control (nonsilencing) 21-mer siRNA 5′-UUCUC-CGAACCGGUCAGCUGTT-3′ (Xeragon-Qiagen, Crawley, United Kingdom) using Oligofectamine reagent (Life Technologies, Inc.;Invitrogen, Paisley, United Kingdom) according to Dharmacon Research instructions.

Immunofluorescence Microscopy. B16 cells grown on glass coverslips 3 days after siRNA transfection were washed three times with PBS and fixed with 3% paraformaldehyde for 20 min at room temperature before permeabilization with 0.1% Triton X-100 for 10 min at room temperature. Slides were incubated for 1 h with mouse Tbx2 monoclonal antibody (62–2) at a dilution of 1:750 and with the p21 (C-19) rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:250, then incubated with the appropriate secondary antibody coupled to FITC or Texas Red (Vector Laboratories, Peterborough, United Kingdom) at 1:100 dilution. Cells were
mounted using Vectashield mounting medium and examined by confocal microscopy.

**Western Blot Analysis.** B16 whole-cell extracts were prepared 3 days after siRNA transfection. Proteins were resolved on 9–15% SDS-polyacrylamide gels as required and then transferred to Hybond C (Amer sham, Amersham, United Kingdom). The membranes were probed with appropriate primary antibodies and detected using peroxidase-conjugated antimouse or antirabbit antibodies and visualized by enhanced chemiluminescence (Amer sham). The primary antibodies used were mouse monoclonal anti-Tbx2 antibody 62–2, anti-p21(C-19) rabbit polyclonal antibody (Santa Cruz Biotechnology), and mouse monoclonal anti-a-tubulin (Clone B-5–1–2; Sigma).

**Electrophoretic Mobility Shift Assays.** Binding reactions were performed with double-stranded 32P-labeled oligonucleotide probes, in vitro transcribed/translated protein, B16 cell extract, antibody, and competitor oligos as described previously (16) and resolved on a 6% polyacrylamide gel.

The sequence of double-stranded oligonucleotides used as probe and competitors are as follows: p21, 5'-ctagaCTCGAGATCGGCAGCTGAGCTCTGAGCAGCTGCCGAAG-3'; p21mut, 5'-ctagaCTCGAGACAGGTGAGGAGGTGTA-3'; p19prom, 5'-GCCTGAGGCCAGCTGAGG-AGGTGTGA-3'; and consensus T-element, 5'-AGGTGAG-3'. An internal control for transfection efficiency (100 ng/transfection). Cells were cultured for 48 h, and then the extracts were assayed for CAT and β-galactosidase activity. CAT values were normalized to the β-galactosidase activity and expressed relative to the empty vector control. All of the transfections were performed in duplicate, and at least three independent experiments were done to confirm reproducibility.

**Culture Conditions and Transfection Assays.** COS 7 cells were grown in DMEM supplemented with 10% fetal bovine serum and 5% antibiotics (penicillin-streptomycin). Cells were plated at 1–2 × 10^5/ml in 35-mm dishes 1 day before transfection. Transfections were performed using FuGene 6 (Roche Molecular Biochemicals, Lewes, United Kingdom) according to the manufacturer’s instructions with 700 ng of the relevant reporter plasmid plus 200 ng of the Tbx2 expression plasmid or an equal amount of empty vector plasmid. The vector pCMV-β-galactosidase containing the cytomegalovirus promoter driving the expression of a lacZ reporter was used as an internal control for transfection efficiency (100 ng/transfection). Cells were cultured for 48 h, and then the extracts were assayed for CAT and β-galactosidase activity. CAT values were normalized to the β-galactosidase activity and expressed relative to the empty vector control. All of the transfections were performed in duplicate, and at least three independent experiments were done to confirm reproducibility.

