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Heterozygous Disruption of the TATA-Binding Protein Gene in DT40 Cells Causes Reduced cdc25B Phosphatase Expression and Delayed Mitosis

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TATA-binding protein (TBP) is a key general transcription factor required for transcription by all three nuclear RNA polymerases. Although it has been intensively analyzed in vitro and in *Saccharomyces cerevisiae*, in vivo studies of vertebrate TBP have been limited. We applied gene-targeting techniques using chicken DT40 cells to generate heterozygous cells with one copy of the *TBP* gene disrupted. Such *TBP*-heterozygous (TBP-Het) cells showed unexpected phenotypic abnormalities, resembling those of cells with delayed mitosis: a significantly lower growth rate, larger size, more G_2/-M- than G_1-phase cells, and a high proportion of sub-G_1, presumably apoptotic, cells. Further evidence for delayed mitosis in TBP-Het cells was provided by the differential effects of several cell cycle-arresting drugs. To determine the cause of these defects, we first examined the status of cdc2 kinase, which regulates the G_2/M transition, and unexpectedly observed more hyperphosphorylated, inactive cdc2 in TBP-Het cells. Providing an explanation for this, mRNA and protein levels of cdc25B, the trigger cdc2 phosphatase, were significantly and specifically reduced. These properties were all due to decreased TBP levels, as they could be rescued by expression of exogeneous TBP, including, in most but not all cases, a mutant form lacking the species-specific N-terminal domain. Our results indicate that small changes in TBP concentration can have profound effects on cell growth in vertebrate cells.

TATA-binding protein (TBP) is a key general transcription factor involved in transcription by RNA polymerases (RNAPs) I, II, and III (23). In RNAP II-mediated transcription from TATA box-containing promoters, the direct interaction between the TATA box and TBP, as a component of TFIID, is the first and potentially rate-limiting step of transcription (11). Thus, this step has been shown to be a target for transcriptional activation and repression (21, 40, 68). Consistent with this, recruitment of TBP to a promoter by a heterologous DNA binding domain can bypass the need of transcriptional activators (8, 33, 49, 80). Moreover, it was reported that the degree of TBP occupancy of promoters in yeast (*Saccharomyces cerevisiae*) is correlated with transcriptional activity (39, 43). Recently, Kuras et al. (38) and Li et al. (44) showed that TBP occupancy is constant at transcriptionally active promoters but that TBP-associated factor II (TAF_{II}) occupancies are variable, suggesting that binding of TBP to DNA is critical for transcription but that the TAF_{II} requirement might be promoter dependent. Once TFIID interacts with the TATA box, either RNAP II holoenzyme is recruited or general transcription factors along with RNAP II are recruited stepwise to promoters and the preinitiation complex is formed (21, 57). For the class of promoters lacking TATA boxes, TBP is not sufficient for transcriptional initiation in vitro and TFIID is required (61, 75). The exact mechanism by which TBP functions on these promoters is not clear yet.

TBP is divided into two domains: the species-specific N-terminal domain and the conserved C-terminal core domain. Studies with in vitro systems and yeast showed that the C-terminal domain of TBP is sufficient for many TBP functions, including DNA binding, complex formation, transcriptional initiation, and yeast viability (23). Although considered species specific, the N-terminal domains of TBPs from vertebrates, such as chickens, mice, and humans, share very high homology. Moreover, several studies have proposed functions for the N-terminal domain in higher eukaryotic systems, i.e., in RNAP II- and III-mediated transcription from certain TATA-containing promoters (42), in RNAP III-mediated transcription of U6 snRNA (53), and in RNAP III-mediated transcription from both TATA-containing and TATA-less promoters (72). These results suggest that, unlike in yeast, the N-terminal domain of TBP might have a function(s) in higher eukaryotes, although genetic confirmation of this is lacking.

In vivo studies of TBP in vertebrate cells have been highly limited, and many questions regarding the roles of TBP in vertebrate systems therefore remain unanswered. Which interactions of TBP with other transcription factors are physiologically significant? How tightly are TBP levels controlled during cell growth? What is the bona fide function of the N-terminal domain? And is TBP required for expression of all genes? Recently, several TBP-related factors have been identified. Their functions are still controversial; in some cases they can replace TBP and direct RNAP II-mediated transcription (22, 48), while in other cases they repress TBP-mediated transcription (54). A recent study identified the *tudor* gene as a direct

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target of TRF1 in *Drosophila melanogaster*, suggesting that TRF1 and TBP could have different promoter preferences (26). This possibility raises questions regarding the strict requirement of TBP in RNAP II-mediated transcription. Could TBP be responsible for transcription of only subsets of genes, as appears to be the case with TAF_{II}s? Genetic depletion studies of several TAF_{II}s in yeast (2, 55) and vertebrate cells (9, 51) suggest that different TAF_{II}s seem to be required for transcription of subsets of genes, although this is still controversial (34, 52). It is unlikely that the function of TBP is as redundant as seems to be the case with certain TAF_{II}s, but these studies emphasize the importance of in vivo analyses of TBP function.

In this paper, we have used gene-targeting techniques in chicken DT40 cells to investigate several in vivo properties of TBP. We generated heterozygous cells with one copy of the *TBP* gene disrupted, which resulted in reduced TBP accumulation. Surprisingly, such *TBP*-heterozygous cells display several abnormalities, in cell shape, cell growth, and cell cycle distribution, which are similar to those of cells with a delayed onset of mitosis, a possibility strengthened by their responses to certain drugs. Strikingly, the delayed mitosis is correlated with inefficient dephosphorylation and thus activation of cdc2 kinase, likely due to reduced cdc25B mRNA expression and the lack of induction that normally occurs in G_2 phase. Rescue experiments indicate not only that all these effects are due to reduced TBP levels, but also that the TBP N-terminal domain is dispensable for some but not all functions. Our study has provided the first genetic analysis of TBP function in vertebrate cells and indicates that expression of a subset of vertebrate genes is critically dependent on TBP levels.

MATERIALS AND METHODS

Plasmid constructs. All plasmids were constructed by standard subcloning procedures. In brief, to construct a targeting vector, a 291-bp *Eco*RI-*Sac*I fragment of exon 3 of the chicken *TBP* gene was replaced by a hygromycin resistance gene under the control of the chicken β-actin promoter, resulting in the deletion of 97 amino acid residues, from 24 to 120. TBP expression constructs were generated by insertion of influenza virus chicken TBP (cTBP), human TBP (hTBP), and influenza virus cTBPΔNH2 cDNAs downstream of the chicken β-actin promoter of the PA-puro vector containing a puromycin resistance gene (78). To generate $cTBP\Delta NH_2$, residues from 1 to 122 of cTBP were removed. An influenza virus hemagglutinin (HA) epitope (flu tag) was fused to the 5′ ends of cTBP and $cTBP\Delta NH_2$ cDNA coding regions by PCR amplication. The first methionine codon of cTBP cDNA was deleted to ensure the expression of influenza virus of cTBP instead of cTBP. The frame between the flu tag and cTBP cDNA was confirmed by sequencing. The sequences of the PCR-amplified cDNAs were also confirmed.

Cell culture and transfections. Chicken DT40 cells were maintained and transfected essentially as described by Wang et al. (78). Cells (1×10^6 to 2×10^6) were transfected with ~30 μg of linearized plasmid by electroporation. Drug selection was started 24 h after transfection with either 1.5 μg of hygromycin (Calbiochem) per ml or 0.5 μg of puromycin (Sigma) per ml as appropriate.

Drug treatment. Approximately 5×10^5 cells were maintained in media containing either colchicine (2 μM), mimosine (200 μM), 5-fluorouracil (5-FU; 500 μM), cisplatin (4 μM), or nocodazol (40 ng/ml). With colchicine, mimosine, and nocodazol, cells were treated for 12 h, and with 5-FU and cisplatin, cells were treated for 48 h.

Southern and Western blot analysis. Genomic DNAs were isolated as described previously (63), and probes were labeled through random priming using chicken cDNA fragments (see Fig. 1B and C). Southern blot analysis was performed as described in the work of Sambrook et al. (63).

For Western blot analysis, cells were harvested, washed with phosphate-buffered saline, lysed in 1× Laemmli buffer, and sonicated. After fractionation on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels and transfer to nitrocellulose membranes, proteins were detected either with anti-influenza virus monoclonal antibody (MAb) 12CA5 (17) or anti-TBP MAb 3G3 (42). Anti-cdc2 and anti-cdc25B antibodies were purchased from Santa Cruz. Anti-TBP MAb 3G3 was a gift from L. Tora. Anti-cyclin D1 and anti-cdk2 antibodies were provided by K. Okamoto. Signals were detected by the enhanced-chemiluminescence method (Amersham).

