

TFIID component TAF7 functionally interacts with both TFIIF and P-TEFb

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Transcription consists of a series of highly regulated steps: assembly of the preinitiation complex (PIC) at the promoter, initiation, elongation, and termination. PIC assembly is nucleated by TFIID, a complex composed of the TATA-binding protein (TBP) and a series of TBP-associated factors (TAFs). One component, TAF7, is incorporated in the PIC through its interaction with TFIID but is released from TFIID upon transcription initiation. We now report that TAF7 interacts with the transcription factors, TFIIF and P-TEFb, resulting in the inhibition of their Pol II CTD kinase activities. Importantly, in *in vitro* transcription reactions, TAF7 inhibits steps after PIC assembly and formation of the first phosphodiester bonds. Further, *in vivo* TAF7 coelongates with P-TEFb and Pol II downstream of the promoter. We propose a model in which TAF7 contributes to the regulation of the transition from PIC assembly to initiation and elongation.

MHC class I genes | regulation | transcription initiation

In eukaryotic cells, expression of protein-encoding genes depends on the ordered recruitment of the general transcription factors (GTF) TFIID, TFIIB, and TFIIA to the promoter, followed by association of Pol II, the mediator, and the remaining GTFs, TFIIF, TFIIE, and TFIIF, to form a preinitiation complex (PIC) (1). Once PIC assembly is complete, transcription initiation ensues; Pol II with the elongation complex dissociate from the PIC (2, 3). A required step during transcription initiation is the phosphorylation of serine 5 in the carboxy terminal domain (CTD) heptad repeat of Pol II by the kinase subunit of TFIIF, CDK7 (4, 5), after which Pol II pauses to ensure proper pre-mRNA capping (6–9). The transition from pausing to elongation is facilitated by the P-TEFb elongation complex, which also mediates efficient elongation (10). P-TEFb consists of two subunits, cyclin T1 and the kinase CDK9, which phosphorylates serine 2 of the CTD, required for productive elongation and the recruitment of complexes involved in mRNA processing (splicing and polyadenylation) (10–15).

Although the general mechanics of transcription have been characterized, relatively little is known about how the transitions from PIC assembly to initiation/pausing to elongation are regulated. Promoter recognition is largely mediated by TFIID, which is composed of the TATA binding protein (TBP) and over a dozen TBP associated factors (TAFs) (16, 17, 18). The largest TFIID component, TAF1, has both acetyltransferase (AT) and kinase activities (19, 20). We demonstrated that TAF1 and its intrinsic acetyltransferase activity are essential for transcription of an MHC class I gene (21). Importantly, MHC class I transcription is inhibited both *in vitro* and *in vivo* by the viral transactivator, HIV Tat, which binds to the TAF1 AT domain, inhibiting its enzymatic activity (22, 23). TAF7, a cellular 55-kDa TFIID component (24, 25), also binds to TAF1 inhibiting its AT activity and repressing MHC class I transcription (26). Significantly, we have demonstrated that TAF7 remains bound to TAF1/TFIID until PIC assembly is complete, whereupon it is released enabling transcription initiation and elongation (27). Thus, TAF7 is an intrinsic regulator of transcription.

