Differential Regulation of Transcription of p21 and Cyclin D1 Conferred by TAF$_{11250}^{1}$

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Abstract
The TATA-binding protein-associated factor TAF$_{11250}$, the largest subunit of TFIIID, was first identified as the cell cycle regulatory protein CCG1. The ts13 Syrian hamster ovary fibroblast cell line, which contains a temperature-sensitive point mutation in TAF$_{11250}$/CCG1, is arrested in G$_1$, following a shift to the nonpermissive temperature. Here we demonstrate that the level of the D-type cyclins, in particular D1, was reduced, whereas the level of the cyclin-dependent kinase inhibitor p21 was stimulated in ts13 cells at the nonpermissive temperature. The levels of expression of cyclins A and E were not affected by temperature shift. We further show that at least part of the regulation of D1 and p21 levels in ts13 cells is mediated at the level of transcription initiation. These results suggest that the effect of the temperature-sensitive mutation TAF$_{11250}$ on cell growth can be mediated through the differential regulation of transcription of specific cell growth regulatory genes, such as cyclin D1 and p21.

Introduction
The regulation of transcription has been shown to be tightly linked to the regulation of the cell cycle. Phosphorylation of specific transcription regulatory factors, such as the protein product of the Rb$^3$ susceptibility gene, by cyclins and their associated cdkks allows for progression of the cell cycle through G$_1$ into S phase (1–3). In particular, the D-type cyclins in association with cdk4 and cdk6, as well as cyclin E-cdk2, are important for phosphorylating Rb (2). It has been proposed that Rb phosphorylation relieves repression of E2F-mediated transcription, resulting in expression of S-phase-specific genes. In addition, cyclin H and its associated kinase cdk7 form part of the basal transcription factor TFIH, which phosphorylates the COOH-terminal domain of the large subunit of RNA polymerase to initiate transcription elongation (4, 5). Cyclin C and cdk8 are also associated with the RNA polymerase holoenzyme, although the exact target(s) for phosphorylation is unknown (6, 7). Moreover, p53, a tumor suppressor and negative regulator of cell growth, is a transcription factor that is able to regulate cell growth in part by stimulating expression of cell growth-inhibitory factors, such as p21 (8–11).

The regulation of transcription has been further linked to the cell cycle by identification of the TATA-binding protein-associated factor TAF$_{11250}$ as the cell cycle control gene CCG1 (12, 13). A ts mutation in TAF$_{11250}$ (ts-TAF$_{11250}$) in the Syrian hamster ovary cell line ts13 results in a block to cell growth in the G$_1$ phase of the cell cycle at the nonpermissive temperature (12, 14). Presumably, the block to the cell cycle is conferred, in part, through TAF$_{11250}$-mediated alterations in gene expression of certain cell cycle control genes (15). Indeed, it has been reported that expression of the cyclin A promoter is reduced in ts13 cells at the nonpermissive temperature, as measured by using a transient transfection assay. The expression of S phase genes, such as DHFR, thymidine kinase, and PCNA, is also affected by the shift to the nonpermissive temperature, although it is not clear whether the observed repression is a direct or indirect effect of the ts-TAF$_{11250}$ (16–19). Interestingly, the ts mutation in TAF$_{11250}$ does not appear to affect the integrity of TFIIID in ts13 cells or affect the level of expression of certain promoters, such as c-fos (15). TAF$_{11250}$ has also been shown to be necessary for conferring transcription mediated by many different transcription factors, including p53, Sp1, and NF-1 in vitro (15, 20–22).

To determine whether the cell cycle block mediated by ts-TAF$_{11250}$ in ts13 cells is conferred by the transcriptional regulation of cell cycle regulatory genes, we have examined the level of certain cyclins, cdkks, cdk inhibitors, and other growth regulatory proteins in ts13 cells following a shift to the nonpermissive temperature. Here, we demonstrate that the levels of D-type cyclins, in particular D1, were reduced, whereas expression of the cdk inhibitor p21 is stimulated at the nonpermissive temperature. We further show that at least part of the regulation of D1 and p21 levels in ts13 cells is mediated at the level of transcription initiation. These results suggest that the effect of TAF$_{11250}$ on cell growth and viability is mediated through the differential regulation of transcription of specific genes, such as cyclin D1 and p21. These results also implicate TAF$_{11250}$ as able to confer regulation of transcription in either a positive or negative manner from specific promoters. Our results suggest that TAF$_{11250}$ regulates the cell cycle through the differential regulation of expression of specific genes.