FACS analysis. Fluorescence-activated cell sorter (FACS) analysis was performed essentially as described by Zhao and Manley (84). In brief, ~5×10^5 cells per ml were harvested and fixed with methanol. After being washed with ice-cold phosphate-buffered saline, fixed cells were stained for cellular DNA with propidium iodide (Sigma). DNA content and cell number were measured with a FACS Calibur (Becton Dickinson), and the ModFit program (Verity Software) was used to analyze cell cycle profiles.

RT-PCR and Northern blot analysis. Total RNA samples from untreated cells and nocodazol-treated cells were prepared basically as described by Chen and Manley (9). RNA was dissolved in water, and the concentration was determined by UV absorbance. RNA (1 μg/μl) was treated with RNase-free DNase, and reverse transcription (RT) was performed according to the method recommended by GibcoBRL. The cDNA obtained was treated with RNase H and amplified by PCR. Two primers were designed to anneal to the 3′ end of the open reading frame and 3′ untranslated region of cdc25B, based on the homologous sequences of human and mouse cdc25B. The sequences of the primers used were 5′-TTCCCNCAGCANCCGAACTT-3′ and 5′-GGGGCAGACAG NNNGACACCACAC-3′. The PCR-amplified ~300-bp DNA fragment was purified and used to prepare a labeled probe for Northern blot analysis by random priming. For Northern blot analysis, RNA samples were resolved on a formaldehyde agarose gel, transferred to nylon membrane, UV cross-linked, prehybridized, and hybridized in hybridization buffer (40% formamide, 0.2 M sodium phosphate buffer [pH 7.2], 1 μM EDTA, 1% bovine serum albumin, 7% SDS) with the labeled probe at 65°C overnight. After the membranes were washed, the signal was detected by autoradiography.

RESULTS

Generation of chicken DT40 TBP-Het cells. To study the in vivo properties of TBP in vertebrate cells, we applied gene-targeting techniques using the chicken B-cell line DT40. DT40 cells have been successfully used in several cases to generate conditional knockout cells, as they show a very high homologous recombination frequency and rapid generation time (9, 70, 78). cTBP shares high homology with TBPs from other vertebrates: the conserved C-terminal domain is identical (except in one residue), and the N-terminal domain is very similar, to those of other vertebrate TBPs, including hTBP and mouse TBP (Fig. 1A) (81). cTBP is a single-copy gene located on chromosome III (65). The genomic structure of cTBP also resembles those of hTBP and mouse TBP: eight exons are separated by seven introns. The N-terminal domain is encoded by exons 2 and 3, and the C-terminal domain is encoded by exons 4 to 8 (Fig. 1B) (7, 65). To construct a gene-targeting vector, we replaced exon 3 with the gene encoding hygromycin resistance (Hyg^r) under the control of the chicken β-actin promoter (Fig. 1B) (Hygro-TBP).

Wild-type DT40 cells were transfected with Hygro-TBP, and 20 hygromycin resistance clones were screened by Southern blot analysis. A cDNA probe corresponding to the internal region of the gene-targeting construct was employed, as any probe that encompassed intron sequences resulted in high background due to highly repeated sequences in the introns (data not shown). Therefore, we used several different strategies of Southern blot analysis to confirm disruption of the *TBP* gene. Out of 20 clones tested, 2 turned out to be *TBP*-heterozygous. Figure 1B and C display representative Southern blot results for one of these two clones. When genomic DNA of heterozygous cells was digested either with *Sac*I or with *Xho*I and *Xba*I and hybridized with probe A, new 6.6- and 6.4-kb bands corresponding to the expected size generated

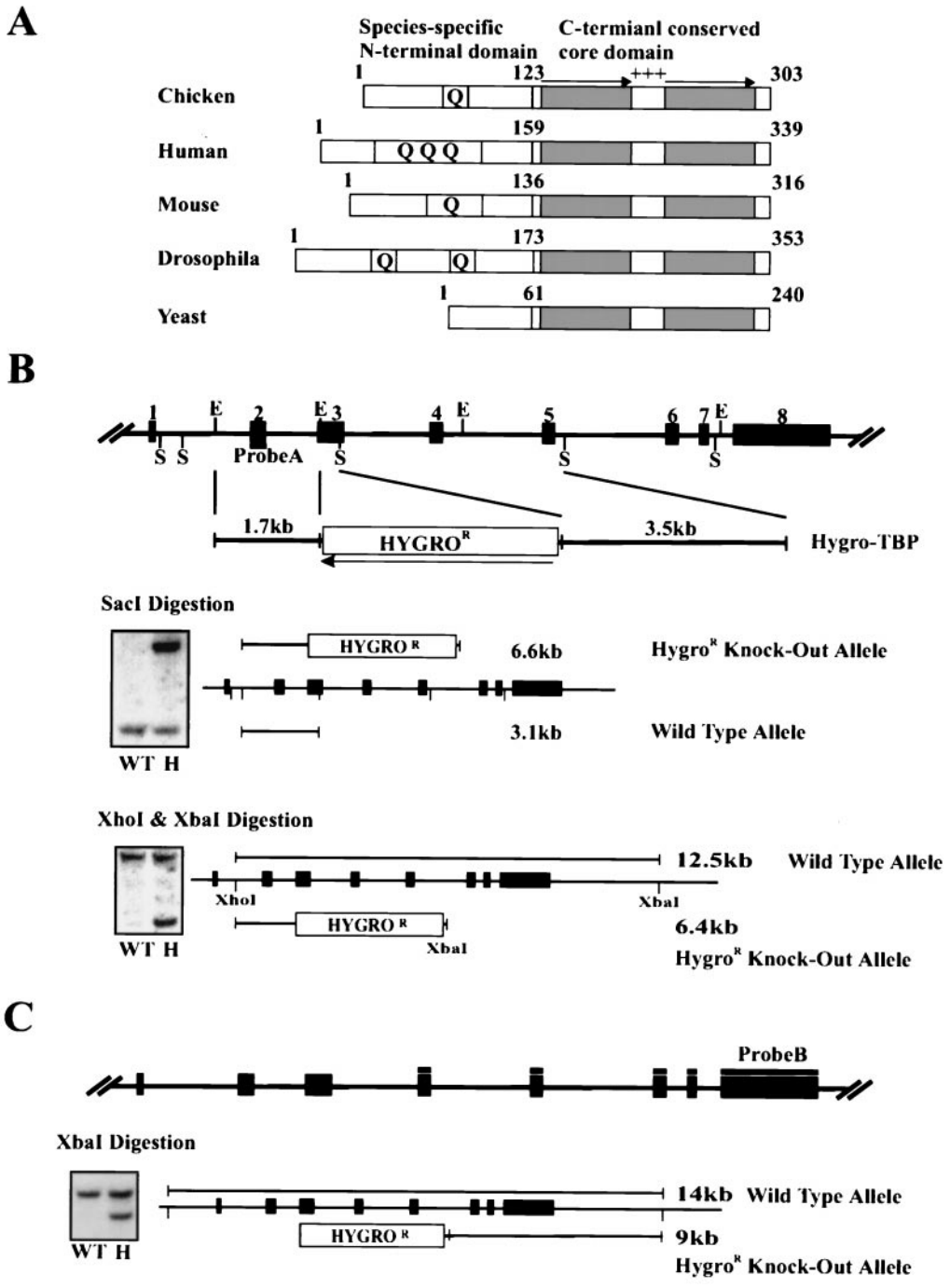

FIG. 1. Generation of chicken DT40 TBP-Het cells. (A) Schematic representation of cTBP and TBPs from other species. Shaded boxes and arrows represent the direct repeats in the C-terminal domain. The positive symbols (+++) between the direct repeats indicate basic residues. Glutamine stretches in the N-terminal domain are shown as boxed Q's. (B) Genomic structure of cTBP and a gene-targeting vector (top diagram) and Southern blot analysis of heterozygous disruption of the *TBP* gene using probe A (gels). Filled boxes and numbers depict exons. Probe A, used in the Southern blot analysis, is also shown. The gene-targeting vector contains a hygromycin resistance gene ($HYGRO^R$) under the control of the chicken β-actin promoter. The arrow indicates the direction of transcription. Diagrams explain the size differences between the wild-type and the disrupted alleles. WT and H indicate the results with wild-type and TBP-Het cells, respectively. (C) Southern blot analysis using probe B. The position of probe B and the size differences of the wild-type and disrupted alleles are indicated.

through homologous recombination appeared, in addition to the 3.1- and 12.5-kb wild-type bands, respectively (Fig. 1B). *Xba*I-digested genomic DNA was hybridized with a different probe, probe B. As shown in Fig. 1C, a 9-kb band, the product of homologous recombination, was observed. The other cell line displayed the same Southern blot profiles (data not shown). Based on these findings, we concluded that we had obtained two *TBP* heterozygous cell lines. Results with one cell

line (designated TBP-Het) are shown, but both behaved indistinguishably in all assays tested (Fig. 1 to 6; data not shown).

