Variations in Intracellular Levels of TATA Binding Protein Can Affect Specific Genes by Different Mechanisms[∇]

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We previously showed that reduced intracellular levels of the TATA binding protein (TBP), brought about by *thp* heterozygosity in DT40 cells, resulted in a mitotic delay reflecting reduced expression of the mitotic regulator *cdc25B* but did not significantly affect overall transcription. Here we extend these findings in several ways. We first provide evidence that the decrease in *cdc25B* expression reflects reduced activity of the *cdc25B* core promoter in the heterozygous (TBP-het) cells. Strikingly, mutations in a previously described repressor element that overlaps the TATA box restored promoter activity in TBP-het cells, supporting the idea that the sensitivity of this promoter to TBP levels reflects a competition between TBP and the repressor for DNA binding. To determine whether cells might have mechanisms to compensate for fluctuations in TBP levels, we next examined expression of the two known vertebrate TBP homologues, TLP and TBP2. Significantly, mRNAs encoding both were significantly overexpressed relative to levels observed in wild-type cells. In the case of TLP, this was shown to reflect regulation of the core promoter by both TBP and TLP. Together, our results indicate that variations in TBP levels can affect the transcription of specific promoters in distinct ways, but overall transcription may be buffered by corresponding alterations in the expression of TBP homologues.

Perhaps the most widely studied general transcription factor has been the TATA binding protein (TBP). TBP was initially thought to be a universal transcription factor, essential for transcription of genes transcribed by all three RNA polymerases (17). However, the isolation of several TBP homologues (5, 10, 12) has provided evidence that this is not the case and that transcriptional regulation, even at the level of this basal factor, is more complex than provided for by a universal transcription factor. TBP consists of two domains, an amino (N)-terminal domain that is species specific and a conserved carboxy C-terminal domain. The conserved core domain directly contacts the TATA box of promoters and nucleates the assembly of other factors required for initiation of transcription (17). The core domain is largely conserved in the more recently described TBP homologues, while the species specific N-terminal domain is either absent or entirely dissimilar (11, 24).

One such homologue is TBP-like protein (TLP; also known as TRF2 or TLF; for a review, see reference 5). For some time it was speculated that TLP regulated transcription by sequestering essential factors (47), preventing their utilization in initiation complexes containing TBP. Indeed, TLP sequences necessary for binding the general transcription factors TFIIA and TFIIB are identical to those found in TBP (5, 11), and TLP, when overexpressed, does repress transcription from the TATA-containing adenovirus major late promoter (39). However, in vitro analysis demonstrated that artificial recruitment of TLP to a promoter by fusion to a Gal-4 DNA-binding domain activated transcription (38), and transcription from the

terminal deoxynucleotidyl transferase promoter lacking a TATA box was stimulated by TLP in transient-transfection assays (39). Chicken DT40 cells lacking TLP are viable, although the G₂ checkpoint is defective in *tlp*-null cells. These cells grow faster than wild-type DT40 cells due to a shortened G₂ phase, and the cells also appear more tolerant to stress (43). However, RNA interference studies established that TLP is essential for embryogenesis in *C. elegans* and *X. laevis* (10, 26, 50). While mice lacking TLP are viable, males are sterile due to defects in spermiogenesis (34, 35, 55). The observation that both TBP and TLP levels increase during spermatogenesis (45) provides further evidence that although structurally similar, TBP and TLP have, at least in part, functionally divergent roles in transcriptional regulation. Taken together, these findings seem to dictate a role for TLP in regulating transcription of a subset of genes required for cell proliferation, perhaps in a species-specific manner.

Another TBP homologue, known as TBP2, TBPL2, or TRF3, was described more recently, in humans, mice, frogs, and zebra fish (3, 24, 41). TBP and TBP2 are ca. 95% identical throughout the conserved C-terminal domain; however, their N-terminal domains are quite divergent, both in the same organisms and between species. TBP2, like TBP, is a component of a multisubunit complex, although the normal complement of TBP-associated factors is not present in the complex associated with TBP2 (41). Like TBP, TBP2 has been shown to bind the TATA box and can mediate RNA polymerase (RNAP) II transcription (3). TBP2 has been shown to be necessary for the embryonic development of both frogs and zebra fish (3, 41). Thus, although TBP2 seems to have specialized functions, it also displays functions that may overlap those of TBP.

TRF-1, a TBP homologue identified only in *Drosophila melanogaster*, has been found to be capable of both sequence-specific DNA binding and transcriptional activation (16, 21).

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Some developmental genes in *Drosophila* require TRF-1 for transcription. In adult flies, however, TRF-1 is largely restricted to neuronal and testicular tissues (16, 21, 46). TRF-1 is associated with a large uncharacterized set of proteins at promoters, termed neuronal TBP-associated factors. TRF-1 also interacts with both TFIIA and TFIIB. The RNAP III specific initiation factor BRF is found together with both TBP and TRF-1 at RNAP III specific promoters (16). Overall, TRF1 seems to function as a general transcription factor for RNAP III, but at different promoters, different times, and in different tissues than TBP.

