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Histone acetylation-independent transcription stimulation by a histone chaperone

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ABSTRACT

Histone chaperones are thought to be important for maintaining the physiological activity of histones; however, their exact roles are not fully understood. The physiological function of template activating factor (TAF)-I, one of the histone chaperones, also remains unclear; however, its biochemical properties have been well studied. By performing microarray analyses, we found that TAF-I stimulates the transcription of a sub-set of genes. The transcription of endogenous genes that was up-regulated by TAF-I was found to be additively stimulated by histone acetylation. On performing an experiment with a cell line containing a model gene integrated into the chromosome, TAF-I was found to stimulate the model gene transcription in a histone chaperone activity-dependent manner additively with histone acetylation. TAF-I bound to the core histones and remodeled the chromatin structure independent of the N-terminal histone tail and its acetylation level *in vitro*. These results suggest that TAF-I remodel the chromatin structure through its interaction with the core domain of the histones, including the histone fold, and this mechanism is independent of the histone acetylation status.

INTRODUCTION

Chromatin remodeling is a crucial step prior to transcription initiation. Various chromatin regulatory factors have been identified. ATP-dependent chromatin remodeling (ADCR) complexes and histone modification enzymes, such as histone acetyltransferases (HATs) and deacetylases (HDACs) are associated with the dynamic regulation of the chromatin structure and gene activity (1). The cooperative function of these two groups is well documented (2). For instance, SWI/SNF, an ATP-hydrolyzing factor of the ADCR complexes, is recruited to the gene promoter region through the recognition of acetylated histones, and it sequentially promotes alteration of the nucleosome structure (3). In addition to these two groups, the histone chaperone family is also involved in chromatin regulation and histone metabolism (4). *In vitro* studies have demonstrated that histone chaperones bind to core histones and facilitate assembly and disassembly of the nucleosome structure in an ATP-independent manner. Nucleosome assembly protein-1 (NAP-1), one of the best-characterized histone chaperones, facilitates assembly and disassembly of nucleosomes containing both canonical and variant histones *in vitro* (5,6). It is reported that NAP-1 facilitates disassembly of nucleosome in concert with ADCR and histone acetylation *in vitro* (7,8). However, there exists little information on the physiological role of histone chaperones and their cooperative function with ADCR complexes and/or histone modifications.

By using the adenovirus (Ad) chromatin-like genome (Ad core) as a template, we found that template activating factor (TAF)-I, a histone chaperone, acts as a stimulatory factor for *in vitro* adenovirus DNA replication (9). Two isoforms exists, namely, TAF-Iα and TAF-Iβ, and these form homo- or hetero-dimers through their dimerization domain (10). TAF-Iβ is encoded by the putative oncogene *SET* (11). TAF-I is implicated in the regulation of several cellular processes including mRNA stability, cell-cycle regulation, signal transduction and apoptosis (12–16). We demonstrated that TAF-I has histone chaperone activity (17,18). Dimerization and the acidic amino acid cluster are essential for the histone chaperone activity and the stimulation of Ad core DNA replication *in vitro* (10,17,18). TAF-I stimulates the *cell-free* transcription from the cellular-type chromatin template *in vitro* (17). Thus, it is quite possible that TAF-I plays a basic role in the assembly and disassembly of the cellular chromatin structure and the regulation of gene activity. It is indicated that TAF-I is a component of an inhibitor of the histone acetyltransferse complex (INHAT) (19). In addition, TAF-I interferes with the DNA binding of transcription factors, such as Sp1 and KLF5, and thereby represses

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transcription (20,21). In contrast to these repressive roles of TAF-I, it has been demonstrated that TAF-Iβ is involved in the stimulation of *Hoxa9* and *KAI1* gene transcription (22,23). It is also reported that TAF-I augments the transcriptional activity of CBP, a HAT/co-activator protein (24). When considered together, these results suggest that TAF-I may regulate transcription positively or negatively in a gene-specific manner, possibly in concert with histone acetylation. However, the molecular mechanism of the gene-specific function of TAF-I and the functional interaction between TAF-I and histone modifications are not well understood.

Here, we have addressed the mechanism of transcription stimulation by TAF-I in concert with histone acetylation. cDNA microarray analyses demonstrated that TAF-I is involved in the positive or negative transcription regulation of a sub-set of genes. The transcription regulation of genes regulated by TAF-I is independent of the level of histone acetylation. Furthermore, TAF-I stimulated transcription from a model gene is integrated in the chromosome in a histone chaperone activity-dependent manner, but independently of the histone acetylation level. Consistent with this finding, we found that the histone N-terminal tail is not required for the histone chaperone activity of TAF-I.