TBP expression in TBP-Het cells is reduced. To test if TBP expression is changed in TBP-Het cells, Western blot analysis using anti-TBP MAb 3G3 (42) was performed. As shown in Fig. 2A, TBP expression in TBP-Het cells was reduced to almost half the level of TBP expression in wild-type cells, implying that TBP expression in chicken DT40 cells is biallelic. Generally, proteins are expressed from both copies of genes, and loss of one copy can be compensated for by the remaining wild-type allele (56). However, in TBP-Het cells, we observed several phenotypic abnormalities (see below), suggesting that a constant level of TBP expression is so critical that the reduced expression due to the heterozygosity of TBP causes haploinsufficiency. To investigate these findings further, we generated several stable cell lines that were *TBP* heterozygous but expressed different forms of TBP under the control of the β-actin promoter. TBP expression constructs designed to express flu-tagged cTBP, hTBP, and a flu-tagged-cTBP C-terminal domain (cTBPΔNH_2) are shown in Fig. 2B. Expression of flu-tagged cTBP, hTBP, and flu-tagged cTBPΔNH_2 was confirmed by Western blot analysis either with anti-TBP MAb 3G3 or with anti-influenza virus MAb 12CA5 (Fig. 2C). Interestingly, when cells expressed flu-tagged cTBP exogeneously, total TBP expression did not exceed that of wild-type cells, as previously observed in HeLa cells (86), presumably because TBP overexpression is deleterious. Expression of hTBP and flu-tagged cTBPΔNH_2 was somewhat lower (Fig. 2C and results not shown).

Abnormalities in cell growth and size of TBP-Het cells. One abnormality of TBP-Het cells was a lower growth rate. Growth curves of wild-type, TBP-Het, and exogeneous flu-tagged-cTBP-, hTBP-, and flu-tagged-cTBPΔNH_2-expressing TBP-Het cells are compared in Fig. 3. Four independent clones were analyzed for each of the three rescue constructs, and all displayed growth rates comparable to those shown. The TBP-Het cells grew significantly more slowly than all other cells. While the doubling times for wild-type and exogeneous flu-tagged-cTBP-and hTBP-expressing cells were approximately 9 to 10 h, that of TBP-Het cells was ~24 h. Interestingly, expression of flu-tagged cTBPΔNH_2 in TBP-Het cells partially restored the growth rate; the doubling time of flu-tagged-cTBPΔNH_2-expressing cells was ~14 h.

As shown in Fig. 4, TBP-Het cells were significantly larger in size than wild-type and exogeneous flu-tagged-cTBP-, hTBP-, and flu-tagged-cTBPΔNH_2-expressing cells. Occasionally gigantic cells that were almost 10 times larger than wild-type cells were observed. The large size of TBP-Het cells was due mostly to the expanded size of nuclei, as confirmed by DAPI (4′, 6′-diamidino-2-phenylindole) staining (data not shown). In addition, a considerable number of dead cells were detected in the TBP-Het population, as shown by trypan blue exclusion (data not shown; see below).

Cell cycle profiles of TBP-Het cells are altered. In an effort to understand the cause of the growth defects of the TBP-Het cells, we first used FACS analysis to determine cell cycle profiles. As shown in Fig. 5, several interesting differences were observed in TBP-Het cells. First, in contrast to wild-type and flu-tagged-cTBP-, hTBP-, and flu-tagged-cTBPΔNH_2-expressing cells, significantly more G_2/M- than G_1-phase cells were present in the TBP-Het population. As DT40 cells are fast growing, ~50% of living cells were in S phase in all of the cell lines. However, while wild-type and all three rescued cell lines contained ~3-fold more G_1- than G_2/M-phase cells, TBP-Het cells exhibited the opposite pattern: ~2-fold more cells were in G_2/M than in G_1 phase. This implies that G_2/M phase is prolonged in TBP-Het cells. Second, in accord with observations from analysis by trypan blue exclusion, a high proportion of sub-G_1, presumably apoptotic, cells was observed in the TBP-Het population, strongly suggesting that TBP-Het cells are more prone to cell death. Interestingly, flu-tagged-cTBPΔNH_2-expressing cells also exhibited a higher proportion of sub-G_1 cells, albeit significantly lower than the proportion in TBP-Het cells, which may explain why cTBPΔNH_2-expressing cells grew more slowly than wild-type cells. Finally, we observed that TBP-Het cells, but none of the other cells, showed an extra peak of tetraploid cells with a DNA content of 8 N. This indicates that TBP-Het cells go through DNA rereplication.

Together, the abnormalities observed in TBP-Het cells, such as expanded cell size, more G_2/M-phase cells, and the presence of products of DNA rereplication, resemble those of cells with delayed mitosis, which can be caused either by defects in cell cycle control factors or by treatment with certain drugs (6, 27, 45). We therefore next examined the response of TBP-Het cells to drugs known to affect cell cycle progression.

TBP-Het cells undergo delayed mitosis. To explore further the idea that TBP-Het cells are subject to delayed mitosis, we examined the effects of several cell cycle-arresting drugs on wild-type, TBP-Het, and flu-tagged-cTBP-, hTBP-, and flu-tagged-cTBPΔNH_2-expressing cells. We reasoned that if TBP-Het cells undergo delayed mitosis, any drug that arrests cells at G_2/M phase would arrest TBP-Het cells more efficiently than the other cells because the former cells have an extra barrier at G_2/M phase. In contrast, a drug that arrests cells at G_1/S phase would not arrest TBP-Het cells as effectively because some proportion of TBP-Het cells would be blocked at the putative barrier at G_2/M phase. Indeed, addition of a low concentration of colchicine, which arrests cells at mitosis, caused >90% of TBP-Het cells to arrest at G_2/M phase, while significant proportions of G_1- and S-phase cells (10 to 20% and 60 to 70%, respectively) remained in wild-type and flu-tagged-cTBP-, hTBP-, and flu-tagged-cTBPΔNH_2-expressing cells (Fig. 6A). In contrast, when mimosine or 5-FU, which arrests cells at G_1/S or S phase, respectively, was used, significant amounts of G_2/M cells (15 to 20%) were detected in TBP-Het cells (Fig. 6B and C) but almost no G_2/M-phase cells were observed in the other cells. Fewer cells were present at the 8 N peak of the TBP-Het cells under these conditions, probably because mimosine and 5-FU also blocked rereplication (S* phase). When a cell cycle-nonspecific apoptosis-inducing drug, cisplatin, was used, all cells, including TBP-Het cells, responded similarly (Fig. 6D). Taken together, these results strongly suggest that TBP-Het cells are subject to delayed mitosis. Interestingly, although TBP-Het cells are more prone to cell death, colchicine, mimosine, and 5-FU treatment did not cause a significant increase in the number of sub-G_1 cells in these cells whereas in all other cells, the sub-G_1 population was dramatically increased. This observation is discussed further below.

cdc2 is hyperphosphorylated in TBP-Het cells. We next wished to determine the mechanism responsible for the ob-