**Growth and Infection of ST.HdhQ111 Cells.** Conditionally immortalized ST.HdhQ111 mouse striatal cells (40) expressing a mutant huntingtin protein and tsA58 SV40 large T antigen were grown at 33°C in DMEM supplemented with 10% fetal bovine serum. Shift to the nonpermissive temperature (39°C) resulted in a loss of SV40 tsA58 protein and a senescence-like cell cycle arrest. To generate retrovirus-producing cell lines, Psi2 packaging cells were transfected with pBabePuro.SV5 (empty vector expressing the SV5 epitope tag only) or pBabePuro.SV5-Tbx2 expression vectors. Two days later, 1 μg/ml puromycin was added. Pooled cell lines were expanded and maintained in 10% fetal bovine serum in DMEM under selective conditions.

Retroviral infections were carried out using viral supernatants from Psi2 cells stably producing SV5-Tbx2 or empty SV5 virus. On the day before infection, 4 ml of growth medium (minus puromycin) was added to a near-confluent 10-cm dish of viral-producing Psi2 cells. Twenty-four h later, the medium was removed and filtered through a 0.45 μm filter. ST.HdhQ111 cells to be infected were plated at 5 × 10^6/10-cm dish. Twenty-four h later, the medium was removed and replaced with 4 ml of viral supernatant containing 10 μg/ml Polybrene. Cells were incubated for 5–6 h at 33°C, then the viral supernatant was removed and growth medium was added. For the senescence bypass assay, cells were moved to the nonpermissive temperature 3 days later. After 11 days, cells were fixed with aceton:methanol (1:1) for 5 min at room temperature, and colonies were visualized with 0.01% crystal violet. Alternatively, ST.HdhQ111 cells expressing SV5 or SV5-Tbx2 at the nonpermissive temperature were selected using 1 μg/ml puromycin. Seven days later, ST.HdhQ111 SV5-expressing cells were pooled and whole-cell lysates were prepared. SV5-Tbx2-expressing cells proliferating at the nonpermissive temperature were isolated and expanded under selective conditions. Lysates for Western blotting were prepared from each individual clone.

**Site-Directed Mutagenesis of the p21 Promoter.** The wild-type p21-CAT plasmid was modified as follows: the T-element (AGGTGAG) located at the initiator region of the wild-type human p21 promoter was mutated to CTCCTGA by site-directed mutagenesis using the Stratagene QuikChange system and the primer pair 5'-CTCGAGCCAGCTGAGCTCTAGGAGGCAGCTGAGG-3' and 5'-CGAGCCCTTGACAGGTGTA-3'.

**Chromatin Immunoprecipitation Assays.** Chromatin immunoprecipitation assays were performed essentially as described previously (41). Briefly, mouse NIH 3T3 cells stably expressing an SV5-tagged Tbx2 cDNA were treated with formaldehyde, and DNA-containing fractions were incubated overnight with different antibodies and collected on protein G beads. Cross-linked products were reversed by heating at 65°C for 4 h and the immunoprecipitated DNA was purified. Primers for PCR amplification of the p21 promoter DNA were 5'-TAAAGGCGCGCCGTCTCTA-3' and 5'-AAAGGCGCTCATGACTGTTGCA-3'. For the HSV1/5 promoter, primers were 5'-CGAAAACTGCCCTACAGCT-3' and 5'-CTCTGGCCACGTCTC-3'.

**Generation of Stable Cell Lines Expressing Epitope-Tagged Tbx2.** The parental cell line ER-NIH3T3, stably expressing the heterodimer of the edysone receptor and the retinoid X receptor, which binds to the edysone response element (encoded by pEGSH) in the presence of the synthetic edysone analogue Ponasterone A (Strategene), was purchased from Stratagene. These cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml each of penicillin and streptomycin, and 400 μg/ml of G418. A plasmid, pEGSH/SV5Tbx2, containing full-length mouse Tbx2 cDNA with sequences encoding the SV5 (PK) epitope tag at its 5' end and the hygromycin-resistant gene was constructed. For stable lines, ER-NIH3T3 cells were transfected with pEGSH/SV5Tbx2 plasmid using FuGENE (Roche Molecular Biochemicals). Transfected cells were allowed to recover for 48 h, and then 400 ng/ml Geneticin (Life Technologies) and 400 ng/ml Hygromycin were added. After 3 weeks, antibiotic-resistant cells were isolated, and 20 stable clonal lines were analyzed for expression levels of recombinant SV5-tagged Tbx2 by Western blotting using the SV5 monoclonal antibody; those lines expressing Tbx2 were chosen for further analysis.