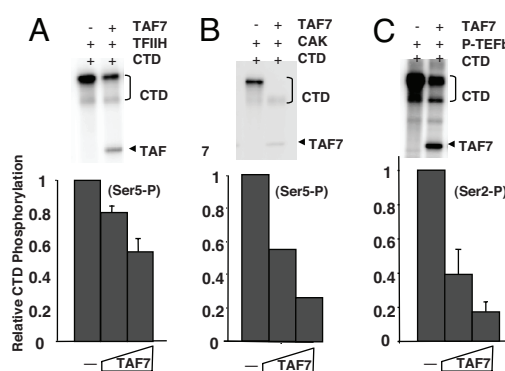


Fig. 1. TAF7 inhibits the kinase activities of TFIIF and P-TEFb. (A) TAF7 inhibits TFIIF-mediated phosphorylation of the Pol II CTD Ser-5. (Upper) Purified TFIIF and 100 ng of GST-tagged Pol II CTD were incubated in the presence or absence of recombinant Flag-tagged TAF7 (F-TAF7) in an *in vitro* kinase assay using [32 P]ATP. Phosphorylation was determined by autoradiography after gel electrophoresis; positions of TAF7 and CTD are indicated based on mobility markers (data not shown). (Lower) Purified TFIIF and 100 ng of GST-tagged Pol II CTD were incubated in the presence of increasing amount of recombinant F-TAF7 (125 and 250 ng), in an *in vitro* kinase assay. Ser-5 CTD phosphorylation was determined by Western blot analysis, using H14 anti-phosphoSer5 antibody, and quantified by densitometry. (B) TAF7 inhibits the CAK-mediated phosphorylation of the Pol II CTD. (Upper) *In vitro* kinase assay with purified CAK, 100 ng of GST-tagged Pol II CTD, and F-TAF7 as in A Upper. (Lower) *In vitro* kinase assay with purified CAK, 100 ng of GST-tagged Pol II CTD, and increasing F-TAF7 as in A Lower. (C) TAF7 inhibits P-TEFb-mediated phosphorylation of the Pol II CTD Ser-2. (Upper) *In vitro* kinase assay with 50 ng of purified P-TEFb, 100 ng of GST-tagged Pol II CTD, and F-TAF7 as in A Upper. (Lower) *In vitro* kinase assay with 50 ng of P-TEFb, 100 ng of GST-tagged Pol II CTD, and increasing F-TAF7 as in A Lower. CTD Ser-2 phosphorylation was determined by Western blot analysis, using H5 anti-phosphoSer2 antibody.

These studies were designed to determine the fate of TAF7 after its dissociation from the PIC. We report that TAF7 functionally interacts with both the general transcription factor TFIIF and the elongation factor P-TEFb. Association of TAF7 with TFIIF inhibits its CDK7 kinase thereby inhibiting TFIIF-mediated phosphorylation of the Pol II CTD Ser-5; binding of TAF7 to the P-TEFb elongation complex inhibits its CDK9-mediated phosphorylation of Pol II CTD Ser-2. Importantly, we show that TAF7 functions *in vitro* to inhibit transcription at steps after PIC assembly and *in vivo* colocalizes with P-TEFb and Pol II downstream of the promoter. Thus, in addition to its role in

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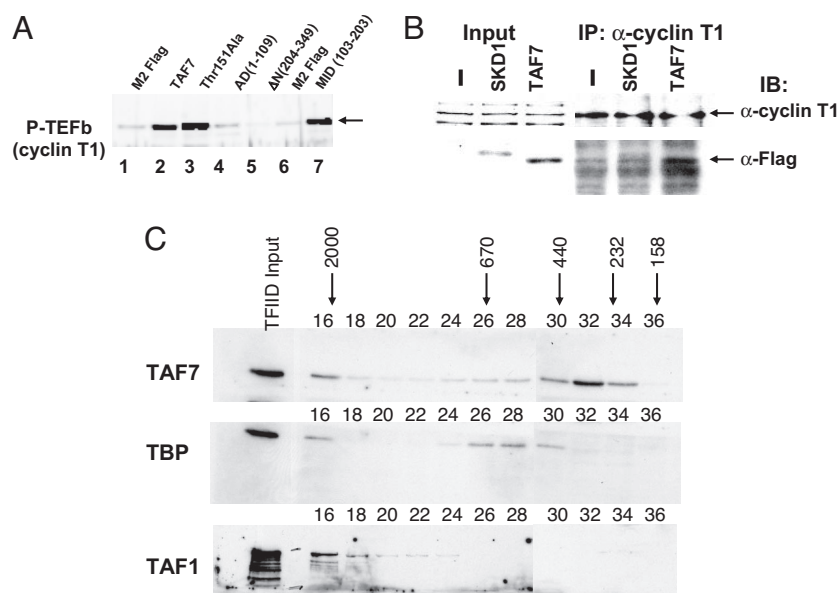