MATERIALS AND METHODS

Cell cultures

Monolayer cultures of HeLa cells and WT (clone 7) and TAF-I KD (clone 4) HeLa cell lines (25) were maintained at 37°C in the minimal essential medium (MEM; Nissui) containing 10% FBS. CHO-AA8-Luc Tet-Off control cell line (CHO-*luc*) (CLONTECH) was maintained at 37°C in the minimum essential alpha medium (α-MEM; GIBCO) containing 10% FBS. In CHO-*luc* cells, the *luciferase* gene transcription is regulated by a tetracycline-responsive VP16 fusion activator (TetR-VP16) (26). In order to investigate the effect of TAF-Iβ under the condition where the luciferase expression does not reach the saturation level, we set the tetracycline concentration (1 μg/ml) so as to keep the luciferase expression at the basal level.

Plasmid DNAs and recombinant proteins

Plasmid DNAs, pCAGGS, an eukaryotic expression vector (27), pCHA-TAF-Iβ and pCHA-TAF-IβΔC3, were prepared as described previously (22). To generate pCHA-TAF-IβPME for expression of TAF-Iβ containing point mutants in its dimerization domain, a DNA fragment was prepared by digestion of pET-14bTAF-IβPME plasmid DNA (10) with NdeI and BamHI. The cohesive ends of this fragment were treated with Klenow fragment to be blunt and ligated into MluI-digested pCHA, the ends of which had also been filled with Klenow fragment. Hexa-histidine- (His-) tagged TAF-Iβ, TAF-IβΔC3 and TAF-IβPME were prepared as described previously (10).

Antibodies

Antibodies used in this study are as follows: For TAF-Iα and β, mouse anti-TAF-I monoclonal antibody (KM1726) and for TAF-Iβ, mouse monoclonal antibody (KM1720) (28); for experimental control of ChIP, mouse anti-Flag (M2) monoclonal antibody; for Hsp90, rabbit polyclonal antibody [kindly provided by Drs Miyata Y. and Nishida E. (Kyoto University)] (29); for TBP, rabbit polyclonal antibody (Santa Cruz); for HA-tag, rat monoclonal (3F10) antibody (Roche), for histone H3, rabbit polyclonal antibody (abcam); for acetylated histone H3 (K9, K14), rabbit polyclonal antibody (Upatate); for histone H4, rabbit polyclonal antibody (abcam); for acetylated histone H4 (K5), rabbit polyclonal antibody (abcam).

Microarray analyses

Total RNA was isolated from WT and TAF-I KD HeLa cell lines using the guanidine method (30). The concentration of RNA in each sample was determined using a spectrophotometer. The RNA was processed and hybridized to the Code Link™ human whole genome array, according to the manufacture's protocols (GE Healthcare). Chips were scanned and analyzed using Microarray software (arrayWoRx^{e}).

RT–PCR

WT and TAF-I KD HeLa cells were treated without or with a HDAC inhibitor, trichostatin A (TSA) at the concentration of 0.1 μM for indicated periods. cDNA was synthesized from the total RNA (2 μg) using Superscript III reverse transcriptase (RT; Invitrogen) and oligo-dT primer. PCR was performed using synthesized cDNAs and a set of specific primers complementary to human *AKAP12*, *KRT17*, *TFAP2C*, *SERPINI1*, *cTnC*, β-*actin*, and *RPL13A* (31) mRNAs by pre-determined PCR cycles under which PCR products are logarithmically amplified. Primer sequences were as follows: *AKAP12* forward direction, 5′-GGGACCATCACCATCACAG-3′; *AKAP12* reverse direction, 5′-CAGTGAGTAGCTGGACAGTG-3′; *KRT17* forward direction, 5′-GAGGATTGGTTCTTCAGCAAG-3′; *KRT17* reverse direction, 5′-TCACATCCAGCAGGATTTTGTA-3′; *TFAP2C* forward direction, 5′-GGCCCAGCAACTGTGTAAAGA-3′; *TFAP2C* reverse direction, 5′-GCAGTTCTGTATGTTCGTCTCCAA-3′; *SERPINI1* forward direction, 5′-GGATTTTGATGCTGCCACTTATC-3′; *SERPINI1* reverse direction; 5′-TTTGGACTTCACTTTCATCATCTTTAG-3′; *cTnC* forward direction, 5′-GTAGAGCAGCTGACAGAAG-3′; *cTnC* reverse direction, 5′- TTGTCAAACATGCGGAAGAG-3′; β-*actin* forward direction, 5′-ATGGGTCAGAAGGATTCCTATGT-3′; β-*actin* reverse direction, 5′-GGTCATCTTCTCGCGGTT-3′; *RPL13A* forward direction, 5′-CATCGTGGCTAAACAGGTACTG-3′; and *RPL13A* reverse direction, 5′-GCACGACCTTGAGGGCAGCC-3′. The PCR products were separated on a 6% PAGE, visualized by staining with EtBr, and quantified with NIH Image.

Luciferase assay

CHO-*luc* cells transfected with plasmid DNAs where indicated were washed with phosphate-buffered saline (PBS) and lysed in a cell lysis buffer [25 mM Tris–HCl (pH 7.9), 10% Glycerol and 0.1% Triton X-100] by five freezing-thawing cycles. The cell lysates and a luciferase substrate (Promega) were mixed, and the luciferse activity was measured by Lumat LB9506 (BERTHOLD). Transfection

was performed using TransIT LT1 transfection reagent (Mirus).