**RESULTS**

**Tbx2 Binds the p21 Promoter.** Recent work suggests that both Tbx2 and the highly related T-box factor Tbx3 can suppress senescence via their ability to bind and repress the p19ARF promoter, thereby preventing the accumulation of p19ARF observed in cells undergoing senescence. Because p19ARF inhibits MDM2-mediated degradation of p53 (42, 43), one scenario that may explain, at least in part, the ability of Tbx2 and Tbx3 to suppress senescence is that inhibition of p19ARF expression prevents p53-mediated up-regulation of the promoter driving expression the p21 cdk inhibitor that has been implicated in control of proliferation, differentiation, and replicative senescence. However, the binding site for Tbx2 and Tbx3 in the p19ARF promoter AGGTGAG (22) does not match the previously identified high-affinity consensus T-box factor binding motif AGGTGAG (44) and may represent a relatively low-affinity site. For T-box factors to suppress senescence efficiently, it therefore may be necessary for the p19ARF-MDM2-p53-p21 pathway to be targeted at multiple points. To investigate this possibility, we first compared the expression of p21 in cells undergoing senescence into those in which senescence is suppressed by Tbx2. The mouse striatum cell line ST.HdhQ111 expresses a temperature-sensitive SV40 T-antigen and undergoes senescence synchronously on switching from 33°C to 39°C (40). These cells have been used previously to demonstrate that Tbx3 can induce a senescence bypass phenotype (20). Western blotting of the parental ST.HdhQ111 cell line revealed a dramatic increase in p21 expression within 8 h after shifting to the nonpermissive temperature (39°C), and this high level of expression was maintained for at least 8 days (Fig. 1A). In contrast to control cells infected with an empty retrovirus, ST.HdhQ111 cells infected with a Tbx2-expressing retrovi-
rus fail to undergo senescence at 39°C (Fig. 1B) as described previously (20), continue to proliferate, and fail to induce p21 expression (Fig. 1C).

The fact that p21 is not induced at 39°C in ST.HdhQ111 cells expressing Tbx2 led us to ask whether the p21 promoter is a direct target for transcriptional repression by Tbx2. Examination of the p21 promoter for motifs matching the consensus T-element (45) identified a perfect match AGGTGTGA close to the p21 initiator that was conserved between the human, mouse, and rat p21 promoters (Fig. 2A). To verify that this element could bind Tbx2, we used it as a radiolabeled probe corresponding to the p21 promoter element together with in vitro transcribed/translated Tbx2. As controls, we competed for binding using oligonucleotides corresponding to the p21 motif and compared its ability to compete with oligonucleotides corresponding to the consensus palindromic T-element as well as the previously identified Tbx2-binding motif in the p19ARF promoter (22). The sequences of the probes and competitors used are shown in Fig. 2A, and the results of the DNA-binding assay are shown in Fig. 2B. The presence of in vitro transcribed/translated Tbx2 resulted in a readily detectable complex that was absent when using unprogrammed reticulocyte lysate. This complex contained Tbx2 as it was supershifted using a specific anti-Tbx2 monoclonal antibody. As expected from the primary sequence, the consensus palindromic T-element competed for Tbx2 binding more efficiently than the oligonucleotide corresponding to the p21 motif, whereas the p19ARF promoter sequence competed ~3-fold more poorly than the p21 promoter element.