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3 The abbreviations used are: Rb, retinoblastoma; cdk, cyclin-dependent kinase; ts, temperature-sensitive.
Results

CCG1/TAF$_{250}$ Confers Differential Regulation of Expression of the D-type Cyclins and p21. The ts-TAF$_{250}$ in ts13 cells has been reported to reduce transcription from specific promoters, in particular the cyclin A promoter, following a shift to the nonpermissive temperature (15). To determine whether expression of endogenous cyclin A, as well as other cell cycle regulatory proteins, was affected by ts-TAF$_{250}$ in ts13 cells, the steady-state levels of certain cyclins, cdk5s, and cdk inhibitors were examined by Western analysis in ts13 cells at the permissive and nonpermissive temperatures (Fig. 1). The levels of cyclins A and E did not vary significantly following a temperature shift to the nonpermissive temperature at both the 12- and 24-h time points. In contrast, the levels of D-type cyclins, which are important for progression of the cell cycle through $G_s$, were reduced. In contrast, the level of the cdk inhibitor p21 increased significantly following the temperature shift.

The differential effects of the temperature shift on the levels of D1 and p21 could be conferred by an effect of TAF$_{250}$ on transcriptional initiation, either directly or indirectly, or could be due to differences in protein and/or RNA stability at 39.6°C. To determine whether the ts mutation in TAF$_{250}$ was important for the observed regulation, a population of ts13 cells expressing wild-type TAF$_{250}$ (ts13-hTAF$_{250}$), able to grow at 39.6°C, as generated by stable transfection of a CMV-TAF$_{250}$ expression vector and used as control. As is shown in Fig. 2, there was no reduction in D1 or increase in p21 in the ts13-hTAF$_{250}$ population that contained a wild-type TAF$_{250}$ at 39.6°C compared to the ts13 cells. It is important to note that the slightly higher level of p21 in the ts13 cells compared to ts13-hTAF$_{250}$ in Fig. 2 is not significant. Thus, the differential effects were not due to changes in protein and/or RNA stability at 39.6°C but instead are conferred by TAF$_{250}$.

Regulation of p21 and D1 Expression in ts13 Cells Is in Part at the Level of Transcription Initiation. The stimulatory and inhibitory effects of TAF$_{250}$ on expression of p21 and D1, respectively, could be conferred at the transcriptional, posttranscriptional, and/or protein level. To determine whether the effects on p21 and D1 protein levels in ts13 cells were regulated at the steady-state level of mRNA, the levels of p21 and D1 mRNAs were determined in ts13 and ts13-hTAF$_{250}$ cells at 34°C and 39.6°C. As is shown in Fig. 3, the steady-state level of D1 RNA was reduced, and the steady-state level of p21 was increased at the nonpermissive temperature in ts13 cells. There were no alterations in the levels of p21 and D1 RNAs in the ts13-hTAF$_{250}$ control cell line. Thus, the differential regulation of p21 and D1 conferred by ts-TAF$_{250}$ was in part, at the level of RNA, consistent with the observed effects on the levels of p21 and D1 proteins.

To determine whether the observed effects were at the level of transcriptional initiation, p21 and cyclin D1 promoter reporter constructs were stably introduced into ts13 and ts13-TAF$_{250}$ cells. After shifting to the nonpermissive temperature, a reproducible, greater than 2-fold stimulation in p21 promoter activity was observed in a population of selected ts13 cells but not in a population of ts13-TAF$_{250}$ cells (Fig. 4A). The stimulation of the p21 promoter following temperature shift required sequences contained within the first kb of the promoter in addition to the TATA box. In contrast to the p21 promoter, the D1 promoter reporter plasmid showed
a significant inhibition of expression following temperature shift that was not observed with the control vector or in the population of ts13-hTAF_{1250} cells. These results suggest that the inhibition of the D1 promoter and the stimulation of the p21 promoter occurs in part at the level of transcription initiation.

As an additional control, a cyclin A promoter-luciferase reporter plasmid, which was shown previously to be inhibited in ts13 cells at the nonpermissive temperature, was tested in the stable ts13 assay (Fig. 4B). Surprisingly, cyclin A promoter activity was stimulated upon switching to the nonpermissive temperature in ts13 cells but not in ts13-hTAF_{1250}. Why the cyclin A promoter was stimulated in the stable assay but repressed in the transient assay is unclear. Because we observed that ts13 cells begin to undergo apoptosis between 24 and 48 h after the temperature shift (data not shown), the differences between the two assays may be due to the different times after temperature shift that luciferase expression was analyzed. Alternatively, the contrasting results may reflect differences in the copy number of the reporter plasmid between the two assays. However, our results suggest that the cyclin D1 promoter is more repressed by the ts mutation in TAF_{1250} at the nonpermissive temperature than the cyclin A promoter.