Chromatin immunoprecipitation (ChIP)

HeLa cells were fixed with 1% formaldehyde at room temperature for 10 min, and ChIP was carried out essentially according to the protocol from Upstate Biotechnology using antibodies where described. The recovered DNA was amplified by PCR with the primer sets. Primer sequences used were as follows: β-*actin* promoter forward direction, 5′-ATGCTGCACTGTCGGCGAAG-3′; β-*actin* promoter reverse direction, 5′-ATCGGCAAAGGCGAGGCTCT-3′; *cTnC* promoter forward direction, 5′-TATGAGCTTGGCATTCGAGGCTT-3′; and, *cTnC* promoter reverse direction, 5′-TGACCTCCACTTTCCAGATAAGG-3′. Transfected CHO-*luc* cells were fixed with 1% formaldehyde at room temperature for 10 min, and ChIP was carried out. A primer set, 5′-CTCGGTACCCGGGTCGAGTAG-3′ and 5′-CCGCGGAGGCTGGATCGGT-3′, was used to amplify the human cytomegarovirus (HCMV) minimal promoter region which directs transcription of the integrated *luciferase* gene. The PCR products were separated on a 6% PAGE, visualized by staining with EtBr, and quantified with NIH Image.

Chromatin reconstitution and micrococcal nuclease (MNase) digestion assay

Chromatin was reconstituted on a plasmid DNA, pUC119-ML2, with core histones prepared from mock-treated cells, hyperacetylated core histones prepared from cells treated with sodium n-butyrate (NaBu), a HDAC inhibitor (32), and tail-less core histones by the salt dialysis method as described previously (17). To generate pUC119-ML2, a DNA fragment containing the region between nucleotide position 4979 and 6242 (where the left terminus of adenovirus type 2 DNA is defined as position 1) was excised from pSmaF (33) by digestion with PstI and HindIII and cloned into PstI and HindIII-digested pUC119. Reconstituted chromatin (50 ng DNA) was subjected to partial digestion with MNase in a digestion buffer [10 mM Tris–HCl (pH 7.9), 70 mM NaCl, 3 mM $CaCl_2$ and 1.5 mM $MgCl_2$] at 37°C. Digested DNA was purified, separated by 1.4% agarose gel electrophoresis, and visualized by SYBR Gold (Molecular probe) staining.

RESULTS

Involvement of TAF-I in the transcription regulation *in vivo*

It has been reported that TAF-I is involved in the expression of various genes. We attempted to confirm this using the microarray technique and TAF-I knock-down (KD) cells. Previously, we had established stable HeLa cell lines in which the expression of TAF-I was knocked down by siRNA (25). Here, we used one of the TAF-I KD cell lines, in which the expression levels of the proteins TAF-Iα and TAF-Iβ were reduced to ∼10% relative to that of the wild-type cell (WT) (Figure 1A). Comparison of the gene expression profile of TAF-I KD with that of WT cells by microarray analysis indicated that several genes are down-regulated or up-regulated due to the reduction of the expression level of TAF-I, suggesting that TAF-I stimulates and represses subsets of genes. Among the 29 172 array probes that we tested, we detected 106 probes that showed more than a 2-fold down-regulation in the signal and 48 probes that showed more than a 2-fold up-regulation in the signal. We selected genes that were well annotated, and summarized the results (Supplementary Table 1). We confirmed the mRNA expression level of some of the genes by using semi-quantitative RT–PCR (Figure 1B, and data not shown). Figure 1B shows the RT–PCR result which indicates that *AKAP12* and *KRT17* are the down-regulated genes and *TFAP2C* and *SERPINI1* are the up-regulated genes. This suggests that the alteration pattern of these genes matches well with the results of the microarray analysis. To study the positive function of TAF-I, we decided to focus on certain down-regulated genes in the TAF-I KD cells. Among the genes examined so far, the *slow/cardiac troponin C* (*cTnC*) gene (34) was observed to be one of the typical genes whose expression level was drastically reduced in the TAF-I KD cells (Figure 1C). To examine whether TAF-Iβ is associated with the *cTnC* gene promoter, we performed ChIP assays by using an antibody against TAF-Iβ (Figure 1D). TAF-Iβ interacted with the *cTnC* promoter but not the β-*actin* promoter region, suggesting that TAF-I regulates the *cTnC* gene expression at the level of transcription by binding to the promoter region.