To demonstrate that Tbx2 isolated from mammalian cells also can bind the p21 promoter, we used nuclear extract from B16 melanoma cells that express Tbx2 (16) in an in vitro DNA-binding assay. Using the p21 element as a probe, three complexes were observed (Fig. 2C), one of which corresponded to Tbx2 as determined by using an anti-Tbx2 monoclonal antibody to inhibit its binding. The bound Tbx2 was competed efficiently by the homologous wild-type p21 element, and interaction with the Tbx2-binding site in the p19ARF promoter was ~5-fold less efficient than binding to the p21 element, which is consistent with the results obtained using in vitro transcribed/translated Tbx2. Surprisingly, however, competition with the consensus T-element that bound in vitro transcribed/translated Tbx2 most efficiently was extremely poor. Thus, binding to the consensus T-element was ~25-fold less efficient than binding to the p21 element and was reduced 5-fold compared with the p19 promoter-binding site. Despite the differences between the in vitro transcribed/translated Tbx2 and Tbx2 obtained from B16 cells, it is clear that the p21 promoter is a target, at least in vitro, for Tbx2. We also note that in addition to the complex containing Tbx2, a faster migrating complex with a similar DNA-binding specificity was also seen. Whether this represents binding of another T-box factor to the p21 promoter or corresponds to an unrelated factor is unclear. The identification of this factor will be the subject of future work.

To determine whether the Tbx2-binding site in the p21 promoter was functional, we cotransfected a wild-type or mutated p21 promoter-CAT reporter into B16 cells and then measured promoter activity. The mutation that was introduced changed the Tbx2-binding site from AGGTGTGA to AGGctcGA (Fig. 3A) and severely inhibited Tbx2 binding in vitro (Fig. 3B). The results obtained (Fig. 3C) indicated that cotransfection of a Tbx2 expression vector could result in an up to 10-fold repression of the wild-type reporter, although repression was
usually in the region of 4–6-fold, whereas mutation of the p21 AGGTGTGA motif to AGGctcGA abrogated the ability of Tbx2 to repress transcription. The degree of repression observed is comparable with that seen on other T-box factor target promoters (16, 18, 46).

To verify that Tbx2 could bind the p21 promoter directly \textit{in vivo}, we performed a chromatin immunoprecipitation assay. Because currently available anti-Tbx2 antibodies do not immunoprecipitate Tbx2 efficiently, we established a 3T3 cell line expressing a low level of SV5 epitope-tagged Tbx2. Using this cell line, we treated cells with paraformaldehyde, extracted and sheared the cross-linked chromatin, and immunoprecipitated Tbx2-bound chromatin using the anti-SV5 antibody. After extensive washing, the cross-linking was reversed and the DNA subjected to PCR using primers specific for either the p21 or \textit{HSP105} promoters. Nonspecific IgG or no antibody was used as a control.

\textbf{Tbx2 Represses Endogenous p21 Gene Expression.} To confirm that the endogenous Tbx2 regulates endogenous p21 expression, we designed a specific siRNA to down-regulate Tbx2 expression in B16 melanoma cells. The efficacy of the siRNA-mediated inhibition of Tbx2 expression was initially monitored by Western blotting using an anti-Tbx2 antibody that revealed that the levels of Tbx2 expressed were down-regulated by \textasciitilde 80\% (Fig. 4A). No effect was observed on tubulin that was used as a loading control or if a control, nonsilencing siRNA was used. Reprobing the same Western blot with an anti-p21 antibody revealed that siRNA-mediated repression of Tbx2 expression also resulted in a substantial increase in p21 expression.