Discussion

In this study, we examined the ability of TATA-binding protein-associated factor TAF_{1250} to regulate transcriptional initiation of specific genes. TAF_{1250} is the largest subunit of TFIID, a basal transcription factor essential for transcription initiation from Pol I, II, and III promoters (23). It was first identified as the gene product of CCG1, important for controlling the cell cycle in the ts13 cells (12, 13). Within 24 h following a shift to the nonpermissive temperature, ts13 cells were arrested in the G_{1} phase of the cell cycle. The identification of a component of the basal transcription factor TFIID as being identical to CCG1 suggests that the regulation of transcription is important for controlling passage of the G_{1} phase of the cell cycle (12). Previous studies have shown that the cyclin A promoter is regulated at the transcriptional level in ts13 cells by the ts mutation in TAF_{1250}, at least in a transient assay (15). Moreover, TFIID complexes isolated from ts13 cells were reduced in their ability to confer transcription from the cyclin A promoter and by Sp1 in vitro. However, the integrity of the TFIID complex was not affected at the nonpermissive temperature, nor was transcription of certain promoters affected.

To determine whether TAF_{1250} can confer regulation expression of specific genes to mediate its effects on cell growth, we examined the levels of expression of a subset of cyclins, cdk, and cdk inhibitors in ts13 cells after a temperature shift to the nonpermissive temperature. In particular, we examined the expression of genes that are important for regulating the G_{1}-S phase of the cell cycle, such as the D-type cyclins and the cdk inhibitor p21. Here, we have shown that expression of the D-type cyclins, especially cyclin D1, was inhibited after temperature shift. In contrast, the level of the cdk inhibitor p21 was stimulated following temperature shift. Northern analysis demonstrated that the regulation of p21 and D1 was conferred at the RNA level, whereas the use of p21 and D1 promoter reporter plasmids demonstrated that the regulation was conferred, in part, at the level of transcriptional initiation.

We also observed, in contrast to previous published results, stimulation of the cyclin A promoter in the stable ts13 assay. Why the cyclin A promoter-luciferase reporter is activated in stably transfected but not in transiently transfected ts13 cells is unclear. Because we observe that ts13 cells begin to undergo apoptosis beginning within 24 h after shift to the nonpermissive temperature (data not shown), the differences between the two assay may be due to the different times post-temperature shift that luciferase expression was analyzed. Alternatively, the contrasting results may reflect differences in the copy number of the reporter plasmid between the two assays. Factors that are important for transcriptional regulation present may be titrated out by the multiple copies of the cyclin A promoter in the transient assay. It is of interest that there is no observable increase in the level of cyclin A protein in ts13 cells at the nonpermissive temperature, whereas there is a 3–4-fold increase in cyclin A-luciferase. By comparison, it would be expected that the significant increase in p21 message and protein level following temperature shift would result in a more significant increase in p21-luciferase expression. These results suggest either that an element in the p21 promoter that is important for the observed increase in ts13 cells is not present in the p21 promoter fragment used in the luciferase reporter or that there is also posttranscriptional regulation specifically of
Fig. 4. A, differential regulation of the p21 and cyclin D1 promoters in ts13 cells and in a population of ts13 cells stably transfected with wild-type TAF11250 (ts13-hTAF11250). Populations of ts13 and ts13-hTAF11250 cells stably transfected with the p21 and D1 reporter plasmids were generated. Luciferase activity was measured 16 h after the shift to the nonpermissive temperature and normalized against total protein. B, comparison of cyclin D1 and cyclin A promoters in response to temperature shift in ts13 and ts13-hTAF11250 cells. Columns, results of two separate experiments with two replicates; bars, SE.

p21. However, if there is posttranscriptional regulation of p21, it must be conferred either directly or indirectly by TAF11250. Clearly, though, the level of p21 RNA is significantly increased following temperature shift regulated in part at the level of transcriptional initiation by TAF11250.

These results suggest that TAF11250 may be involved in conferring differential transcriptional regulation of the D1 and p21 promoters, as well as the cyclin A promoter. The observed stimulation of the p21 promoter is mediated by sequences upstream of the TATA box, as well as the TATA box itself. Thus, it is possible that the alterations in the conformation of TFII D, mediated by the ts mutation in TAF11250, affects transcription conferred by specific upstream transcription factors. It is possible that either the activity of a positive factor is stimulated in the context of the p21 promoter and repressed in the context of the D1 promoter. Alternatively, it is possible that the activity of a specific repressor bound to the p21 and/or D1 promoters is affected by the ts mutation in TAF11250. The p21 promoter appears to be a target of both growth and differentiation signal transduction pathways. It is stimulated in response to DNA damage through p53, in response to EGF and IFN-γ through signal transducers and activators of transcription 1, in response to transforming growth factor β1 through Sp1, and in response to muscle differentiation through MyoD.