TAF-I stimulates the model gene transcription in a histone chaperone activity-dependent manner

By microarray analyses, we found that TAF-I stimulates the transcription of a sub-set of genes, such as *cTnC*. Further, to quantitatively analyze the effect of TAF-I on the transcription from the chromatin template, we exploited the properties of the cell line CHO-*luciferase* (CHO-*luc*); in CHO-*luc* the model reporter gene is integrated in the chromosome such that the *luciferase* gene transcription is regulated in the context of the chromatin structure. By using this system, we found that the luciferase expression is stimulated by TAF-Iβ in a dose-dependent manner (Figure 2A and B). We did not detect the TAF-I-dependent transcription stimulation when the luciferase reporter plasmid was transiently co-transfected with a TAF-I expression vector (data not shown), indicating that TAF-I-dependent stimulation is specific for the gene integrated in the chromosome.

To examine whether this stimulatory effect of TAF-I is dependent on its histone chaperone activity, we prepared histone chaperone activity-deficient TAF-Iβ mutants (Figure 2A–C). Since the acidic amino acid cluster and dimerization of TAF-I are essential for the remodeling of the Ad core and histone chaperone activity, we used two mutants -one lacking the C-terminal acidic amino acid cluster region, termed TAF-IβΔC3, and the other containing point mutations in the coiled-coil dimerization domain, termed TAF-IβPME (10,11). The acidic amino acid cluster is essential for the INHAT activity, while dimerization of TAF-Iβ is not completely required (19). In contrast, neither the acidic amino acid cluster nor the dimer formation is essential for the I_2PP2A activity of TAF-Iβ (16). These suggest that the dimerization domain is crucial for the maximal chaperone