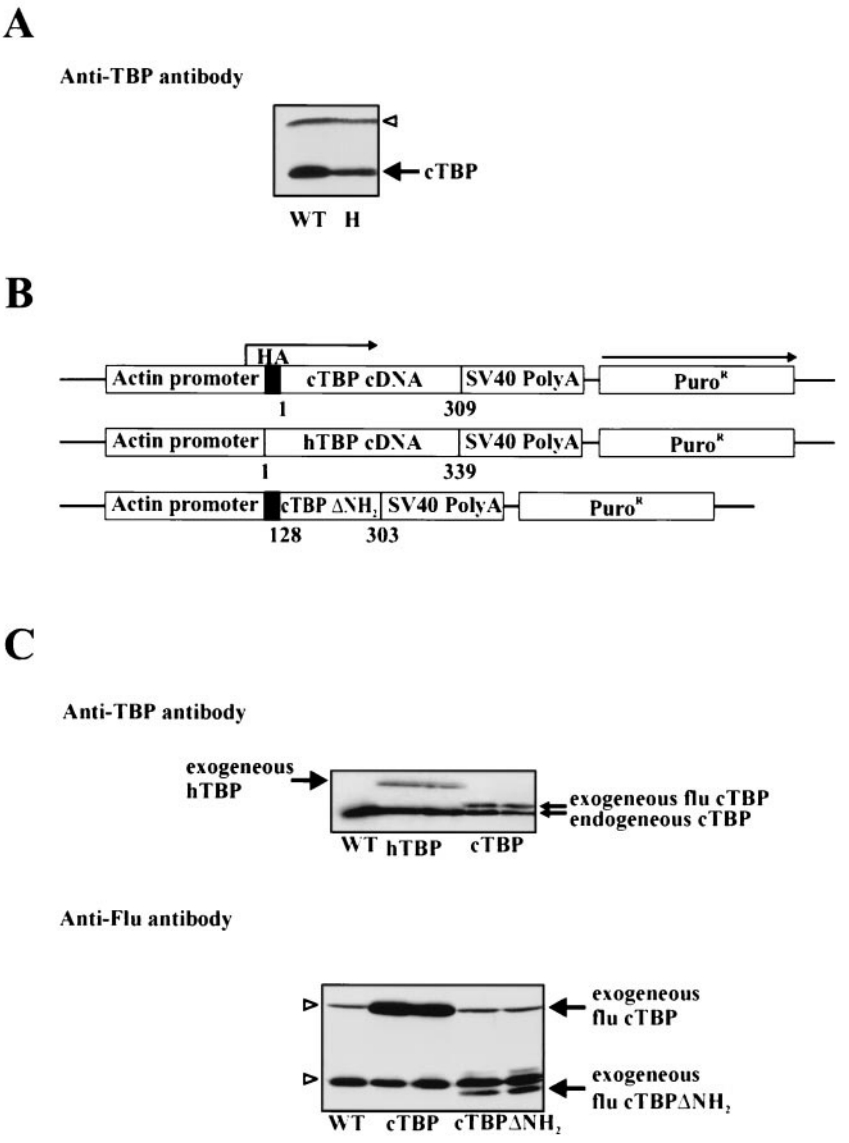

FIG. 2. Western blot analysis of TBP expression. (A) TBP expression in TBP-Het cells is reduced. TBP expression in wild-type (WT) and TBP-Het (H) cells was examined with anti-TBP MAb 3G3. The arrow indicates the position of TBP, and the open arrowhead points to a nonspecific band used as a loading control. (B) TBP expression constructs. Exogeneous flu-tagged-cTBP, hTBP, and flu-tagged-cTBPΔNH_2 expression is under control of the chicken β-actin promoter. HA represents the influenza virus HA epitope. A puromycin resistance marker ($Puro^R$) was used for selection. The arrows indicate the directions of transcription. SV40 PolyA, simian virus 40 polyadenylation signal. (C) Exogeneous expression of flu-tagged cTBP, hTBP, and flu-tagged cTBPΔNH_2 in TBP-Het cells. Expression of proteins was analyzed using either anti-TBP MAb 3G3 or anti-influenza virus MAb 12CA5. Locations of flu-tagged (flu) cTBP, hTBP, and flu-tagged cTBPΔNH_2 are indicated by arrows. The open arrowheads in the lower panel represent nonspecific bands. WT, results with whole-cell extracts from wild-type cells; hTBP, results with hTBP-expressing cells; cTBP, results with flu-tagged-cTBP-expressing cells; cTBPΔNH_2, results with flu-tagged-cTBPΔNH_2-expressing cells.

served delayed mitosis in TBP-Het cells. It is well known that the G_2/M transition is regulated by cdc2 kinase in association with B-type cyclin (4, 10, 32). While cdc2 levels remain constant throughout the cell cycle, cdc2 activity is regulated by phosphorylation and dephosphorylation. Cdc2 phosphorylated by the kinases Wee1 and Myt1 is inactive. At the G_2/M boundary, the phosphatase cdc25 dephosphorylates cdc2, resulting in activation of cdc2. As TBP-Het cells display reduced transition through mitosis, we examined the status of cdc2 and cyclin B in these cells by Western blotting. Strikingly, although no changes in cyclin B were detected (results not shown), almost half of the cdc2 was in an inactive, hyperphosphorylated form in unsynchronized TBP-Het cells while in all other cells, including the cTBPΔNH_2-rescued cells, most of the cdc2 was hypophosphorylated (Fig. 7A). Figure 7B confirms that the positions of the cdc2 upper and lower bands from TBP-Het cells match, respectively, those of the hyperphosphorylated cdc2 species from G_1/S-phase wild-type cells arrested with mimosine and those of the hypophosphorylated cdc2 species in G_2/M-phase cells arrested with nocodazol. We also examined the levels of

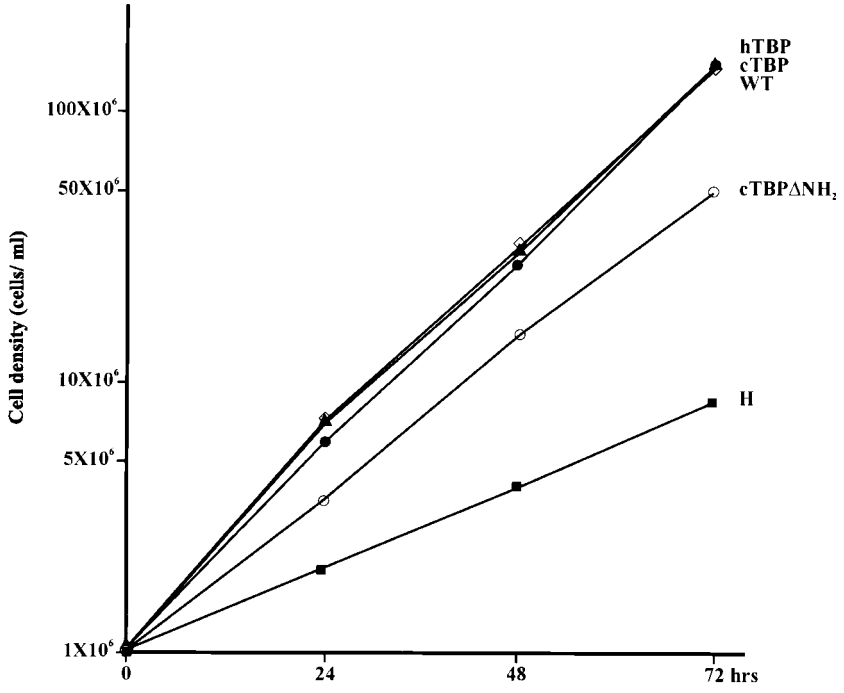

FIG. 3. TBP-Het cells grow more slowly than wild-type and flu-tagged-cTBP-, hTBP-, and flu-tagged-cTBPΔNH_2-expressing cells. Numbers of cells at indicated time points were determined by counting with a hemacytometer. Filled circles, wild-type cells (WT); filled squares, TBP-Het cells (H); filled triangles, flu-tagged-cTBP-expressing cells; open squares, hTBP-expressing cells; open circles, flu-tagged-cTBPΔNH_2-expressing cells. The results shown are averages of results from four independent experiments.

cyclin D1 and cdk2, cell cycle regulators involved in G_1/S phase, but as shown in Fig. 7A (lower gels), no significant differences were observed. Therefore, inefficient activation of cdc2 might be the cause of the delayed mitosis observed in TBP-Het cells. But how could reduced TBP levels result in enhanced cdc2 phosphorylation?

Levels of cdc25B are significantly lower in TBP-Het cells. A possible explanation for the increased cdc2 phosphorylation is that TBP-Het cells have reduced cdc25 activity. Unlike in *S. cerevisiae* and fission yeast (*Schizosaccharomyces pombe*), where only one form of cdc25 has been found, three forms of cdc25 have been reported in vertebrate cells. Among the three forms, cdc25B and cdc25C are involved in the G_2/M transition (24, 39) whereas cdc25A is involved in the G_1/S transition (25, 29). It has been suggested that cdc25B works as a "trigger" to initiate dephosphorylation of cdc2 (39). Once a fraction of cdc2 is dephosphorylated, it phosphorylates and activates cdc25C. Activated cdc25C, in turn, dephosphorylates cdc2 more efficiently, forming a cdc2-cdc25C positive feedback loop (24, 28, 67). High levels of cdc25B, but not C, are sufficient to trigger premature mitosis (31), and studies with transgenic mice indicate that cdc25B overexpression can induce cell proliferation and hyperplasia (47, 83). As none of the cdc25 family has been cloned in chicken cells, we first examined cdc25B

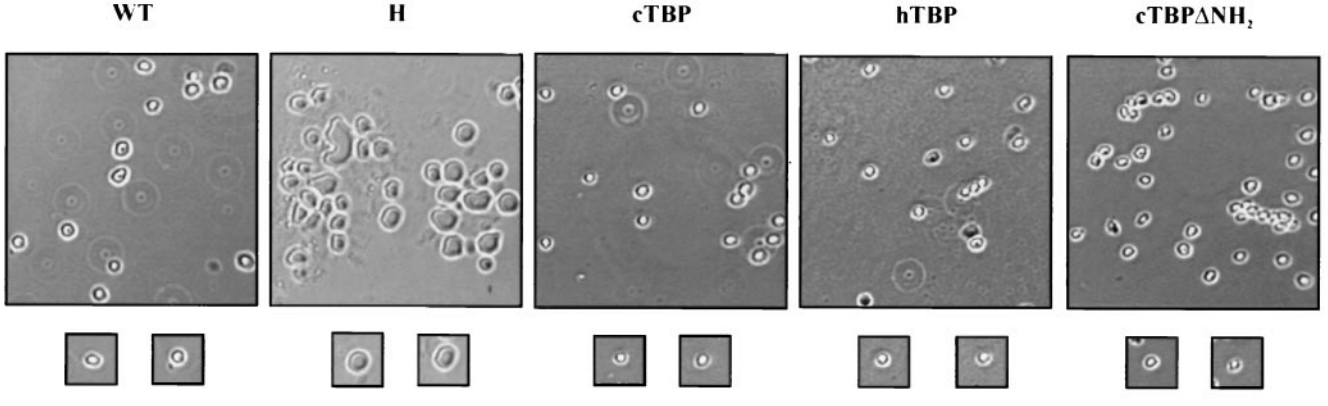

FIG. 4. TBP-Het cells are larger than wild-type (WT) and flu-tagged-cTBP-, hTBP-, and flu-tagged-cTBPΔNH_2-expressing cells. Magnification, ×57.75. In the lower panels, cells are shown in boxes of the same size to facilitate comparison of cell size. H, TBP-Het cells.