Fig. 2. TAF7 interacts with P-TEFb *in vitro* and *in vivo* and exists in a TFIID-independent complex. (A) TAF7 binds to P-TEFb through its central domain in *in vitro* pull-down assays. Purified recombinant P-TEFb (200 ng) was incubated with F-TAF7 or each of the F-TAF7 mutants immobilized on Sepharose beads. TAF7-bound fractions were assayed for retention of P-TEFb by immunoblotting, using anti-cyclin T1 antibody. (B) TAF7 binds to P-TEFb *in vivo*. Extracts from cells transiently transfected with either F-TAF7, pcDNA3, or Flag-SKD1, an irrelevant control protein, were immunoprecipitated with anti-cyclin T1 antibody. Immunoprecipitates were analyzed for cyclin T1 (Upper) or F-TAF7 (Lower) by Western blot. (Upper) Anti-cyclin T1. Total extract from cells transfected with vector (lane 1), Flag-SKD1 (lane 2), or F-TAF7 (lane 3) before immunoprecipitation and immunoprecipitated cyclin T1 (lanes 4–6). (Lower) Anti-Flag M2 antibody, detection of F-TAF7 coimmunoprecipitated from extracts of cells transfected with vector control (lane 4), Flag-SKD1 (lane 5), or F-TAF7 expression vector (lane 6). (C) TAF7 exists in a TFIID-independent form. C8166 whole cell extracts were fractionated on an FPLC column and fractions analyzed by Western blotting as indicated. Arrows refer to the indicated molecular sizes. Purified TFIID was included as a marker. TFIID consistently appears in the excluded volume in column fractionation of whole cell extracts. Fractions 16–28 and 30–36 were run in parallel but on two separate SDS/PAGES.

transcription initiation as a TFIID component, TAF7 also functions in the transition from PIC assembly to initiation and elongation. We propose a model in which TAF7 regulates the orderly progression of events in transcription, preventing transcription elongation until the steps of transcription initiation are completed and the transcription elongation complex (TEC) is fully assembled.

Results

TAF7 Functionally Interacts with TFIID and P-TEFb and Inhibits Their Phosphorylation of the Pol II CTD. We demonstrated that TAF7 is released from the TFIID complex upon transcription initiation (27). The present studies were designed to determine whether TAF7 associates with other components of the transcription complex after its dissociation from TFIID. Because TAF7 was released from a partially assembled preinitiation complex with TFIIB, TFIIF, and Pol II (27), none of these factors is likely to be a TAF7 interacting partner. Therefore, we considered the possibility that TAF7 functionally interacts with TFIID and/or P-TEFb (7). To assess the functional interaction of TAF7 with TFIID or P-TEFb, its effect on their Pol II CTD kinase activities was determined. TAF7 inhibited the CTD kinase activities of TFIID (Fig. 1A). TFIID consists of a complex of 10 subunits, 3 of which including the CDK7 kinase form a subcomplex called CAK (28). TAF7 inhibited the phosphorylation of the Pol II CTD by the CAK complex, demonstrating that TAF7 acts directly on CAK (Fig. 1B). Remarkably, TAF7 also inhibited P-TEFb phosphorylation of the Pol II Ser-2 CTD (Fig. 1C). In contrast, recombinant CREB has no effect on CDK9 kinase phosphorylation of the CTD (29) [supporting information (SI) Fig. S1D]. Thus, TAF7 functionally interacts with both TFIID and P-TEFb, inhibiting their respective CTD kinase activities.

Because both TFIID and P-TEFb also phosphorylate TAF7 (Fig. 1), we considered the possibility that TAF7 acts as a

competitive inhibitor. However, phosphorylation of SPT5, another P-TEFb substrate (30), is enhanced in the presence of TAF7 (Fig. S1A). Furthermore, TAF7 does not inhibit the activity of CDK2, another CDK family member (Fig. S1B). Finally, neither CTD nor TAF7 alone has any kinase activity (Fig. S1C). Thus, TAF7 inhibition of TFIID and P-TEFb CTD kinase activities is specific. Thus, TAF7 regulates TAF1 acetyl transferase activity and function and the CTD kinase activities of both TFIID and P-TEFb.