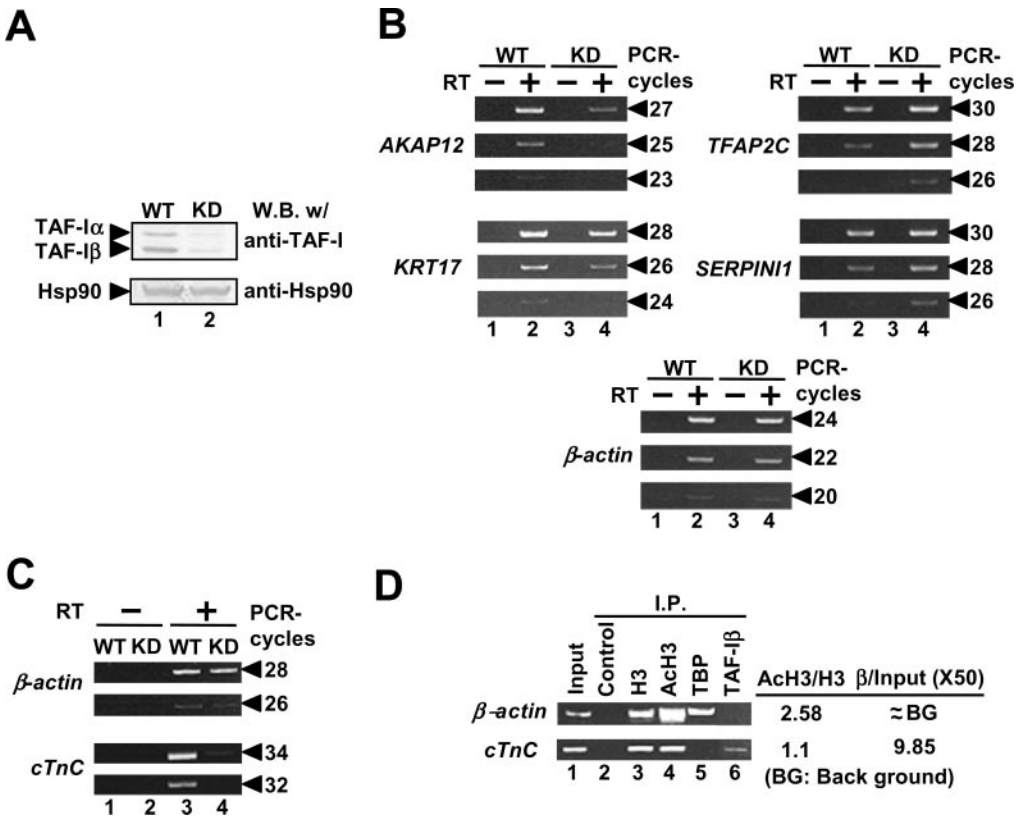

Figure 1. Involvment of TAF-I in the *cTnC* transcription in HeLa cells. (**A**) The level of TAF-I in TAF-I KD HeLa cell lines. Cell extracts prepared from WT (clone 7) and TAF-I KD (clone 4) cells were separated by a 10% SDS–PAGE, and TAF-I proteins and Hsp90 for a loading control were detected by immunoblotting with anti-TAF-I (KM1726) (upper panel) and anti-Hsp90 (lower panel) antibodies, respectively. (**B**) Semi-quantitative RT–PCR for endogenous genes. Total RNA was isolated from WT and TAF-I KD cell lines and analyzed by RT–PCR for semi-quantitative detection of transcripts from the β-*actin*, *AKAP12*, *KRT17*, *TFAP2C* and *SERPINI1* genes. cDNAs were synthesized without (lanes 1 and 3) and with (lanes 2 and 4) the reverse transcription. PCR products amplified by 23, 25 and 27 PCR cycles for the *AKAP12*, 24, 26 and 28 PCR cycles for the *KRT17*, 26, 28 and 30 PCR cycles for the *TFAP2C* and the *SERPINI1*, and 20, 22 and 24 PCR cycles for the β-*actin*, respectively, were loaded onto a 6% polyacrylamide gel. After separation, PCR products were visualized by staining with EtBr. (**C**) Semi-quantitative RT–PCR of *cTnC* mRNA. Total RNA was isolated from WT and TAF-I KD cell lines and analyzed by RT–PCR for semi-quantitative detection of transcripts from the β-*actin* and *cTnC* genes. cDNAs were synthesized without (lanes 1 and 2) and with (lanes 3 and 4) the reverse transcription. PCR products amplified by 26 and 28 PCR cycles for the β-*actin*, and 32 and 34 PCR cycles for the *cTnC*, respectively, were loaded onto a 6% polyacrylamide gel. After separation, PCR products were visualized by staining with EtBr. The *cTnC* gene is mainly expressed and functions in cardiac ventricle and slow skeletal muscle. The *cTnC* gene is also expressed but at the low level in HeLa cells, in which neither a biological meaning nor a regulatory mechanism of the *cTnC* gene expression is yet known. (**D**) ChIP assay. Association of TAF-Iβ with the *cTnC* gene in HeLa cells was examined by ChIP assays. ChIP assays were performed with indicated antibodies. The anti-Flag tag antibody was used as a non-specific control antibody. Immunoprecipitated DNA was amplified with PCR using a set of primers specific for the β-*actin* and the *cTnC* promoter regions. PCR products were analyzed by a 6% polyacrylamide gel, and visualized by staining with EtBr. Input DNA was purified from 1/50 of cell lysates used for immunoprecipitation. The band intensity was quantitatively measured with NIH image, and ratios (AcH3/H3 and TAF-Iβ/Input) are shown. BG indicates that no differences were observed between values obtained with control antibody and those with anti-TAF-Iβ antibody.

activity but not essential for the INHAT and the I_2PP2A activities. In contrast to TAF-Iβ WT (wild-type), neither TAF-IβΔC3 nor TAF-IβPME stimulated luciferase expression, suggesting that the stimulation of the *luciferase* gene transcription is dependent on the histone chaperone activity of TAF-Iβ. Next, we examined the chromatin remodeling activity of TAF-Iβ, and compared this activity of TAF-Iβ WT with that of each TAF-Iβ mutant by using the nuclease sensitivity assay that employed recombinant His-tagged TAF-Iβ derivatives (Figure 2D and E) (10). The chromatin structure was reconstituted using purified cellular core histones on a plasmid DNA *in vitro* (17). After incubation with each TAF-Iβ protein, the reconstituted chromatin was subjected to nuclease sensitivity assays by using micrococcal nuclease (MNase). While the reconstituted chromatin was highly insensitive to digestion by MNase, TAF-Iβ WT enhanced the nuclease sensitivity of the reconstituted chromatin (Figure 2E, compare lanes 2–4 and 5–7); this was in good agreement with the previous report, which stated that TAF-Iβ WT efficiently remodels the chromatin structure (17). In contrast, the nuclease sensitivity of chromatin was slightly induced by TAF-IβPME (Figure 2E, compare lanes 5–7 and 11–13). The nuclease sensitivity was not significantly induced by TAF-IβΔC3 (Figure 2E, lanes 8–10), while TAF-IβΔC3 showed slightly higher activity than TAF-Iβ PME. These results suggest that the chromatin remodeling activity of TAF-Iβ is dependent on both dimer formation and the acidic cluster region. It is likely that the hydrophilic surface of the dimerized coiled-coil region has a slight chromatin remodeling activity as shown by the experiments using the Ad core (35). When these results are considered together, it may be suggested that TAF-Iβ