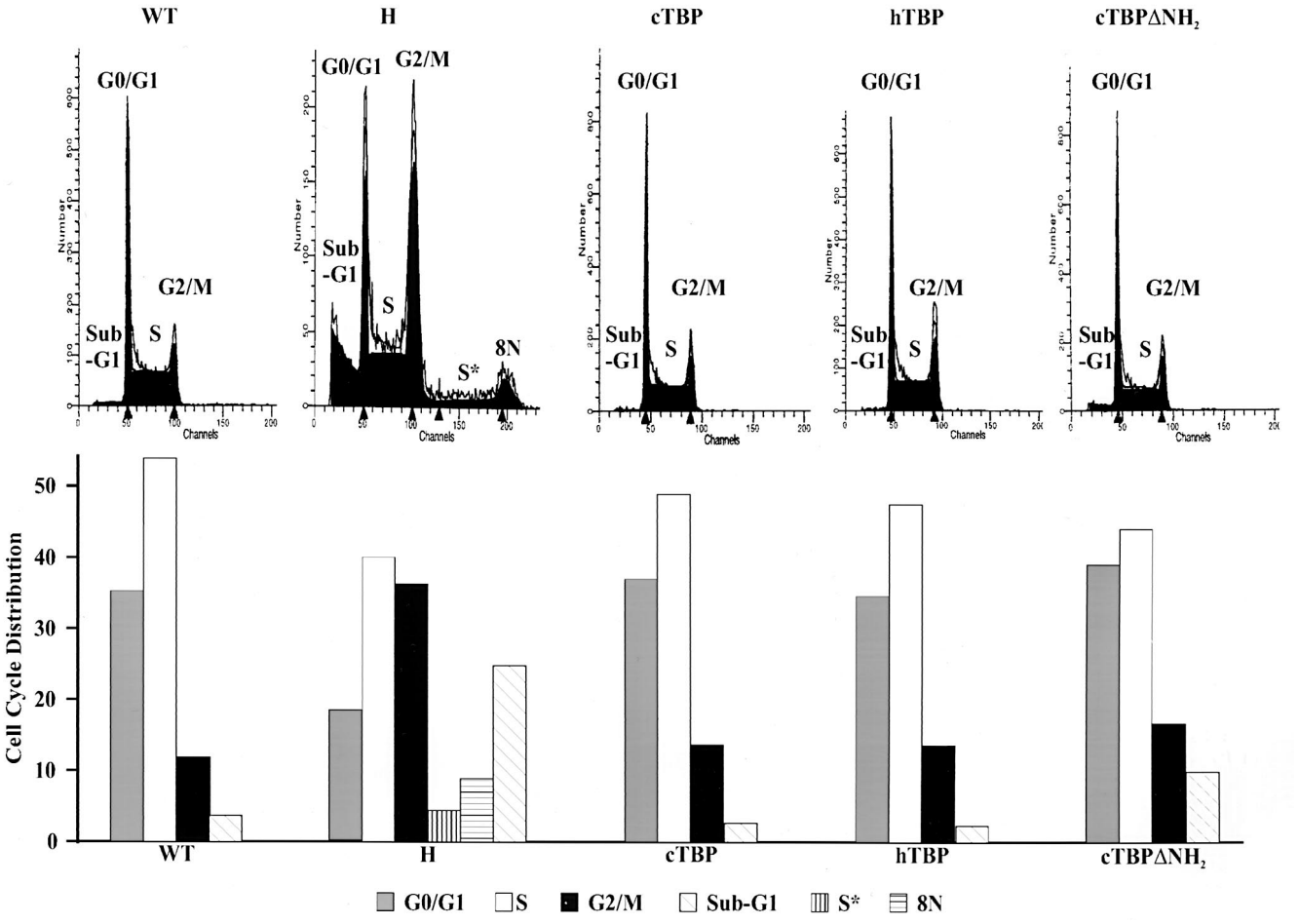

FIG. 5. Cell cycle profiles of TBP-Het cells are altered. DNA content and cell number are plotted on the horizontal and vertical axes, respectively. The positions of cells at G_0/G_1, S, G_2/M, and sub-G_1 phases and, for TBP-Het cells (H), the positions of cells at rereplication (S* phase) and tetraploid cells with the product of rereplication (8N) are indicated. Bar graphs in the lower panel indicate the percentages of cells in each phase. FACS analysis was performed at least five times with similar results. WT, wild-type cells.

protein levels by Western blot analysis using an antibody that recognizes human, rat, and mouse cdc25B. We first compared total protein expression patterns in all cell lines (Fig. 8A). Whole-cell lysates analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie staining showed somewhat more intense staining for TBP-Het cells, consistent with the larger cell size, but qualitatively the patterns among all cells were similar. We next performed Western blot analysis with the anti-cdc25B antibody. Strikingly, compared with the other cell lines, TBP-Het cells accumulated significantly reduced levels of cdc25B (almost fivefold lower, as estimated by densitometry) (Fig. 8B, upper gel). Flu-tagged-cTBP-, hTBP-, and flu-tagged-cTBPΔNH_2-expressing cells in fact appeared to express slightly higher levels of cdc25B than did wild-type cells. Levels of cyclin D1 and actin were similar, as shown in Fig. 8B (lower gels).

Expression of cdc25B has been reported to increase in G_2 phase (3, 18, 39). We therefore decided to test if TBP heterozygosity affects possible induction of cdc25B at G_2 phase. As shown in Fig. 8C, when cells were arrested with nocodazol, a significant induction of cdc25B expression was observed in wild-type and flu-tagged-cTBP-expressing cells, as previously observed in HeLa cells (18). In sharp contrast, cdc25B levels were again low in TBP-Het cells and, importantly, no increase was observed in the nocodazol-treated cells. Cyclin D1 levels again remained unchanged.

If the reduced amount of cdc25B protein detected in TBP-Het cells was a direct consequence of reduced TBP expression, then cdc25B mRNA levels should likewise be reduced. To test this, we first designed PCR primers complementary to sequences conserved between human and mouse cdc25B cDNA sequences (see Materials and Methods). These were used in RT-PCR with total RNA prepared from normal wild-type cells and wild-type cells arrested at G_2/M phase with nocodazol. As shown in Fig. 9A, a ~300-bp RT-dependent DNA, which agrees with the size expected from the human (299-bp) and mouse (296-bp) cdc25B cDNAs, was obtained, but only with total RNA prepared from G_2/M-arrested wild-type cells. Using this DNA fragment as a probe, we performed Northern blot analysis with total RNA samples from TBP-Het cells and the other cell lines (Fig. 9B). Consistent with the Western blot results, cdc25B mRNA levels were significantly lower in TBP-