TBP itself is essential for viability in all eukaryotic cells, and genetic analyses have therefore been limited, especially in metazoans. To analyze the effects of reduced TBP levels, our lab previously generated a line of DT40 cells heterozygous for *tbp* (TBP-het) (48). While overall transcription was relatively unaffected and TBP-het cells were viable, phenotypic irregularities demonstrated the importance of wild-type levels of TBP during the cell cycle. TBP-het cells were found to be significantly larger than wild-type DT40 cells, and the doubling time for the heterozygotes was about 24 h, more than twice that of wild-type DT40 cells, reflecting a delayed M phase. Intriguingly, the mitotic delay phenotype was attributed to a significant reduction in expression of the M-phase inducer phosphatase *cdc25B* (48). Providing a possible explanation for this, analysis of the murine *cdc25B* promoter revealed a cell cycle-regulated repressor (CCR) element that partly overlaps the TATA box (27). Thus, the reduced levels of TBP were hypothesized to decrease competition with the putative repressor, making the *cdc25B* promoter especially sensitive to reduced TBP levels.

In the present study we have investigated further the consequences of *tbp* heterozygosity. We first examined in more detail the effect of lowered TBP levels on *cdc25B* expression. We inserted the human *cdc25B* promoter into a reporter vector and performed luciferase assays after stable transfection of both wild-type DT40 and TBP-het cells. The results demonstrated a significant downregulation of *cdc25B* promoter activity in the TBP-het cells. Significantly, mutations introduced into the portion of the CCR that did not overlap the TATA box restored expression in TBP-het cells but had only minimal effects in wild-type cells, supporting the idea that the *cdc25B* promoter is indeed regulated by competitive binding of the CCR repressor and TBP. We also investigated the expression of the two TBP homologues in TBP-het cells. We found that TLP expression was upregulated in TBP-het cells and that this reflects increased activity of the core TLP promoter. This was confirmed by transient-transfection assays in HeLa cells, which indicated that TBP and TLP overexpression affected the TLP promoter in opposite ways. Significantly, chromatin immunoprecipitation (ChIP) assays revealed the presence of both TBP and TLP on the TLP promoter, indicating that TLP interacts physically with its own promoter. We identified the *tbp2* gene in chickens and found that as in other organisms, TBP2 is closely related to the conserved core domain of TBP. We also found that transcription of the *tbp2* gene was upregulated significantly in TBP-het cells. These results provide a plausible explanation for the lack of global effects on transcription caused by lower levels of TBP and point to a significant cross-regulation among the different TBP isoforms.

MATERIALS AND METHODS

PCR methods. RNA was isolated from DT40 cells using TRIzol reagent (Invitrogen). Reverse transcription-PCR (RT-PCR) was performed using specific primers and Superscript One-Step PCR (Invitrogen). Real-time PCR assays used cDNA synthesized using the Superscript First-Strand synthesis kit (Invitrogen) and Syber Master Mix (Applied Biosystems) on an ABI prism 7300 real-time PCR machine. Dilutions of an actin standard were used to normalize real-time PCR results. PCR was performed on genomic DNA isolated using TRIzol reagent and amplified with *Pfu* polymerase (Stratagene). The primer sequences used are available upon request.

Plasmids and constructs. All cloning was done using standard subcloning methods. Luciferase reporter vectors were derived from the PGL3 Promoter Vector (Promega). The simian virus 40 promoter was replaced with the promoters under study by digesting the vector with KpnI and HindIII and inserting the appropriate promoter. The promoterless construct was made by blunt ending the KpnI and HindIII sites and ligating the vector to itself. An antibiotic-resistant cassette derived from loxP vectors (1) was inserted into the BamHI site of the PGL3 vector. Expression vectors were made by subcloning full-length cDNA with a sequence encoding the hemagglutinin epitope fused to the 5' end into pExpress (1). The cDNA expression cassette from pExpress was then subcloned into suitable loxP vectors. Mutagenesis of the *cdc25B* promoter was done by DpnI-mediated site-directed mutagenesis (14). Briefly, mutant DNA was amplified by using complementary primers designed to change the first four nucleotides of the CCR. Parental DNA was digested with DpnI. Mutant plasmids were amplified in DH5 α . All plasmids were sequenced by GeneWiz.

Cell culture and transfections. DT40 cells were maintained as previously described (52) in RPMI 1640 (Sigma) supplemented with chicken serum and fetal bovine serum (HyClone). HeLa cells were maintained in Dulbecco modified Eagle medium (Gibco) supplemented with fetal bovine serum (HyClone). Lipofectamine 2000 (Invitrogen) was used to transfect approximately 4×10^5 DT40 cells with 800 ng of linearized or supercoiled DNA. Selection of stably transfected cells took place 48 h after transfection in either 0.5 μ g of puromycin/ml, 30 μ g of Blasticidin/ml, or 1.5 mg of G418 (Gibco)/ml. Transient transfections of HeLa cells were done by using Lipofectamine 2000 to transfect 5×10^5 cells using 300 ng of reporter vector and 400 ng of the appropriate protein expression vector. An empty protein expression vector was used to bring DNA totals for transient transfections up to 800 ng. Nocodazole was used at a concentration of 40 ng/ml for 16 h. A Becton Dickinson fluorescence-activated cell sorter was used to confirm cell cycle arrest. Cells were harvested and fixed with methanol. Propidium iodide (Sigma) was used to stain cellular DNA after cells were washed.