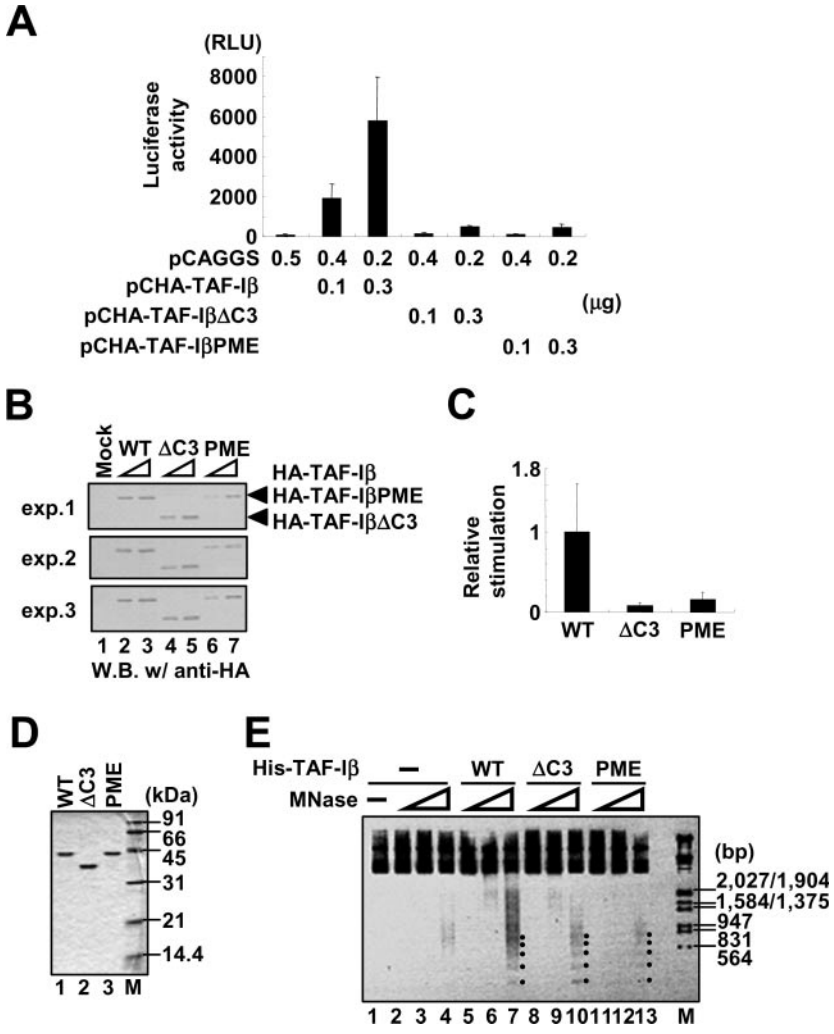

Figure 2. Stimulation of the model gene transcription by TAF-I in the histone chaperone activity-dependent manner. (**A**) Luciferase assays. CHO-*luc* cells were transiently transfected with indicated amounts of pCAGGS, pCHA-TAF-Iβ, pCHA-TAF-IβΔC3 and pCHA-TAF-IβPME, incubated in the presence of 1 μg/ml tetracycline, and collected at 48 h post transfection. pCHA vector (28) was generated from a control vector by pCAGGS (27) so as to express an HA-tagged protein. Cell lysates were prepared, and the luciferse expression was examined. The protein concentration of cell lysates was determined by the Bradford method, and the luciferase activity was normalized by the protein concentration. Results are represented as mean values ± SDs from three independent experiments. (**B**) The expression level of each HA-tagged TAF-I derivatives. Cell extracts (total amount of protein; 1.5 μg) prepared from CHO-*luc* cells transfected with each amount of DNA for expression of TAF-I mutants [see (A), under the graph] were separated by a 10% SDS–PAGE, and each HA-tagged TAF-I protein was detected by immunoblotting with anti-HA antibodies. Results of three independent experiments are shown. (**C**) Representation of relative transcription stimulatory activities of TAF-Iβ derivatives. The band intensity of each TAF-I derivatives (B) were quantitatively measured with NIH image. The luciferase activity (A) was normalized by each protein expression level, and the relative activity is summarized, where the average of experiments using the TAF-Iβ WT is set as 1. Results are represented as mean values ± SDs from three independent experiments. (**D**) Purified recombinant proteins. Recombinant His-tagged TAF-Iβ (WT), TAF-IβΔC3 (ΔC3), TAF-IβPME (PME) (400 ng each) generated in the bacterial expression system were purified according to the manufacture's instruction, separated by a 15% SDS–PAGE, and visualized by staining with Coomassie brilliant blue. Lane M shows molecular weight markers. (**E**) MNase sensitivity assay. Reconstituted chromatin (50 ng DNA) was incubated at 30°C for 30 min without (lanes 1–4) or with His-tagged TAF-Iβ (1.2 μg, lanes 5–7), His-tagged TAF-IβΔC3 (960 ng, lanes 8–10) and His-tagged TAF-IβPME (1.2 μg, lanes 11–13). The mixture was then subjected to partial digestion without (lane 1) or with MNase (0.0003 U for lanes 2, 5, 8 and 11, 0.0015 U for lanes 3, 6, 9 and 12, 0.0075 U for lanes 4, 7, 10 and 13) at 37°C for 6 min. DNAs were purified, separated by electrophoresis on a 1.4% agarose gel and visualized by SYBR Gold staining. Lane M shows DNA size markers. The nucleosome-derived DNA ladders are shown by bullets.

remodels the chromatin structure of the model gene and stimulates its transcription in CHO-*luc* cells.