TAF7 Physically Interacts with P-TEFb. The experiments described above demonstrate a functional association between TAF7 and both TFIID and P-TEFb. To examine the physical interactions, we performed *in vitro* pull-down assays with recombinant Flag-tagged TAF7 and either purified P-TEFb or purified TFIID. A weak association of TAF7 with the CAK subunit of TFIID was observed in the pull-down assay (data not shown). TAF7 efficiently recovered P-TEFb as revealed by anti-cyclin T1 immunoblotting, indicating that TAF7 stably interacts with P-TEFb *in vitro* (Fig. 2A; compare lanes 1 and 2). Similar results were obtained in an anti-CDK9 immunoblot (data not shown). The interaction domain of TAF7 with P-TEFb was mapped using a set of deletion mutants to the central 103- to 203-aa segment of TAF7 [MID (103–203)] (Fig. 2A, compare lanes 6 and 7), which is within the same domain that interacts with TAF1 (24). However, mutation of Thr-151 in the TAF1 binding domain (24) has no effect on TAF7 binding to P-TEFb. Thus, TAF7 binds P-TEFb *in vitro*.

The *in vivo* interaction of TAF7 with P-TEFb was determined by immunoprecipitation after transfection of Flag-TAF7 into 293 cells. Consistent with the *in vitro* findings, immunoprecipitation of endogenous cyclin T1 coimmunoprecipitated TAF7 from Flag-TAF7-transfected 293 extracts but not control protein from control extracts (Fig. 2B Lower, lanes 5 and 6).