Luciferase assays. Cells were lysed in reporter lysis buffer and assayed by using the luciferase assay system (Promega). Fluorescence was detected using a luminometer (Berthold). Each luciferase assay was done in triplicate with three to five pools of cells, each independently transfected with the appropriate promoter construct, and the results were averaged. Equal numbers of HeLa cells were lysed using passive lysis buffer (Promega), and the extracts were used to assay luciferase activity. Protein concentrations of cell extracts were used for normalization when assaying luciferase in DT40 cells, rather than cell number, because of differences in cell sizes. Each luciferase assay was also normalized by using values from the same cells transfected with the promoterless construct.

ChIP assays. The ChIP protocol was adapted from the fast protocol described by Nelson et al. (37). HeLa cells were cross-linked with 1.4% formaldehyde, typically for 20 min at room temperature. Cross-linking was stopped with 125 mM glycine for 5 min at room temperature, and cells were harvested and washed twice with cold phosphate-buffered saline. Cells were lysed with immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 0.5% [vol/vol] NP-40, 1% [vol/vol] Triton X-100), and chromatin was sheared by sonication with a Branson Sonifier 250 (output: 2.5, 10 s at constant duty cycle, six times with 20-s intervals on ice). Lysates were cleared by centrifugation at $12,000 \times g$ for 10 min at 4°C. Antibodies (anti-RNA polymerase II [N-20; catalog no. sc-899, 4 μ g], anti-TBP [N-12; catalog no. sc-204, 4 μ g], and anti-TLP [C-16; catalog no. sc-10105, 8 μ g]; all from Santa Cruz Biotechnology) were added to samples (equivalent to 4 million cells each), followed by incubation in an ultrasonic water bath for 1 h at 4°C. Chromatin was cleared by centrifugation at $12,000 \times g$ for 10 min at 4°C, and protein G-Sepharose (20 μ l per sample) was added. Tubes were rotated overnight at 4°C, beads were collected by centrifugation and washed five times with immunoprecipitation buffer, and antibody-protein-DNA complexes were eluted by adding elution buffer (1% [vol/vol] sodium dodecyl sulfate, 100 mM NaHCO $_3$). Reversal of cross-linking was performed overnight at 65°C with an NaCl and RNase A solution, followed by proteinase K treatment for 90 min at 45°C. DNA was phenol-chloroform ex-

FIG. 1. Chicken *cdc25B* mRNA is expressed at reduced levels in TBP-het cells. (A) RT-PCR of *cdc25B* mRNA using specific primers on RNA derived from wild-type and TBP-het cells with or without nocodazole treatment. (B) Real-time PCR of *cdc25B* cDNA from wild-type and TBP-het cells, with or without nocodazole treatment. (C) Real-time PCR of *cdc25B* cDNA derived from wild-type cells, TBP-het cells, and TBP-het cells expressing either full-length exogenous TBP or TBP lacking the N terminus (dN).

tracted and ethanol precipitated and eventually used as a template for PCR. PCR were performed for 30 cycles using [³²P]dCTP and primers corresponding to the NF1, β -actin, and TLP promoters and to an intergenic region used as a control [$\sim$ 4 kb downstream the β -actin poly(A) site]. PCR products were resolved on an 8% native polyacrylamide gel, exposed by using a phosphorimager (Storm 860), and quantified by ImageQuant 5.2. Signals were normalized to the control (intergenic amplification) after subtraction of the protein G-Sepharose background signal. Experiments were repeated four independent times with similar results.

RESULTS

Cdc25B promoter activity in DT40 cells is affected by TBP levels. We first wanted to investigate further the repression of *cdc25B* expression in TBP-het cells. Our previous experiments to measure *cdc25B* transcript levels in TBP-het cells were done by RT-PCR with primers designed from consensus sequences within mouse and human transcripts (48). The database of full-length cDNAs from DT40 cells (6) allowed us to design specific primers for *cdc25B* to verify and extend these previous findings. Humans have three variant *cdc25B* transcripts, produced by alternative splicing (2). The sequence of *cdc25B* cDNAs in chickens most closely resembles variant 2 (data not shown; accession number AJ720997). Using primers specific for chicken *cdc25B*, we performed RT-PCR on total RNA from both wild-type and TBP-het cells (Fig. 1A). Consistent with our previous results, *cdc25B* mRNA was barely detectable

in TBP-het cells (Fig. 1A, lane 3), whereas wild-type cells expressed *cdc25B* mRNA at significantly higher levels (lane 1). To determine how this might be related to the mitotic delay phenotype of the TBP-het cells (48), both cell lines were treated with nocodazole to arrest cells during mitosis, and *cdc25B* mRNA levels were again measured by using RT-PCR. Fluorescence-activated cell sorting analysis confirmed that the nocodazole-treated cells were in fact arrested in G₂/M phase (data not shown). As expected, nocodazole treatment resulted in higher levels of *cdc25B* mRNA in both wild-type and TBP-het cell lines (lanes 2 and 4). However, *cdc25B* mRNA levels in TBP-het cells remained significantly lower than in the wild-type cells.