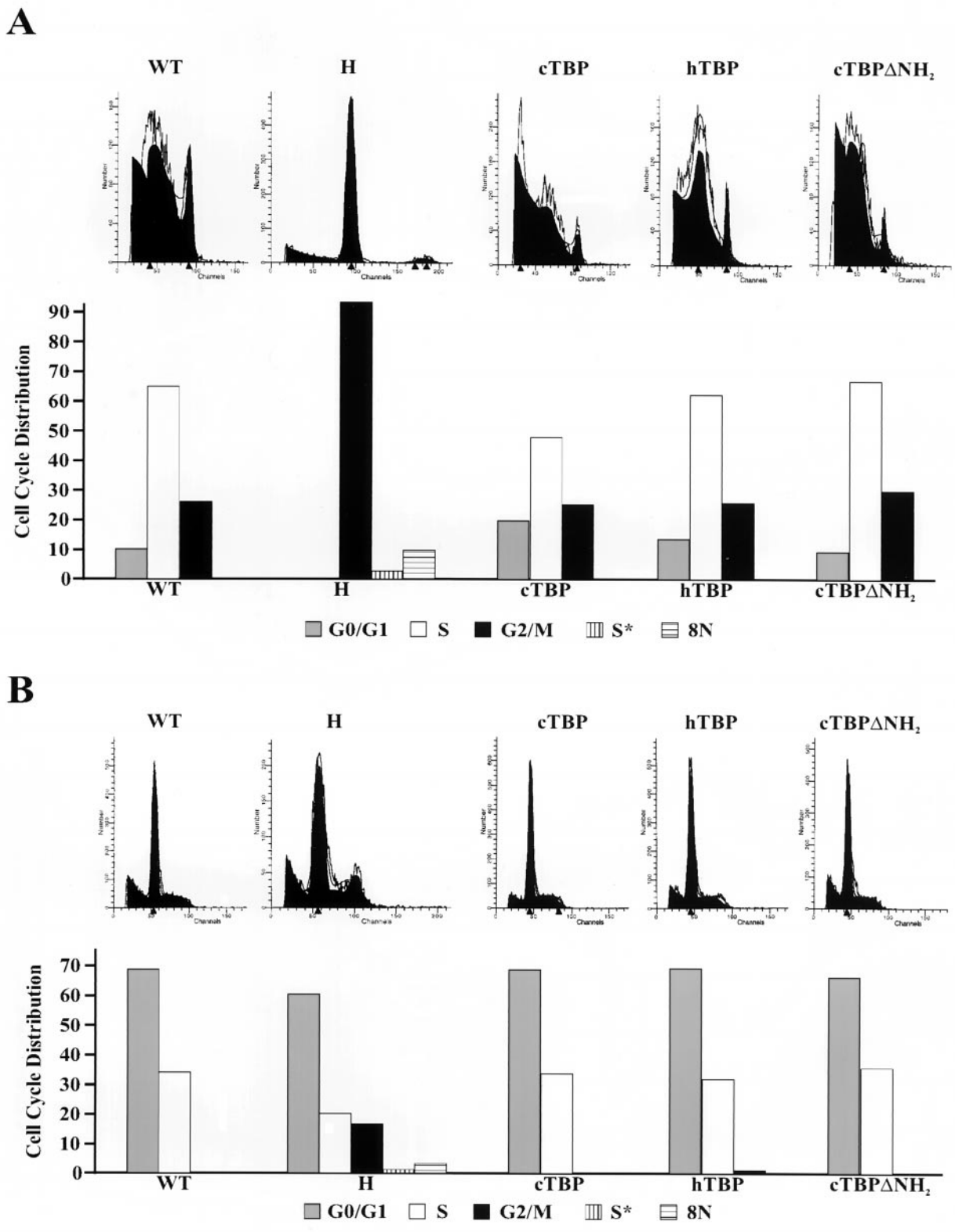

FIG. 6. TBP-Het cells undergo delayed mitosis. Shown are cell cycle profiles of cells subjected to colchicine treatment (A), mimosine treatment (B), 5-FU treatment (C), and cisplatin treatment (D). FACS analyses of cells treated with the indicated drug are shown in the upper plots, and the percentages of cells at each phase are depicted as bar graphs below. With cisplatin treatment, as over 95% of cells were dead and no significant number of cells at each phase was observed, no bar graphs are given. Each experiment was performed at least three times with similar results. WT, wild-type cells; H, TBP-Het cells.

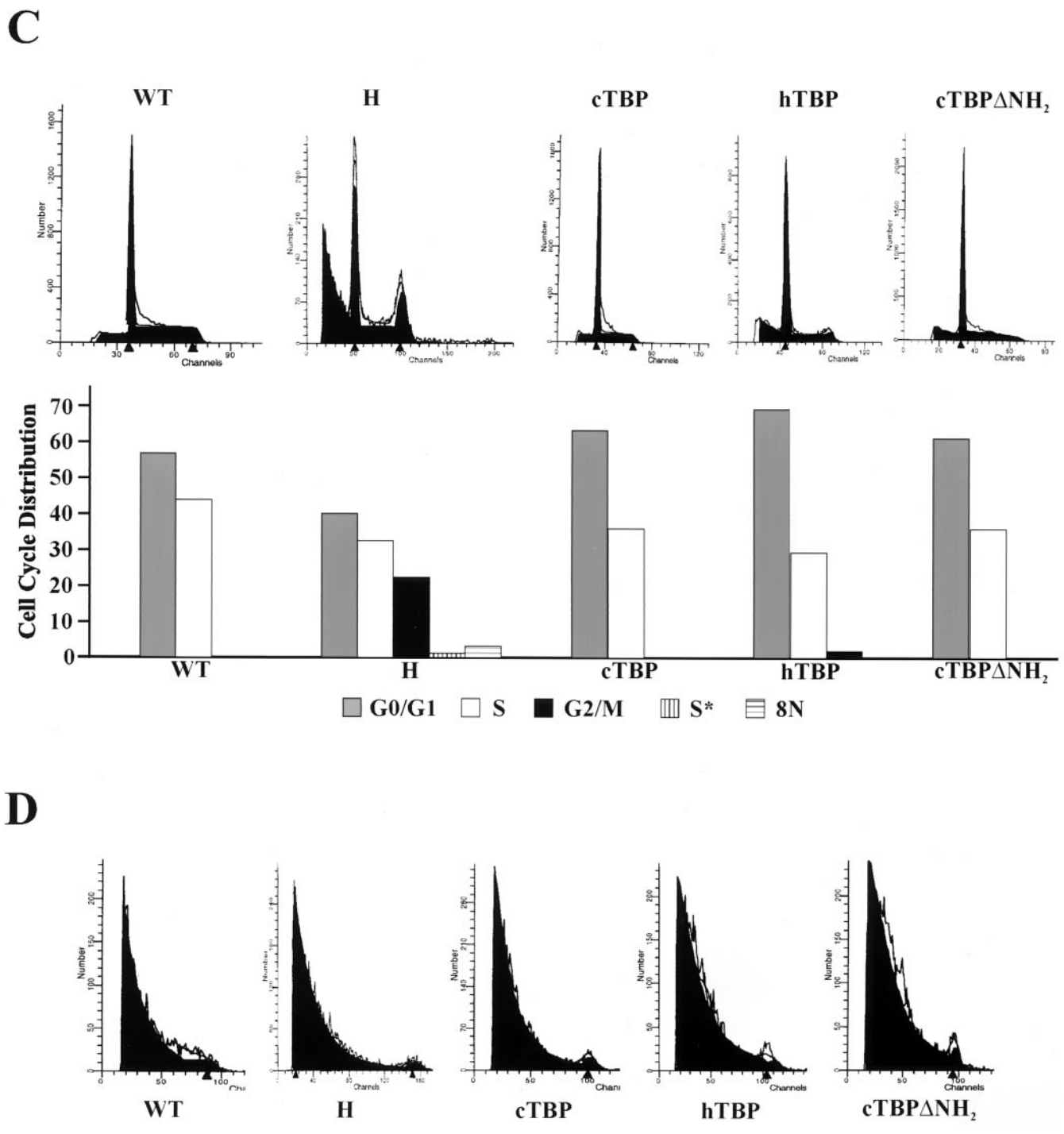

FIG. 6—*Continued.*

Het cells than in wild-type and flu-tagged-cTBP-, hTBP-, and flu-tagged-cTBPΔNH_2-expressing cells. Levels of 28S and 18S rRNAs, U6 snRNA, and actin mRNA were examined and showed no significant differences (Fig. 9C). In consideration of these findings together, the lower level of cdc25B expression combined with its failure to be induced at G_2 phase is likely responsible for the accumulation of hyperphosphorylated, inactive cdc2, which in turn leads to the delayed onset of mitosis observed in TBP-Het cells.

DISCUSSION

***TBP* heterozygosity causes delayed mitosis.** In this paper, we report that DT40 cells heterozygous for *TBP* exhibit significant

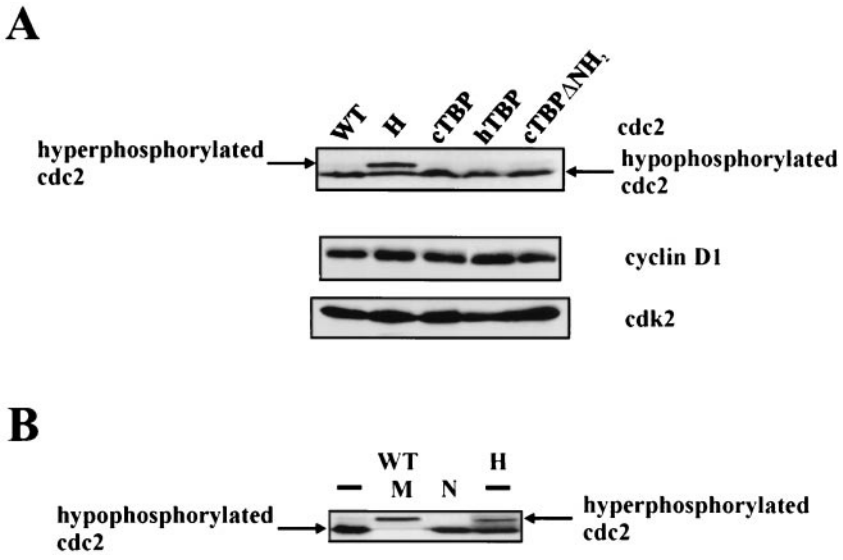

FIG. 7. Cdc2 is hyperphosphorylated in TBP-Het cells. (A) Western blot analysis was performed using whole-cell extracts from each cell line with either anti-cdc2, anti-cyclin D1, or anti-cdk2 antibodies. Positions of hyperphosphorylated and hypophosphorylated cdc2 are indicated. WT, wild-type cells; H, TBP-Het cells. (B) Whole-cell extracts from wild-type cells untreated (−) or treated with either mimosine (M) or nocodazol (N) were analyzed by Western blotting. Results with whole-cell extract of TBP-Het cells are shown in the next lane for comparison.

phenotypic abnormalities, including slower cell growth, expanded cell size, elevated numbers of G_2/M-phase cells, and a high proportion of apoptotic cells. These defects appear to involve decreased expression of the cdc25B phosphatase and resultant interference with the G_2/M-phase transition and importantly can all be rescued by expression of exogenous TBP. Delayed mitosis caused by *TBP* heterozygosity cannot be explained by general inhibition of transcription, because expression of most proteins was not significantly changed in TBP-Het cells.