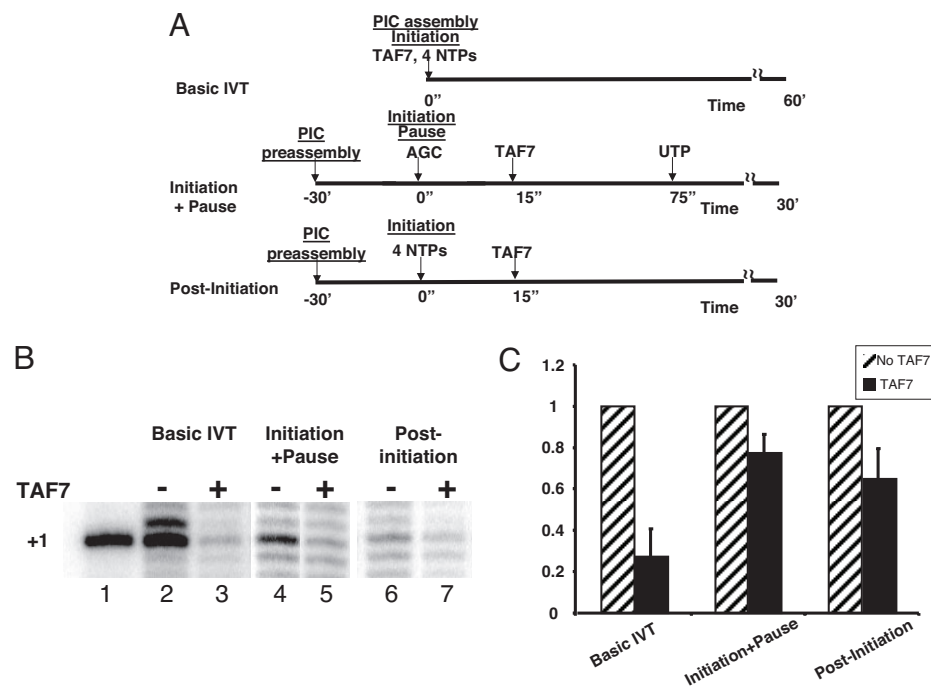


Fig. 3. TAF7 regulates multiple distinct steps in transcription initiation. (A) Scheme of experimental protocol. Basic IVT, TAF7, and the four NTPs are present during PIC assembly and initiation; Initiation+Pause, PIC preassembly occurs in the absence of NTPs or TAF7. TAF7 is added after transcription is initiated with AGC; UTP is added at 75 sec to permit clearance and elongation. Post-Initiation, PIC preassembly occurs in the absence of NTPs or TAF7, which is added after transcription is initiated with all four NTPs. (B) TAF7 regulates *in vitro* transcription. Lane 1, marker; lanes 2–3, HeLa nuclear extract in the presence or absence of purified TAF7 preincubated for 15 min in presence of rNTPs. Transcription was initiated by the addition of the MHC class I –313CAT plasmid. In lanes 4–7, –313CAT DNA and extracts were preincubated for 30 min in presence of 40 μ M ATP to permit PIC assembly. Transcription was initiated by addition of ATP/CTP/GTP mix (lanes 4 and 5) or rNTP (lanes 6 and 7). Control TKEG buffer (–) or purified F-TAF7 (+) were added 15 seconds after initiation of transcription and incubated for 30 min. Analysis of –313CAT transcripts was by primer extension. [In previous studies (27), addition of TAF7 was after promoter clearance so did not affect transcription.] (C) Quantitation of results of three experiments, showing averages and SEM. Addition of TAF7 in each of these conditions results in significant inhibition of transcription: IVT, $P < 0.003$; pause, $P < 0.03$; initiation, $P < 0.04$.

The observed TAF7 inhibition of P-TEFb and TFIID kinase activities predicted that TAF7 exists in a TFIID-independent form *in vivo*. To test this prediction, native cell extracts were fractionated by FPLC gel filtration and assayed for the elution pattern of TAF7, TBP, and TAF1 (Fig. 2C). As expected, TFIID components (TBP, TAF1, and TAF7) were detected in high molecular weight fractions (fractions 16–18); a second peak of TBP was detected in fractions 26–30, corresponding to free TFIID. Importantly, TAF7 eluted in a distinct peak in fractions corresponding to molecular mass between 230 and 440 kDa (fractions 30–32). Thus, TAF7 is found in a form independent of TFIID. Detecting the 55-kDa TAF7 in fractions corresponding to 230 kDa suggests that it is in a complex with other proteins. A TFIID-independent TAF7 fraction was also observed in MOLT4 whole cell extracts (Fig. S2). Whether TAF7 is associated with cyclin T1 or CDK9 or other proteins in the 230-kDa fraction remains to be determined.

These experiments provide the first demonstration that TAF7 exists in at least two distinct forms *in vivo*—a TFIID-independent form and the well known TFIID complex.

TAF7 Regulates Both PIC Assembly and Transcription Initiation *in Vitro*. TAF1, TFIID, and P-TEFb are critical for PIC assembly, transcription initiation, and elongation. We have shown that TAF7 functionally interacts with all three. Because TFIID and P-TEFb are involved in transcription initiation and elongation, we postulated that TAF7 functions in the regulation of transcription at steps after PIC assembly. To address this question, *in vitro* transcription assays were performed in which TAF7 was added at one of three times: before PIC assembly, at transcrip-

tion initiation, or after initiation (schematized in Fig. 3A). The presence of TAF7 during the entire *in vitro* transcription assay is predicted to block transcription at all steps of transcription initiation: inhibition of TAF1 AT activity and of TFIID and P-TEFb kinase activities (Basic IVT). Indeed, addition of TAF7 before PIC assembly significantly reduced transcription (Fig. 3B, basic IVT, lanes 2 and 3). Transcriptional repression in the presence of TAF7 is specific; addition of irrelevant proteins to the *in vitro* transcription reaction had no effect (Fig. S3).

The effect of TAF7 on transcription after initiation was determined as follows: After PIC assembly, transcription was initiated by the addition of ATP, GTP, and CTP to allow the incorporation of the first three nucleotides in the nascent RNA and pausing of Pol II. (The sequence at the transcription start site is gctcAGCTTCT; transcription initiates at A). Then, TAF7 was added, followed by UTP to allow promoter clearance and elongation (initiation plus pause). Consistent with a role for TAF7 after initiation of transcription, TAF7 significantly reduced transcription even when added after phosphodiester bond formation (Fig. 3B, Initiation+Pause, lanes 4 and 5). Finally, to examine the effect of TAF7 after promoter clearance, transcription was initiated with the 4NTPs after PIC assembly, followed by addition of TAF7 (Fig. 3A, Post-Initiation). Even added after PIC assembly and promoter clearance, TAF7 is still able to significantly reduce transcription (Fig. 3B, lanes 6 and 7). The results of three independent experiments are summarized in Fig. 3C. The effects of TAF7 after transcription initiation, although modest, are reproducible and significant. Thus, TAF7 functionally targets components of the transcription machinery other than TAF1/TFIID, consistent with a functional role for its inhibition of the kinase activities of TFIID and P-TEFb.