We next wished to quantitate the amounts of *cdc25B* mRNA in the above cells, as well as provide evidence that the repressive effect was indeed due to lower TBP levels. To this end, cDNA was synthesized from RNA extracted from wild-type and TBP-het cells, as well as from TBP-het cells stably expressing either full-length exogenous TBP or TBP-dN (a TBP derivative lacking the N terminus [see reference 38]), and analyzed by using real-time PCR (Fig. 1B and C). Unsynchronized wild-type DT40 cells expressed 2.7 times the amount of *cdc25B* mRNA as the TBP-het cells (Fig. 1B, columns 1 and 3). When both cell lines were treated with nocodazole to induce G₂/M arrest, wild-type cells expressed more than four times as much *cdc25B* as did TBP-het cells (Fig. 1B, columns 2 and 4). Cells expressing exogenous TBP or TBP-dN produced 2.0 times and 1.3 times more *cdc25B* mRNA, respectively, than did the TBP-het cells (Fig. 1C, columns 3 and 4). The above data confirm that TBP levels indeed affect *cdc25B* transcript levels in DT40 cells and suggest that the TBP N terminus is important for full activity.

We next wanted to determine whether the changes in *cdc25B* mRNA levels were due to alterations in *cdc25B* promoter activity. Several attempts to isolate the *cdc25B* promoter from chicken genomic DNA were unsuccessful, and its sequence is not present in the available chicken genomic databases. We therefore isolated the human *cdc25B* promoter (-330 to $+20$), confirmed that the CCRR was conserved and correlated in position with the murine promoter (data not shown), and cloned it into a luciferase reporter vector. Pools of transfected cells stably expressing luciferase driven by this promoter, or by a promoterless control, were derived from wild-type and TBP-het cells. Luciferase expression from the *cdc25B* promoter was relatively high in wild-type cells, and treatment of the cells with nocodazole increased expression, with levels 3.0 times higher than in untreated cells (Fig. 2A, columns 1 and 2). Strikingly, TBP-het cells expressed luciferase barely above the background levels observed with the promoterless control (Fig. 2B, columns 1 and 2), reflecting a nearly 10,000-fold decrease in enzyme activity from levels in wild-type cells. In addition, nocodazole treatment of the TBP-het transformed cells did not cause a significant increase in luciferase levels (column 3). The remarkable magnitude of the decrease in luciferase expression in the TBP-het cells likely reflects in part some property of the enzyme reporter system. Indeed, analysis of luciferase mRNA levels by RT-PCR revealed a smaller decrease, of three- to fourfold (Fig. 2C, lanes 1 and 2), which is comparable to the decrease in *cdc25B* mRNA levels (Fig. 1A, lanes 1 and 3). Nonetheless, these results, which were

FIG. 2. Cdc25B promoter activity is repressed in TBP-het cells. (A) Luciferase driven by the cdc25B promoter. Columns 1 and 2 show luciferase expression in pools of wild-type cells stably transfected with the cdc25B promoter construct, unsynchronized and treated with nocodazole, respectively. Columns 3 and 4 show luciferase expression from the same construct in TBP-het cells, with or without nocodazole treatment. (B) Luciferase values of TBP-hets from the cdc25B promoter construct compared to a promoterless control. The results for activity in wild-type cells were omitted to reduce the scale and highlight comparison between the cdc25B promoter and promoterless control vector in TBP-het cells. (C) RT-PCR of luciferase mRNA driven by the cdc25B promoter in unsynchronized and nocodazole-treated cell lines indicated. +TBP cells are a derivative of TBP-het cells that express exogenous flu-tagged TBP.

observed reproducibly with multiple independent pools of transformed cells, indicate that the reduced levels of cdc25B mRNA in TBP-het cells reflect a reduction in core promoter activity.

Mutations in the CCRR element relieve the repressive effect of lower TBP levels. We next sought to address whether our hypothesis that DNA binding competition between the CCRR and TBP underlies the sensitivity of the cdc25B promoter to TBP levels. A prediction of this idea is that mutations that disrupt the CCRR element would at least partly alleviate the effect of reduced TBP levels on cdc25B promoter activity in TBP-het cells, but the same mutations would have less effect in wild-type cells. Mutations were therefore introduced into the CCRR element in the four nucleotides that are not part of the TATA box (Fig. 3A), the mutant promoters were inserted into luciferase vectors, and pools of stably transformed wild-type and TBP-het cells were isolated and analyzed for luciferase activity. Mutant m2 luciferase levels did not differ significantly

FIG. 3. Mutations of the CCRR rescue cdc25B promoter activity in TBP-het cells. (A) Sequence of mutations introduced into the CCRR. Mutated promoters were then cloned into the same vector as the wild-type cdc25B promoter and stably transfected into TBP-het cells. (B) Luciferase assays from wild-type and mutant promoters in TBP-het cells (columns 2 to 7) compared to the unmutated promoter in wild-type cells (column 1). (C) Mutant promoters driven luciferase activity in wild-type cells.

from those found in TBP-het cells with an unmutated promoter, being barely above background levels (Fig. 3B, columns 2 and 3). Strikingly, however, the other four mutant promoters drove luciferase expression in TBP-het cells between 54 and 84% of the levels driven by the unmutated promoter in wild-type cells (Fig. 3B, columns 4 to 7).