The extreme sensitivity of DT40 cells to TBP levels may be a property reflective of vertebrate cells. Yeast cells are relatively insensitive to TBP levels, and inactivation of TBP in yeast does not result in a cell cycle phenotype (12, 13; K. Struhl, personal communication). Results reported by Zhou et al. (85) are also consistent with the existence of species-specific differences. Those authors showed that the heterozygous disruption of the *TBP* gene in *Drosophila* did not cause a detectable phenotype and specifically had no effects on expression of the *snail* and *dorsal* genes (although see below). With these findings taken together, we suggest that vertebrate cells are more sensitive than other cells to *TBP* gene dosage.

Depletion of yeast $TAF_{II}90$ or human $TAF_{II}150$ results in cell arrest at G_2/M phase (1, 50). However, the mechanism for this arrest appears different, as expression of other gene products, such as SPC98 (a component of the spindle body) and APC2 (a component of the anaphase-promoting complex) in the case of $yTAF_{II}90$ (41) and cyclin B1 and cyclin A in the case of $hTAF_{II}150$, was found to be affected, while in TBP-Het cells, we suggest that reduced cdc25B expression is responsible for delayed mitosis. On the other hand, depletion of $hTAF_{II}250$ and $yTAF_{II}145$ induces G_1 arrest, presumably due to effects on the transcription of D-type cyclin, and G_1 and B-type cyclin genes, respectively (15, 69, 77). Depletion of TAF_{II}s does not always lead to cell cycle arrest. For example, depletion of $TAF_{II}31$ in chicken cells induces apoptosis but no specific cell cycle arrest (9).

Several cases of upregulation of TBP levels related to cellular transformation have been reported. Hepatitis B virus X protein, a transcriptional activator thought to be important for hepatitis B virus transformation, appears to increase cellular TBP levels through a Ras-dependent pathway (30). Activation of protein kinase C by the tumor-promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has also been reported to increase cellular TBP levels (19). Moreover, increased TBP mRNA was observed in lung and breast carcinomas, leading to the suggestion that increased cellular TBP levels might be important in the transformation process (76). This possibility is consistent with our observation that reduced TBP levels inhibit mitosis and cell growth. The effects of *TBP* heterogygosity and upregulation of TBP related to transformation together strongly suggest that delicate regulation of TBP expression may play a critical role in growth control in vertebrate cells. Indeed, TBP expression appears to be regulated during embryonic development of *Xenopus laevis*. At early stages, when transcription is repressed, TBP was found to be barely detectable, but at the midblastula stage, when transcriptional repression is relieved, TBP levels were upregulated (73). Also, TBP levels increase significantly during late spermatogenesis in mice (64), and TBP levels have also been shown to vary tissue specifically in mice, being especially high in the testis, small intestine, and pituitary gland (59). These results together strongly suggest that TBP expression can be regulated during development and differentiation and that this regulation may be perturbed in disease. Our findings that reduced TBP levels can affect gene expression and cell growth are consistent with this and provide a possible mechanism.

This is the first study to examine the consequence of heterozygous disruption of a gene encoding a general transcription factor in vertebrates, so it is unknown if other such factors

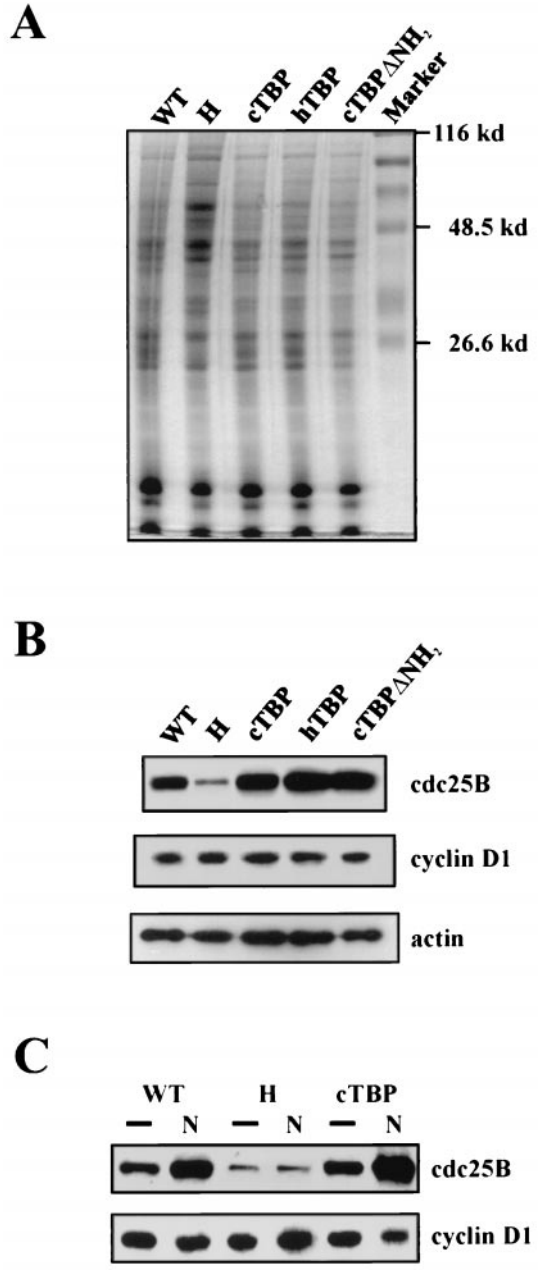

FIG. 8. Levels of cdc25B are significantly lower in TBP-Het cells. (A) Whole-cell extracts from each cell line were subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. Locations of protein markers are indicated on the right. WT, wild-type cells; H, TBP-Het cells. (B) Whole-cell extracts from each cell line were analyzed by Western blotting using anti-cdc25B antibody. Expression levels of cyclin D1 and actin were examined as controls. (C) Cdc25B expression is not induced at G_2 phase in TBP-Het cells. Whole-cell extracts from each cell line that were either untreated (−) or treated with nocodazol (N) were analyzed using either anti-cdc25B antibody (upper gel) or anti-cyclin D1 antibody (lower gel).

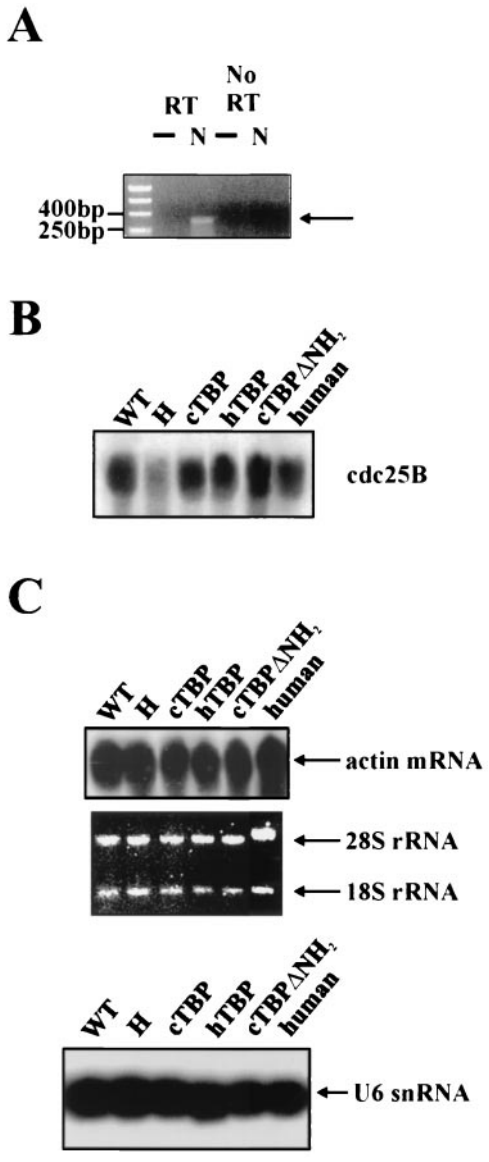

FIG. 9. cdc25B mRNA levels are lower in TBP-Het cells. (A) RT-PCR was performed using total RNAs from wild-type cells that were either untreated (−) or treated with nocodazol (N). No RT represents the results of a control PCR without RT. The amplified ~300-bp DNA fragment is indicated. WT, wild-type cells; H, TBP-Het cells. (B) Northern blot analysis was performed with total RNA samples from each cell line with the cdc25B probe obtained from the experiment whose results are shown in panel A. (C) Levels of 28S and 18S rRNAs were determined by formamide agarose gel electrophoresis and ethidium bromide staining. Northern blot analyses for U6 snRNA and actin mRNA are shown.

are similarly sensitive to dosage effects or if this property reflects the central role of TBP. However, a number of genes encoding proteins involved in transcriptional regulation display haploinsufficiency and can often be associated with one or more genetic disorders (14, 20). Particularly intriguing examples are provided by the related coactivator proteins CREB-binding protein and p300. Heterozygous disruption of either gene in mice causes reduced protein levels and significant growth defects (36, 71, 82), and in humans, loss-of-function mutations in one allele of CREB-binding protein are associated with a disorder known as Rubinstein-Taybi syndrome (60).