As an alternative assay, we used immunofluorescence in B16 melanoma cells to determine the effects of siRNA on both Tbx2 and p21 expression. Using an anti-Tbx2 antibody and the control siRNA, Tbx2 can be detected readily in the nucleus of all cells examined (Fig. 4B), although some variation between individual cells in the absolute level of fluorescence detected is seen. Staining with anti-p21 antibody revealed a low level of staining. In contrast, using the Tbx2-specific

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Fig. 3. Tbx2 represses the p21 promoter. \textit{A}, the sequence of the p21 probe and mutated site used for the DNA-binding assay. \textit{B}, bandshift assay using the consensus T-element probe and \textit{in vitro} transcribed/translated (ITT) Tbx2 together with the indicated consensus, wild-type (WT), and mutated (mut) p21 oligonucleotides. Only the bound DNA is shown. \textit{C}, Tbx2 represses the p21 promoter. B16 cells were transfected with a WT p21-chloramphenicol acetyltransferase (CAT) reporter or a reporter in which the AGGTGTGA element was mutated to AGGctcGA in the presence of a cotransfected vector expressing Tbx2. CAT activity was determined 24 h posttransfection. \textit{D}, Tbx2 binds the p21 promoter \textit{in vivo}. Chromatin immunoprecipitation of Tbx2 was performed using a 3T3 cell line expressing a low level of SV5-tagged Tbx2. After cross-linking and shearing the chromatin to \textasciitilde 500 bp, chromatin was immunoprecipitated using the SV5 antibody. After extensive washing, the cross-linking was reversed and the DNA subjected to PCR using primers specific for either the p21 or \textit{HSP105} promoters. Nonspecific IgG or no antibody was used as a control.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Fig. 4. Tbx2 represses the endogenous p21 gene. \textit{A}, small interfering RNA (siRNA)-mediated down-regulation of Tbx2 results in up-regulation of p21 expression. B16 melanoma cells were treated with siRNA specific for Tbx2 or a control siRNA. Three days after treatment, cells were analyzed by Western blotting using antibodies specific for Tbx2, p21, and tubulin. \textit{B} and \textit{C}, immunofluorescence using anti-Tbx2 or anti-p21 antibodies on B16 cells (B) or MCF-7 cells (C) treated for 3 days with siRNA specific for Tbx2, or a control siRNA. Cells were viewed using a 63 \times Ph3 plan apochromat objective.

Downloaded from cancerres.aacrjournals.org on May 1, 2017. © 2004 American Association for Cancer Research.
siRNA, Tbx2 expression is extinguished in up to 80% of the cells examined, consistent with the results of the Western blot, although in some cells Tbx2 expression is maintained at levels comparable with that seen in the control cells. Resistance to the effects of siRNA in 10–20% of the cell population is observed routinely using siRNA to repress expression of specific genes. Examination of p21 expression in the cells treated with the Tbx2 siRNA revealed that, in those cells in which Tbx2 expression had been extinguished, p21 expression was elevated to a level in excess of that observed in any cell treated with the control siRNA. p21 levels also remained low in those cells that apparently were resistant to the Tbx2-specific siRNA. Thus, the results from both Western blotting and immunofluorescence are consistent with Tbx2 acting to repress the expression of p21. Similar results using immunofluorescence were obtained using an MCF-7 breast cancer cell line that is known to overexpress Tbx2 (Ref. 21; Fig. 4C). However, in this case the efficiency of siRNA-mediated down-regulation of Tbx2 was not as elevated as that seen in B16 cells, and we were unable to achieve a decrease in Tbx2 levels that was high enough to enable the effects on p21 to be determined using Western blot analysis.

**DISCUSSION**

In addition to their role in development, there is a growing body of evidence implicating Tbx2 and Tbx3 in cancer. In particular, both factors are able to suppress senescence, with the evidence to date indicating that overexpression of Tbx2 or Tbx3 results in binding and repression of the promoter of the gene encoding p19ARF (20–22), a key regulator of the p53 pathway. We show here that in addition to p19ARF, the endogenous Tbx2 protein is a direct repressor of the p21 promoter through an element located close to the p21 transcription start site. Because p21 is implicated in the control of cellular senescence, the ability of Tbx2 to suppress expression of both p19ARF and p21 may provide an explanation for a powerful and cooperative antisenescence signal. Importantly, given that T-box factors share a highly related DNA-binding specificity, it is likely that other members of the family will also act to regulate p21 expression either positively or negatively, depending on whether the given T-box factor is a transcription activator or repressor. Although the transcription regulatory status of most T-box factors is unknown, Tbx3, the most closely related T-box factor to Tbx2, is also a transcriptional repressor and has been shown to cooperate with Ras in cellular transformation. Because the growth arrest associated with acute, high-level oncogenic Ras activity requires p21 (36), we anticipate that, like Tbx2, Tbx3 also acts to repress expression of specific genes targeted by this important transcription factor.