These results are consistent with the idea that disruption of the CCRR element can compensate for reduced levels of TBP. However, because these mutations were adjacent to the TATA box, it was conceivable that they affected core promoter activity by another mechanism. To address this issue, we set out to determine whether these mutations would affect promoter activity in the presence of normal levels of TBP and, to this end, repeated the experiments in wild-type DT40 cells. Wild-type cells transfected with the m2-driven luciferase vector expressed 1.5 times less than cells containing the unmutated promoter (Fig. 3C, columns 1 and 2). Luciferase levels from the m3, m4, and m5 promoters were only very slightly higher than those from the wild-type promoter, between 10 and 20% higher (Fig. 3C, columns 3 to 5), and luciferase levels driven by promoter m6 were 1.8 times that from the wild-type promoter (column 6). The fact that these mutations all had only minimal effects in wild-type cells indicates that they did not significantly affect core promoter activity and strongly supports the conclusion

FIG. 4. TLP is expressed at higher levels in TBP-het cells and in nocodazole-treated DT40 cells. (A) RT-PCR of TLP mRNA with RNA isolated from wild-type cells (Wt -), TBP-het cells (Het -), wild-type cells treated with nocodazole (Wt +), and TBP-het cells treated with nocodazole (Het +). (B) Real-time PCR analysis of TLP cDNA from wild-type cells, wild-type cells treated with nocodazole, TBP-het cells, TBP-het cells treated with nocodazole, TBP-het cells expressing exogenous TBP, and TBP-het cells expressing TBP-dN (columns 1 to 6, respectively). (C) Western blots using anti-TLP and anti-actin antibodies with whole-cell lysates of wild-type and TBP-het cells.

that the striking increases in activity observed in the TBP-het cells indeed reflect reduced competition between the CCRR and TBP.

TLP is upregulated during G₂ phase and in TBP-het cells.

The data presented above provide strong support for the idea that transcription of a specific gene, *cdc25B*, is indeed very sensitive to levels of TBP. Previous results, however, indicated that overall transcription was largely unaffected in TBP-het cells (42). While this difference undoubtedly reflects, at least in part, the unusual structure of the *cdc25B* promoter, we wondered whether additional factors might ensure that cells are resistant to fluctuations in TBP levels. Specifically, might expression of TLP or TBP2 be enhanced when TBP levels are decreased? To address this, we first assessed the levels of TLP mRNA in DT40 and TBP-het cells. Indeed, RT-PCR revealed that TBP-het cells expressed TLP mRNA at about three times the level found in wild-type cells (Fig. 4A, lanes 1 and 2; real-time PCR in Fig. 4B, columns 1 and 3). Western blots confirmed that the upregulation of TLP mRNA in TBP-het cells was reflected in increased levels of TLP protein (Fig. 4C). To investigate whether the TBP-het phenotype (i.e., mitotic delay) might contribute to the elevated TLP levels, we treated both wild-type and TBP-het cell lines with nocodazole. Although expression of TLP mRNA was more than doubled in nocodazole-treated wild-type cells (Fig. 4A, lanes 1 and 3, and Fig. 4B, columns 1 and 2), TBP-het cells exhibited no further increase in TLP levels (Fig. 4A, lanes 2 and 4, and Fig. 4B,

columns 3 and 4). These results suggest that TLP mRNA is expressed at maximal levels in TBP-het cells and that the upregulation reflects at least in part the cell cycle phenotype. TBP-het cells have an extended G₂ phase, and TLP has previously been implicated in the G₂ checkpoint (43).

To extend these results, we examined the effects of exogenous TBP expression on TLP mRNA levels. Stable expression of full-length TBP reduced TLP mRNA levels in TBP-het cells to levels equivalent to those found in wild-type cells (Fig. 4B, column 5). TLP mRNA levels in TBP-het cells expressing TBP-dN were 1.3 times higher than levels in wild-type cells (column 6), possibly reflecting the only partial restoration of the mitotic delay phenotype (42). We were unable to perform the converse experiments, because in multiple attempts we failed to obtain cells overexpressing TLP (results not shown), suggesting that its expression must be very tightly regulated. In any case, the restoration of wild-type levels of TLP mRNA when exogenous TBP was supplied suggests that the observed TLP overexpression was a direct effect of reduced TBP levels.

TBP levels control TLP promoter activity. The data described above indicate that TBP levels can influence the expression of TLP mRNA and protein in DT40 cells. We next wanted to determine whether this change in expression levels results from a direct effect on the activity of the TLP promoter and also whether such regulation extends to human cells. Comparison of the human and chicken TLP promoters indicates that while both promoters have multiple binding sites for heat shock factors, they otherwise share little homology (data not shown). To investigate whether the activity of the two TLP promoters is sensitive to TBP levels in DT40 cells, we first cloned the human (-280 to +90) and chicken (-320 to +72) TLP promoters into luciferase reporter vectors and stably transfected the vectors into wild-type DT40 cells, TBP-het cells, and (in the case of the human promoter) TBP-het cells expressing full-length TBP or TBP-dN. Luciferase activity was then measured in pools of stably transformed cells. Strikingly, the TBP-het cells expressed luciferase at very high levels, nearly nine times greater than in wild-type cells from vectors containing the human TLP promoter (Fig. 5A, columns 1 and 2). Luciferase expression from the chicken TLP promoter was 10 times higher in TBP-het cells than in the wild type (Fig. 5B, columns 1 and 3). TBP-het cells expressing full-length exogenous TBP actually expressed 30% less luciferase from the human promoter than did the wild-type cells (Fig. 5A, column 3). TBP-het cells expressing TBP-dN (column 4) expressed roughly twice as much luciferase as wild-type cells (column 1), but still four times less than TBP-het cells not supplied with exogenous TBP (column 2). As observed with endogenous TLP expression, nocodazole increased luciferase expression from the chicken TLP promoter 2.5-fold in wild-type cells but did not further increase luciferase activity in TBP-het cells (Fig. 5B, columns 2 and 4). The human TLP promoter also expressed elevated levels of luciferase in wild-type cells treated with nocodazole (data not shown). Together, these results indicate that the TLP core promoter itself is sensitive both to TBP levels and to the cell cycle and suggest that, despite limited sequence similarity, this response is conserved among vertebrates.