Mapping experiments are now in progress to map the cis-acting elements that are important for conferring activation of p21 and repression of D1 promoters. As discussed
above, it is possible that at least one of the elements may lie outside the 1.3 kb of the p21 promoter that were used in the luciferase reporter construct. However, rough mapping of the p21 promoter site, which is transcriptionally up-regulated by the ts-TAF250 mutation, places at least one of the regulatory elements between −61 bp (p21 ΔSmα) and −1.3 kb (p21 Δ11.1). The binding sites for several transcription factors and/or signaling pathways involved in regulating p21 transcription have been mapped to this region, including signal transducers and activators of transcription 1, transforming growth factor β, and Sp1 (24–26). Whether the ts-TAF250 mutation affects any of these transcriptional regulatory pathways or whether novel sites are involved is under investigation. Furthermore, whether the sequences around the TATA box in the D1 and/or p21 promoters also contribute to the observed regulation is also unclear, but this question is under investigation.

It is important to note that the effects observed in ts cells at the nonpermissive temperature may not be directly mediated by TAF250. It is possible that other factors are affected by TAF250, which, in turn, differentially affect the D1 and p21 promoters. However, the increase in p21 protein levels and promoter activity have been observed within 6–8 h following temperature shift (data not shown), suggesting that the observed promoter regulation is an immediate, if not direct, effect of TAF250.

Materials and Methods

Plasmid Constructs. The p21 promoter constructs were provided by X-F. Wang (Duke University Medical Center, Durham, NC; Ref. 24); the D1 promoter construct was provided by M. Strauss, Max-Delbrück-Haus, Berlin, Germany; and the cyclin A-luciferase plasmid was provided by E. Wang (University of Washington School of Medicine, Seattle, WA). The promoter-negative, pG-L-based luciferase vector was used as a negative control.

Cell Lines and Transfections. The tk-ts13 were derived from the Syrian hamster fibroblast BHK cell line and have a mutation of the TAF250 gene (27). The tk-ts13 cells were cultured in DMEM with 10% fetal bovine serum at 34°C in 5% CO2. The nonpermissive temperature for ts13 cells (39.6°C) was used to generate stable cell lines expressing the reporter plasmid and wild-type TAF250. Cells were transfected using the calcium phosphate method. ts13 cells were transfected with either the pCMVhtTAF250 (supplied by S. Ruppert, Genentech, San Francisco, CA) and selected at 39.6°C or pSVE hygromycin expression vectors and selected at 34°C in the presence of 400 μg/ml of hygromycin (Boehringer Mannheim). For the temperature-shift experiments, the cells were plated at 2.5 × 105 cells per plate, cultured at 34°C for 24 h, shifted to 39.6°C, and cultured for 16 h prior to cell harvest.

Luciferase Assays. Cells were lysed in 0.25 vol Tris-HCl by three freeze/thaw cycles, and lysates were normalized by protein levels using the Bradford assay. Ten μl of lysate were mixed with luciferase assay buffer [20 mM tricine, 1.07 mM MgCO3·4 Mg(OH)2·5H2O, 1 mM EDTA, 33.3 mM DTT, 270 μM CoA, 470 μM d-Luciferin, and 530 μM ATP] and read on an Autolumat LB953.

Northern Blot Analysis. RNA was isolated by fractionation over cesium chloride gradients as described (28). RNA was resolved on a 1% agarose formaldehyde 3-(N-morpholino)-propanesulfonic acid gel, transferred by capillary action to the nitrocellulose, and UV cross-linked. Hybridization was performed in 5× SSC, 5× Denhardt’s reagent, 50% formamide, 1% SDS, and 100 μg/ml of denatured salmon sperm DNA at 50°C for 16 h.

Western Blot Analysis. The Western analysis was performed using standard techniques. Anti-D2, anti-D3, and anti-p21 antibodies are rabbit monoclonal antibodies, whereas the anti-cyclin A, anti-cyclin E, and anti-cyclin D1 antibodies are mouse monoclonal antibodies (Santa Cruz Biotechnologies). The immune complexes were detected by a secondary antibody and visualized with an ECL reaction kit (Amersham Corp.).

References