Next, to determine whether over-expression of TAF-Iβ affects histone acetylation around the model gene promoter region, we examined the histone acetylation level in the model gene promoter region by ChIP assays. In CHO-*luc* cells that over-express each TAF-Iβ derivative, the amounts and the acetylation levels of histone H3 and H4 were not significantly altered (Figure 3A). These results indicate that TAF-Iβ does not affect the histone acetylation of the model gene promoter. Next, to determine whether TAF-Iβ is associated with the model gene promoter region, we carried out ChIP assays using a TAF-Iβ antibody (Figure 3B). We normalized the level of DNA that co-immunoprecipitated with each TAF-Iβ mutant by estimating the expression level of each protein (Figure 3C and D). TAF-Iβ WT was associated with the model gene promoter, while TAF-IβPME showed lower efficiency with regard to this association as

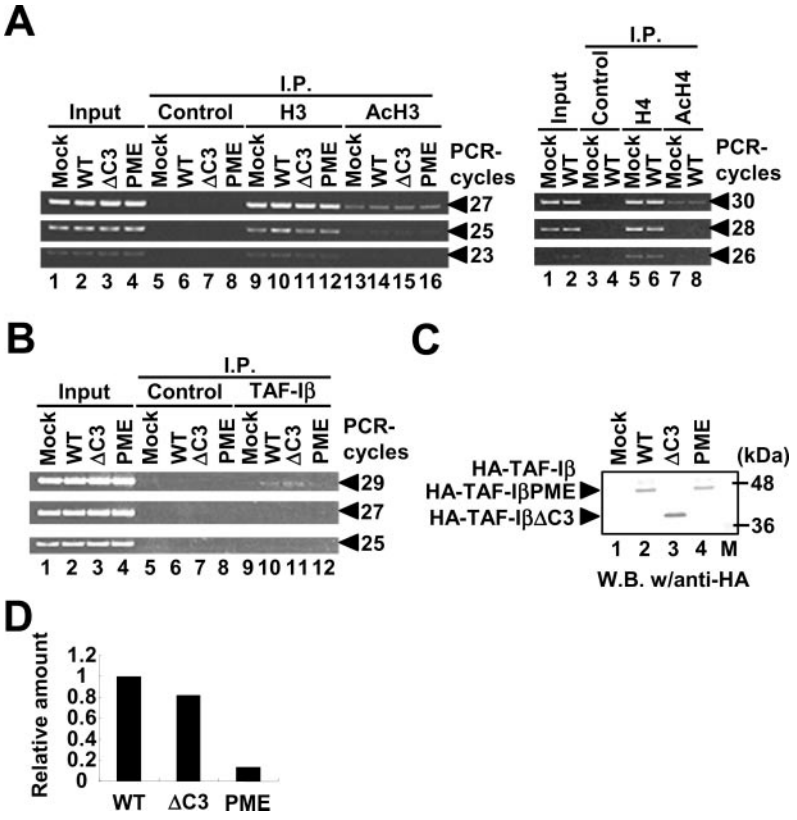

Figure 3. Association of TAF-I with the model gene promoter. (**A** and **B**) ChIP assays. CHO-*luc* cells were transfected with 10 μg of pCAGGS (Mock), pCHA-TAF-Iβ (WT), pCHA-TAF-IβΔC3 (ΔC3) and pCHA-TAF-IβPME (PME) plasmids. Cells were fixed with 1% formaldehyde at 48 h post transfection. Cell lysates were prepared, and immunoprecipitation was performed with indicated antibodies. DNA was purified, and the HCMV minimal promoter region of the *luciferase* gene was amplified by PCR using immunoprecipitated DNA as a template. PCR products amplified by 23, 25, 27 and 29 PCR cycles [(A); left panel and (B)], and 26, 28 and 30 PCR cycles [(A); right panel] were analyzed by a 6% polyacrylamide gel and visualized by staining with EtBr. Input DNA was purified from 1/50 of cell lysates used for immunoprecipitation. (**C**) The expression level of each HA-tagged TAF-I mutant protein. Cell extracts (1/150 of the input for ChIP assay) were separated by a 10% SDS–PAGE, and each HA-tagged TAF-I protein was detected by immunoblotting with anti-HA antibodies. Lane M shows molecular weight markers. (**D**) Representation of the relative chromatin binding activity of TAF-Iβ derivatives. The band intensity of each TAF-I derivatives (C) was quantitatively measured with NIH image. The level of DNA co-immunoprecipitated with each TAF-I derivatives (B) was normalized by each protein expression level, and the relative activity is summarized, where the average of experiments using TAF-Iβ WT is set as 1.

compared to WT (Figure 3B, lanes 10 and 12, and D). TAF-IβΔC3 was also associated with the model gene promoter with lower efficiency than TAF-IβWT (Figure 3B, lanes 10 and 11, and D). These results suggest that dimmer formation but not the acidic amino acid cluster of TAF-Iβ is essential for its association with chromatin. It is interesting to note that the acidic cluster is not required for the chromatin binding of TAF-I. In other words, the hydrophilic surface of the dimerization domain would be important for the chromatin binding of TAF-I. Nevertheless, it is concluded that the histone chaperone/chromatin remodeling activity of TAF-I is important for stimulation of the transcription of the model gene.