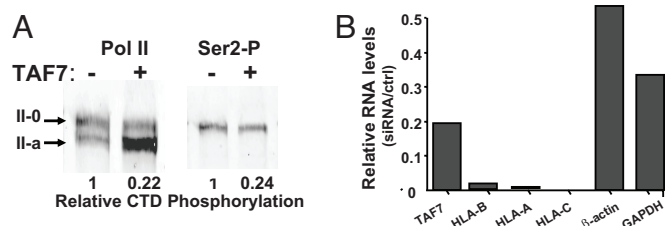


Fig. 4. TAF7 regulates promoter activity *in vivo*. (A) TAF7 inhibits Pol II CTD phosphorylation *in vivo*. Nuclear extracts from either 293T-TAF7 (+) or control 293T (-) cells were analyzed by Western blotting with either an antibody specific for the Ser-2 CTD phosphorylated form of pol II (Right) or an antibody reactive with all forms of Pol II CTD (Left). (Quantitation) (Left) Phosphorylated Pol II (II-0) is expressed relative to nonphosphorylated Pol II (II-a). (Right) The amount of Ser-2-P is expressed relative to the total amount of Pol II in the same sample (Left). (B) Depletion of TAF7 results in reduced levels of endogenous MHC class I RNA. Human 293T cells were transiently transfected with TAF7 siRNA or scrambled siRNA, as described; levels of TAF7, HLA class I RNAs, actin, and GAPDH RNAs were determined by real time PCR. Results are presented relative to control siRNA.

TAF7 Regulates Promoter Activity *in Vivo*. The biological implications of TAF7 inhibition of Pol II CTD phosphorylation were examined *in vivo* in 293T cells stably transfected with TAF7 (293/F-TAF7). Both TAF7 protein and RNA levels were increased in the 293/F-TAF7 cells relative to control 293 cells (Fig. S4 A and B). Importantly, the fraction of total phosphorylated Pol II species was markedly reduced in the 293/F-TAF7 cells relative to controls (Fig. 4A; compare two left lanes); CTD Ser-2 Pol II was also reduced by 75% in the TAF7-transfected cells (Fig. 4A; compare Ser-2 in Right to total Pol II in Left). These results clearly demonstrate that over-expression of TAF7 leads to inhibition of CTD phosphorylation *in vivo* and *in vitro*.

Because TAF7 regulates the functions of TAF1, TFIIF and P-TEFb and has a major effect on MHC class I expression *in vitro* (Fig. 3) (26, 27), we predicted that both overexpression and underexpression of TAF7 would perturb transcription *in vivo*: Overexpression would sustain inhibition of TAF1, TFIIF, and P-TEFb functions, whereas under-expression would allow premature initiation and promoter clearance, resulting in abortive transcripts. In either case, MHC class I expression would be reduced. Indeed, overexpression of TAF7 decreased MHC class I promoter activity in a transient transfection assay (Fig. S5). To determine whether ablated expression of TAF7 affected transcription, human 293T cells were transiently transfected with TAF7 siRNA, resulting in a reduction of TAF7 RNA to 19% of the control level (Fig. 4B). As predicted, RNA levels of the endogenous MHC class I genes HLA-A,B,C were dramatically reduced after TAF7 siRNA treatment (Fig. 4B); siRNA to a control transcript did not affect HLA-A RNA levels (Fig. S6B). A TAF7 shRNA construct stably introduced into HeLa cells similarly decreased TAF7, HLA-A, and HLA-C RNA levels (Fig. S6). Because the siRNA and shRNA sequences and the transfected cell lines were different, the observed effects of TAF7 knock-down on HLA class I RNA levels are unlikely due to off-target effects. Taken together, these findings demonstrate that TAF7 actively contributes to regulating transcription of MHC class I genes *in vivo*.