Given the regulation of the p19ARF and p21 promoters by Tbx2 and Tbx3 in cells in culture, an effect on proliferation in animals in which these genes are defective or disrupted might be expected. It has been suggested that in individuals haploinsufficient for Tbx3, ulnar-mammary syndrome may develop in part through hypoplasia of tissues resulting from inappropriate apoptosis or differentiation at key stages of development (13–15). Moreover, Tbx3-null mouse embryos exhibit reduced liver size and massive cell death of the yolk sac endoderm (13) that may be explained by a failure to regulate correctly the p19ARF–MDM2-p53–p21 pathway in the absence of Tbx3. Whether Tbx2 also may play a role in the control of proliferation or apoptosis during development will be revealed when the phenotype of Tbx2-null mice is examined.

Finally, the differential binding of in vitro transcribed/translated Tbx2 compared with that found in B16 cells is intriguing. One possible explanation is that Tbx2 DNA-binding specificity is affected by post-translational modifications that occur in vivo but that are absent from the in vitro transcribed/translated Tbx2. Alternatively, and perhaps more likely, the Tbx2 complex identified in B16 extracts may represent a Tbx2 heterodimer with an unidentified factor that can recognize efficiently sequences flanking the p21 Tbx2 AGGTGTGTA motif but that cannot bind the AGGTGTGTA motif itself. Thus, although Tbx2 homodimers produced from the in vitro transcribed/translated reaction would bind the consensus T-element better, a heterodimer with a non-T-box dimerization partner would prefer the p21 element. In this respect, the target gene specificity of VegT and Eomesoderm in can be induced to resemble that of Xbra by introduction of a single amino acid substitution in the DNA-binding domain that would be predicted to mediate protein–protein interaction rather than protein–DNA interaction (47). Moreover, both Tbx5 and Tbx2 have been shown to bind cooperatively to target genes through interaction with the homeodomain factor Nkx2.5 (48, 49), whereas Tbx19 (T-Pit) activates the pro-opiomelanocortin promoter synergistically with the homeodomain protein PitX1 (50). It may be that a similar mechanism operates in the regulation of the p21 promoter, in which case only those T-box factors with the ability to interact with the appropriate factor will be able to regulate p21 expression. The ability of T-box factors to bind their target sequences with high affinity as heterodimers may explain why all of the known T-box factor target sites identified to date represent T-element half sites (51) and also would provide a mechanism for specificity because the choice of dimerization partner would dictate the nature of the target sites. It is also possible that the low levels of transcriptional repression by Tbx2 and other T-box factors achieved in transfection assays may arise through the limited availability of any dimerization partner required for efficient recruitment of Tbx2 to target promoters. The identification of T-box factor dimerization partners is clearly a priority if we are to identify the repertoire of genes targeted by this important transcription factor family.

**ACKNOWLEDGMENTS**

We thank Marc Macdonald for the ST.HdlQ118 cell line.

**REFERENCES**


Tbx2 Directly Represses the Expression of the p21WAF1 Cyclin-Dependent Kinase Inhibitor

Sharon Prince, Suzanne Carreira, Keith W. Vance, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/5/1669

Cited articles
This article cites 51 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/5/1669.full.html#ref-list-1

Citing articles
This article has been cited by 27 HighWire-hosted articles. Access the articles at:
/content/64/5/1669.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.