We next investigated the possibility that TBP and/or TLP can regulate expression from the TLP core promoter in human

FIG. 5. The human TLP promoter is affected by both TBP and TLP levels. (A) The human TLP promoter driving luciferase in stably transformed wild-type DT40 cells, TBP-het cells, TBP-het cells expressing exogenous TBP, and TBP-het cells expressing TBP-dN (columns 1 to 4, respectively). (B) The human TLP promoter driving luciferase in stably transformed wild-type (columns 1 and 2) and TBP-het cells (columns 3 and 4). The results in columns 2 and 4 are after 16 h of nocodazole treatment.

cells. To this end, we transiently cotransfected the human TLP promoter-luciferase reporter vector along with plasmids encoding either flu-tagged TBP or flu-tagged TLP into HeLa cells (Fig. 6A). Significantly, luciferase expression in cells expressing exogenous TBP was reduced to a level 2.4 times lower than in cells transfected with the reporter vector alone. In sharp contrast, overexpression of TLP strongly stimulated the TLP promoter to produce more than 10 times the luciferase activity than did cells without exogenous TLP expression (Fig. 6A, column 3). The same experiment using a vector containing the chicken TLP promoter yielded very similar results when TLP was cotransfected, causing a marked increase in luciferase expression (Fig. 6B, column 3). However, exogenous TBP had only a modest effect (1.2-fold) on transcription driven by the chicken TLP promoter (column 2). These results indicate that TLP stimulates transcription from its own promoter, whereas excess TBP slightly inhibits the TLP promoter in humans and has little to no effect in chickens. These findings extend the results obtained in DT40 cells and indicate the existence of significant cross-regulatory interactions between TLP and TBP.

Both TBP and TLP are present on the TLP promoter. We examined whether TBP and/or TLP interact with the TLP promoter. To this end, a ChIP analysis using HeLa cell DNA and antibodies to RNAP II, TBP, and TLP was performed. After cross-linking of proteins and chromatin with formaldehyde, RNAP II-, TBP-, and TLP-containing complexes were isolated by immunoprecipitation. The resulting coimmunopre-

FIG. 6. TBP and TLP affect the TLP promoters of human and chicken in different ways. (A) Human TLP promoter. Luciferase values from transient cotransfection into HeLa cells of human (h)TLP promoter-luciferase vector, hTLP promoter vector and TBP expression vector, and hTLP promoter vector and TLP expression vector (columns 1 to 3, respectively). (B) Chicken TLP promoter. Transient transfection into HeLa cells of chicken TLP (cTLP) promoter-luciferase vector, cTLP promoter vector and TBP expression vector, and cTLP promoter vector and TLP expression vector (columns 1 to 3, respectively).

cipitated DNA was amplified by PCR using primers specific for the promoter regions of the *neurofibromatosis type 1* (NF1), β -actin, and *tbp* genes and for an intergenic region used as a control. The NF1 promoter was tested because it has previously been shown to interact with TLP after transfection of a TLP-green fluorescent protein encoding construct and an NF1 promoter-containing plasmid (9). Although the TLP antibodies were relatively weak, evidence that TLP associates with both the NF1 promoter and, most importantly, its own promoter was obtained (Fig. 7A, lane 5, and Fig. 7B, columns 6 and 9). We were also able to amplify the NF1 and TLP sequences after TBP immunoprecipitation, showing that TBP bound to the endogenous NF1 and TLP promoters (Fig. 7A, lane 4, and Fig. 7B, columns 5 and 8). Indeed, after normalization TLP was present in quantities roughly equivalent to the amount of TBP at the TLP promoter. Providing evidence for the specificity of the association, TLP was not present on the TATA-containing β -actin promoter (Fig. 7A, lane 5, and Fig. 7B, column 3). The presence of both TBP and TLP on the human TLP promoter further supports the existence of cross regulation by these two proteins and also provides evidence that TLP autoregulation reflects a direct interaction of TLP with its own promoter.