Rereplication and apoptosis in TBP-Het cells. Delayed mitosis can lead to DNA rereplication and apoptotic death. DNA

rereplication caused by delayed mitosis is naturally blocked by p53, while apoptosis is p53 independent (6, 74). As DT40 cells do not express p53 (66), TBP-Het cells can undergo DNA rereplication and also exhibit a high proportion of apoptotic cells. Although TBP-Het cells seem to be more prone to cell death, drug treatments (except cisplatin) did not result in significant increases in apoptosis in these cells, unlike in the wild-type and rescued cell lines. Interestingly, under certain conditions, mimosine has been shown to prevent neuronal cell death (16, 58) and apoptosis induced by RNAP II blockage (5). The mechanism involved in this protection is not understood, except that it was suggested to be mediated by p53-independent induction of the cdk inhibitor $p21^{Waf1/Cip1}$. It will therefore be informative to investigate expression of $p21^{Waf1/Cip1}$ in wild-type and TBP-Het cells untreated and treated with mimosine.

TBP dosage sensitivity of cdc25B expression. Protein expression is not generally affected in TBP-Het cells. In contrast to cdc25B, all other genes tested, including products of RNAP I, II, and III, were unaffected by reduced TBP expression, suggesting that transcription of only a subset of genes is sensitive to reduced TBP dosage. In general, most genes do not show haploinsufficiency from loss-of-function mutations in one copy because the remaining copy still expresses at least a minimum threshold level required for protein function. In contrast, genes that show haploinsufficiency do not express enough protein over the threshold from one copy, at least not for all functions, thus causing phenotypic defects (56, 79). In TBP-Het cells, expression of most genes, including those encoding cdc2, cyclin D1, cdk2, and actin, is not changed because reduced TBP expression is still sufficient to allow transcription from their promoters at wild-type levels. Alternatively, in some cases it may be that transcriptional initiation, i.e., the TBP-dependent step, is not rate limiting for protein expression. In contrast, for cdc25B, the reduced TBP levels are lower than the threshold required for efficient transcription, rendering cdc25B expression sensitive to TBP dosage. Why is the cdc25B promoter especially sensitive to TBP levels?

Transient-expression studies showed that responses to overexpression of TBP depend on the type of promoters. In *Drosophila* Schneider cells, transcription from TATA-containing promoters were significantly enhanced by TBP overexpression while TATA-lacking promoters were unaffected by TBP overexpression (11). Also, in mammalian cells, overexpression of TBP can further enhance the effect of certain activators, such as VP16, whereas it inhibits others, such as Spl (62). These studies suggest that TBP levels can differentially affect the activities of various promoters. However, the in vivo situation seems to be more complicated.

As mentioned earlier, *TBP* heterozygosity per se in *Drosophila* did not result in any severe defects (85). However, when combined with *dorsal* heterozygosity, the double heterozygote displayed reduced expression of the Dorsal target gene, *snail*. Significantly, the defect caused by this double heterozygosity was more severe than those caused by double heterozygosity of *dorsal* and either *$TAF_{II}110$* or *$TAF_{II}60$*, both of which have been suggested to be required for transcriptional activation by Dorsal. At least two not mutually exclusive explanations for this requirement can be suggested. *TBP* heterozygosity may have slightly reduced expression of *snail* per se, to levels that were not distinguished by the reporters used. However, once *snail* expression was further decreased by *dorsal* heterozygosity, the additive effects may have resulted in the observed defect in *snail* expression. Alternatively, *TBP* heterozygosity may lower the expression of $TAF_{II}110$ and/or $TAF_{II}60$ or another factor required for snail activation, including Dorsal, and this lower expression level, coupled with *dorsal* heterozygosity, may have reduced *snail* expression.

Similar explanations can be provided for the reduced cdc25B expression in TBP-Het cells. Reduced TBP levels may result directly in lower expression of cdc25B itself. Alternatively, the reduced TBP levels may reduce expression of a factor required for cdc25B transcription and thus affect cdc25B expression indirectly. Why cdc25B expression is so sensitive to TBP dosage will be determined only by detailed studies of the cdc25B promoter. Expression of cdc25C is under the control of a TATA-less and Inr-less promoter, a structure common to several cell cycle regulatory genes (35, 46). How TFIID is recruited to such promoters is not known, and it is conceivable that they might be especially sensitive to TBP and TFIID levels. However, this may not be the entire explanation, as cdc2 and cyclin B promoters contain similar structures (35), yet expression of these genes was not detectably affected in TBP-Het cells. Indeed, a more intriguing possibility emerges from the very recent cloning and characterization of the murine cdc25B promoter (34a). Although the promoter contains recognizable TATA and Inr elements, it also possesses an apparently novel repressor element that overlaps the TATA box. Thus, as suggested by Kornes et al. (34a), it is conceivable that a competition between a repressor and TBP or TFIID might help regulate cdc25B expression, and this could certainly be quite sensitive to TBP levels.

We observed significant induction of cdc25B mRNA and protein expression in wild-type cells arrested by nocodazol. This induction seems to be important, as TBP-Het cells not only express a low level of cdc25B but also have lost the ability to induce cdc25B expression during G_2 phase. Consistent with this, rescue by expression of exogeneous TBP restored both of these defects in cdc25B expression. In HeLa cells, expression of cdc25B also appears to be regulated in a cell cycle-specific manner during G_2 phase (3, 18). However, Lammer et al. (39) reported that cdc25B levels remain relatively constant throughout the cell cycle and that activation of cdc25B at late S and early G_2 phase is regulated primarily by phosphorylation. Our results provide strong support for the idea that cdc25B levels are transcriptionally regulated during the cell cycle, at least in DT40 cells. The inability of TBP-Het cells to activate cdc25B expression in G_2 phase is intriguing. It indicates that whatever is responsible for the reduced basal cdc25B transcription in TBP-Het cells also prevents the cdc25B promoter from responding to an activation signal.

The N-terminal domain of TBP plays a role in vertebrate cells. As mentioned in the introduction, expression of only the C-terminal core domain of TBP allows efficient cell growth in yeast (23). Nevertheless, in higher eukaryotic cells, the possible involvement of the N-terminal domain in RNAP II- and III-mediated transcription has been suggested (42, 53, 72). Consistent with species-specific differences, the N-terminal domain of yeast TBP is significantly shorter than those of TBPs from higher eukaryotes and also lacks the glutamine stretches found

in metazoan TBPs. Our data provide evidence that the TBP N-terminal domain is required for some but not all TBP functions in chicken DT40 cells. Although cell sizes and levels of cdc2 dephosphorylation and cdc25B expression were comparable in all cells, cTBPΔNH_2-expressing cells grew more slowly than wild-type cells, albeit much faster than TBP-Het cells, and showed a detectable population of sub-G_1 cells. These differences were unlikely due to the lower protein stability of cTBPΔNH_2, as cTBPΔNH_2 and hTBP, which was fully active, accumulated to comparable levels. These findings indicate that, as with *TBP* heterozygosity itself, only a subset of genes are susceptible to the absence of the N-terminal domain of TBP. While expression of genes responsible for cell size and cdc25B transcription must have been unaffected by the loss of the N-terminal domain, full expression of one or more genes involved in cell growth rate control and in preventing cell death seem to require the N-terminal domain of TBP. This is the first evidence showing that deletion of the N-terminal domain of TBP can cause physiological abnormalities in vivo, implying that, unlike in yeast, the N-terminal domain of TBP can have an important function(s) in vertebrate cells. Nevertheless, as the most serious defects caused by *TBP* heterozygosity were rescued by expression of cTBPΔNH_2, the C-terminal domain of cTBP appears to be sufficient for most TBP functions.

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