Interestingly, the depletion of TAF7 did not result in a global inhibition of transcription, because the levels of actin and GAPDH were only modestly reduced, suggesting that TAF7 regulates a subset of mammalian promoters, consistent with the observation in yeast that loss of TAF7 affects only a subset of genes (31).

TAF7 Plays a Critical Role in Cell Viability and Growth. Because transcription of many cell cycle genes depends on TFIID, we

Table 1. TAF7 deficiency compromises cell growth

Construct	Absence of colonies, % seeded wells	Avg cells per well, no.
Control	4.2	21.8 ± 1.9
shRNA1	22.9	10.6 ± 1.6, $P = 1.4 \times 10^{-4}$
shRNA2	17.8	NT

Human 293 cells, stably transfected with two different TAF7 shRNA retroviral vectors, shRNA1 or shRNA2, or the control vector, were seeded into 100 microtitre wells at an average density of five cells per well. After TAF7 shRNA induction, the number of wells with colonies and the size of each colony were determined by microscopic examination. In the absence of induction of TAF7 shRNA, transfectant growth was comparable to a control cell line transfected with an empty vector (data not shown).

considered the possibility that TAF7 levels may play a critical role in cell viability and growth. As assessed by limiting dilution analysis in HeLa tet-off cells stably transfected with an inducible TAF7 shRNA, TAF7 depletion reduced both cell viability and growth (Table 1). Thus, TAF7 contributes to the regulation of sustained cell viability and growth.

TAF7 Is Associated with Both the PIC and Transcription Initiation/Elongation Complex *in Vivo*. Because TAF7 interacts with P-TEFb, an elongation factor that moves with the elongation complex, we postulated that, *in vivo*, TAF7 would be associated with coding regions of the gene. To test this prediction, we examined the *in situ* localization of TAF7 by *in vivo* ChIP assays of the class I gene in spleen and brain (Fig. 5). These two tissues differ in MHC class I expression by >2 orders of magnitude (32). In the high-expressing spleen, TAF7 was associated with both the promoter and downstream sequences as far as exon 5 (Fig. 5A). The extent of TAF7 association paralleled that of Pol II. In contrast, the transcription factors CREB and c-jun were found only at the promoter (Fig. 5A and data not shown). Consistent with the high level of transcription of the class I gene in spleen, acetylated H4 histone—a marker of active chromatin—was detected throughout the gene. In brain, where class I transcription is barely detectable, the levels of TAF7, Pol II, and acetylated H4 histone were correspondingly low. No TAF7 was detected either to sequences beyond the poly(A) addition site or to sequences 700 bp 5' to the promoter in either tissue (data not shown). Thus, association of TAF7 with the promoter and coding sequences is specific and dependent on active transcription.

To examine the relationship between TAF7 and the elongation complex, the correlation between TAF7 and P-TEFb migration along the gene was determined by ChIP analysis of spleen cells with antibodies to TAF7, cyclin T1, and Pol II. Because the abundance of the elongation complex varies along the gene, the abundances of TAF7 and cyclin T1 were normalized to the level of Pol II association at each point along the gene. This normalization also corrects for differences between the antibodies and efficiency of PCR primers at the various positions within the gene. As shown in Fig. 5B, the relative abundance of TAF7 paralleled that of cyclin T1 through the exon 2/intron 2 junction. Thus, the occurrence of TAF7 within the class I coding sequences correlates with the abundance of Pol II and the level of transcription. From these experiments, we conclude that TAF7 is associated with initiation/elongation complexes *in situ* in tissues.

Discussion

Transcription depends on the temporal and spatial integration of preinitiation complex assembly, initiation, elongation, and termination. Regulation of the transitions from PIC assembly to initiation to elongation is necessary to assure proper rates of productive transcription. Here, we report that the TFIID com-

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