TBP2 is expressed at higher levels in TBP-het cells. We next investigated whether the unusual levels of TBP and TLP in the TBP-het cells would have any effect on transcription levels of the second TBP homologue, TBP2, in TBP-het and wild-type DT40 cells. Whereas *tbp* and *tlp* genes are both located on chromosome III, we discovered a sequence on chromosome V

FIG. 7. TLP associates with its own promoter and with the NF1 promoter but not with the β -actin promoter. (A) ChIP analysis of HeLa cells using anti-RNAP II (lane 3), anti-TBP (lane 4), and anti-TLP (lane 5) antibodies. PCR was performed using primers for the β -actin, NF1, and TLP promoters and for an intergenic sequence as a control. (B) Graph of signal strength of PCR results for each antibody after normalization (see Materials and Methods). The results for the β -actin (columns 1 to 3), NF1 (columns 4 to 6), and TLP (columns 7 to 9) promoters are shown.

that when translated conceptually closely resembles the sequence of the conserved core domain of TBP (recently identified in Ensemble as TBPL2). While the overall identity of the predicted TBP2 sequence compared to TBP was 67%, comparison of only the conserved core domain revealed 93% identity (not shown). RT-PCR using primers specific for the sequences encoding the N-terminal domain of TBP or the N-terminal domain of TBP2 revealed that while TBP2 mRNA was undetectable in wild-type cells, it was present in TBP-het cells (Fig. 8A). To quantitate expression of TBP2 mRNA in these cell lines, real-time RT-PCR was performed with RNA derived from wild-type and TBP-het cells. Although absolute levels of TBP2 mRNA were relatively low in both cell lines, TBP-het cells expressed more than three times as much TBP2 mRNA as did wild-type DT40 cells (Fig. 8B, columns 1 and 3). When the cells were treated with nocodazole, no significant differences in TBP2 mRNA levels were seen in either cell line, indicating that TBP2, unlike TLP, is not upregulated in G_2 phase. Our data provide evidence that TBP2 functions, at least in part, as a redundant mechanism that compensates for lower levels of TBP.

DISCUSSION

In this study we have provided new insights into how altered levels of the TBP can influence expression of *cdc25B* and two TBP homologues. We first extended our previous results by

FIG. 8. TBP2 mRNA is upregulated in TBP-het cells. (A) RT-PCR of TBP and TBP2 mRNA with primers from the region encoding the diverged N-terminal domain in wild-type (Wt) and TBP-het cells. (B) Real-time PCR of TBP2 cDNA derived from wild-type cells, wild-type cells treated with nocodazole, TBP-het cells, and TBP-het cells treated with nocodazole (columns 1 to 4, respectively).

showing that *cdc25B* promoter activity was specifically diminished by reduced levels of TBP in DT40 cells, providing support for the idea that this reflects competition with a repressor for binding to the *cdc25B* TATA box. We also report that reduced levels of TBP enhance, either directly or indirectly, the expression of the two TBP homologues, TLP and TBP2, and that TLP can positively autoregulate its own promoter. Complete understanding of the consequences of altered TBP levels is important not only to the ongoing investigation of transcription mechanisms but also because of the possible role of TBP in human diseases, including neurodegenerative diseases (49) and cancer (25).

Our data indicate a strong correlation between the CCRR described by Korner et al. (27), *cdc25B* expression, and levels of TBP. *Cdc25B* is directly implicated in several types of cancer (18, 19, 22, 23, 54), and in fact *cdc25B* protein levels are often used as indicators of a patient prognosis (22, 42). Patients with *cdc25B*-positive tumors exhibit a significantly lower disease-free survival rate (22). Furthermore, immunostains of brains from patients with Alzheimer's disease show increased levels of *cdc25B* expression (51). Given that TBP is overexpressed in certain tumors (25), it will be of interest to determine whether *cdc25B* levels are correspondingly elevated in such tumors. Another possibility is that mutations in the *cdc25B* promoter region (i.e., in the CCRR element) cause upregulation of *cdc25B*. Identification of the protein and/or protein complex that binds to the CCRR element and represses *cdc25B* expression is an important goal and may be a key to possible gene therapy techniques for cancers that overexpress this important mitotic regulator.

The CCRR site of the *cdc25B* promoter directly overlaps the TATA box. There are precedents for repressor binding sites that overlap the TATA box, inhibiting assembly of the initiation complex by directly competing with TBP. The basal transcription apparatus of *Archaea* contains proteins that correspond to the eukaryotic RNA polymerase II and TBP. A protein, Lrs 14, represses transcription from its own promoter by binding to a site that overlaps the TATA box (4). In *Dro-*

sophila, the homeodomain protein Engrailed binds not only to a specific consensus sequence but also to the TATA box, repressing transcription from promoters that are activated by other homeotic proteins (40). Another example of a repressor exhibiting steric hindrance of TBP-DNA binding is the proline-rich homeodomain protein PRH, which binds directly to the TATA box of an engineered simian virus 40 promoter, as well as to TBP itself (15). The promoter of the *osteocalcin* gene contains glucocorticoid receptor (GR) response elements that when occupied by the GR directly prevents binding of TBP to the TATA box (44). The TATA box in the *osteocalcin* promoter is a "weak" TATA box, and replacement with a canonical TATA box (TATAAAA) increases transcription from this promoter (36). It will be of interest to learn how altered levels of TBP affect expression from these types of promoters.

Our results indicate that disrupting the binding site for the putative Cdc25B repressor by mutation of the four nucleotides of the CCRR that are not part of the TATA box can increase transcription from the *cdc25B* promoter when TBP levels are limiting. The fact that the same mutations had much smaller effects in wild-type cells, where TBP levels are higher, strongly supports our idea that a competition between TBP and the CCRR modulates *cdc25B* transcription. Whether the relatively small differences in expression from the various mutant promoters we observed reflect differential binding of the repressor to the mutated CCRR sequences and/or whether the sequences flanking the TATA box also have the potential to affect TBP (31, 53) or TFIIB (7, 8, 13, 29) binding cannot be determined until the CCRR protein is identified and characterized.

The first TBP homologue we investigated, TLP, also seems to be a significant component in cell cycle regulation and apoptosis in DT40 cells (43). No role for TLP has yet been discovered in human disease. However, the function of TLP appears to vary between species (10, 26, 34, 43), which may limit the use of model systems in elucidating potential role(s) that TLP plays in mammals. Nevertheless, the study of this transcription factor is important because TLP directly regulates transcription of a subset of genes. For example, TLP upregulates the *NF1* gene and represses transcription of *c-fos* (9). In addition to our findings that TLP interacts with its own promoter, we have recently identified a high-affinity recognition motif for TLP, which has suggested a number of additional target genes (S. D. Bush, P. Richard, and J. L. Manley, unpublished data). The results reported here support the idea that TLP and TBP may reciprocally regulate the transcription of a larger subset of genes than currently described.

The more recent identification of TBP2 has introduced another layer of complexity to core promoter function. One interesting fact is that while the core domains of TBP and TBP2 are nearly identical, their N termini are quite distinct. The upregulation of TBP2 in TBP-het cells may allow resolution of the role of the N-terminal domain of TBP. Given that the upregulation of TBP2 that occurs in TBP-het cells is not sufficient to prevent the *tbp* (+/−) phenotype, it seems likely that the N-terminal domain of TBP is uniquely necessary for at least some regulatory functions. It will be interesting to determine whether expression of exogenous TBP2, to even higher levels than occur from the upregulation of the endogenous gene, can result in a partial rescue of the phenotype as seen

with TBP-het cells expressing TBP-dN (42), or if overexpression of TBP2 can fully rescue TBP-het cells. The N-terminal domain of TBP has been implicated in transcription from TATA-less promoters (30) and has been shown to reduce the binding of TBP to promoters containing a TATA box (28, 32). It is doubtful that TBP2 can fully replace TBP, since no successful knockout of TBP has been documented and, in fact, mice lacking most of the N-terminal of TBP exhibit a lethal defect midway through gestation (20). The upregulation of TBP2 that occurs in TBP-het cells is not sufficient to compensate for the effect of lower TBP levels on transcription of *cdc25B*. However, the possibility that TBP2 has functions that overlap those of TBP is not unreasonable. For example, overall transcription is not severely affected in TBP-het cells (48), perhaps indicating a compensatory role for TBP2 and explaining why TBP2 levels increase when TBP levels decrease.

Comparison of the human TBP2 and TLP promoters shows no similarity in sequence or in putative transcription factor binding sites. Neither promoter has a TATA box in the −30 position, although TLP does have two upstream sequences that correspond to canonical TBP binding sites. Indeed, an intriguing possibility is that TBP binding to these or to related sequences contributes to the downregulation of the two promoters that is alleviated by *tbp* heterozygosity. However, the levels of upregulation (TLP mRNA is expressed to a significantly higher level than TBP2 mRNA) lends credence to the idea that the mechanism(s) regulating the expression of these two TBP homologues likely differ. This concept is reinforced by the upregulation of TLP that occurs in cells arrested in mitosis, while TBP2 expression is unaffected in such cells. TLP has also been implicated in regulation of the G₂ cell cycle checkpoint, as well as in the response to cellular stress, such as UV and heat shock (43), while a role for TBP2 in the cell cycle or the stress response has not been established. Experiments to examine the regulation of TBP2 during all phases of the cell cycle will help to clarify the role of this TBP homologue and could be especially informative using TBP-het cells.

In summary, our data have provided additional insights into the functions of the three TBP-related proteins that are conserved throughout metazoa. Two of the three, TBP and TLP, have previously been implicated in the G₂ cell cycle checkpoint (33, 43). As we have shown here, *cdc25B* transcription is directly affected by TBP levels, likely reflecting competitive binding with the CCRR. The TLP upregulation we documented is likely at least in part a result of the cell cycle phenotype exhibited by TBP-het cells. While the elevated levels of TBP2 suggest it may be involved in a transcriptional backup mechanism that compensates for irregular TBP expression, it has yet to be determined whether this expression ameliorates the effect of lower levels of TBP on transcription of specific genes. Indeed, it is intriguing that its upregulation is insufficient to compensate for reduced TBP levels in *cdc25B* transcription. Whether this reflects insufficient levels of TBP2 to compete effectively with the CCRR even after upregulation, or a sequence difference, which would likely be in the N-terminal domain, remains to be determined. It will be of interest, for example, to determine whether downregulation of TBP2 in TBP-het cells further reduces *cdc25b* transcription and/or exacerbates the *tbp* (+/−) phenotype. In any event, our data have

shown that reduced TBP levels affect transcription of several specific genes and do so by distinct mechanisms.

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