TAF-I and histone acetylation additively stimulates the transcription

Next, we examined the effect of histone acetylation on the model gene transcription stimulated by TAF-Iβ (Figure 4). CHO-*luc* cells expressing TAF-Iβ WT were incubated without or with increasing concentrations of TSA, a class I HDAC inhibitor (36). The luciferase expression was stimulated by the over-expressed TAF-Iβ (1st and 2nd bars). The luciferase expression was slightly stimulated by the addition of 3 nM of TSA (1st and 3rd bars), significantly stimulated in combination with over-expressed TAF-Iβ (1st–4th bars), and greatly stimulated by the addition of 10 nM of TSA (1st and 5th bars). Under this condition however the over-expressed TAF-Iβ was not stimulatory (5th and 6th bars). The expression levels of both endogenous and exogenous TAF-Iβ were not affected by TSA treatment (Figure 4, upper panel). These results indicate that the model gene transcription is stimulated by TAF-Iβ additively or slightly more than additively with histone acetylation when the basal transcription level of the model gene remains at a low level. The stimulatory effect of TAF-Iβ decreased when the model gene transcription was sufficiently stimulated by a high level of histone acetylation or the transcription activator TetR-VP16 (data not shown). Based on these results, it is presumed that TAF-I is associated with genes expressed at an intrinsically low level. This possibility is supported by the observation of the endogenous *cTnC* gene (Figure 1D), since the transcription of the *cTnC* gene is maintained at a low level in HeLa cells as compared with that in the cardiac ventricle and slow skeletal muscle cells (34). We compared the acetylation level of histone H3 and the amount of TBP (TATA-binding protein) at the *cTnC* promoter with those at

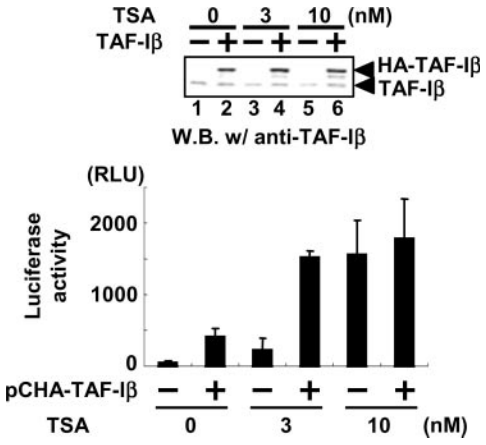

Figure 4. Effects of over-expressed TAF-Iβ and TSA. CHO-*luc* cells were transiently transfected with 0.5 μg of pCAGGS (−) or a combination of 0.4 μg of pCAGGS and 0.1 μg of pCHA-TAF-Iβ (+). After the addition of TSA at 24 h post transfection, cells were incubated further for 24 h, and the luciferase expression was examined. The luciferase activity was normalized by the protein concentration. Results are represented as mean values ± SD from three independent experiments (lower panel). The level of endogenous and exogenous TAF-Iβ was examined with western blotting using an anti-TAF-Iβ antibody (upper panel).

the β-*actin* promoter using ChIP assays. The results of ChIP assays clearly demonstrated that both the acetylation level of histone H3 and the amount of TBP at the *cTnC* promoter are lower than those of the β-*actin* promoter (Figure 1D). These results suggest that the transcription of the *cTnC* gene ossurs at a basal level, and TAF-I may be involved in the basal transcription step.

To confirm this finding using the model gene, we examined the effect of histone acetylation induced by TSA on the transcription level of the endogenous *cTnC* gene in TAF-I KD cells. If TAF-I functions as INHAT on the *cTnC* gene, TSA may restore the *cTnC* transcription level in the KD cells more efficiently than that in the WT cells. In contrast, if TAF-I positively affects the HAT function, TSA may restore the *cTnC* transcription level in the WT cells more efficiently than that in the KD cells. Total RNA was prepared from the cells incubated with TSA and subjected to semi-quantitative RT–PCR (Figure 5A), and the results are summarized in Figure 5B. In both WT and TAF-I KD cells, the *cTnC* transcription was up-regulated during the incubation periods in the presence of TSA, whereas the *ribosomal protein L13A* (*RPL13A*) gene transcription remained unaltered (31). The transcription level of the *cTnC* in the WT cells was higher than that in the TAF-I KD cells at all time points, suggesting that TAF-I functions positively in the *cTnC* transcription. Furthermore, the stimulation profiles of both WT and TAF-I KD cells were parallel, suggesting that the *cTnC* gene transcription is stimulated by TAF-I additively with histone acetylation.

Association of TAF-Iβ with chromatin independent of the histone acetylation level *in vivo*

It has been reported that TAF-Iβ represses the *EB1* gene transcription in a manner similar to INHAT (37). In this gene regulation, the association of TAF-Iβ with the promoter region is repressed by histone acetylation. By using the model

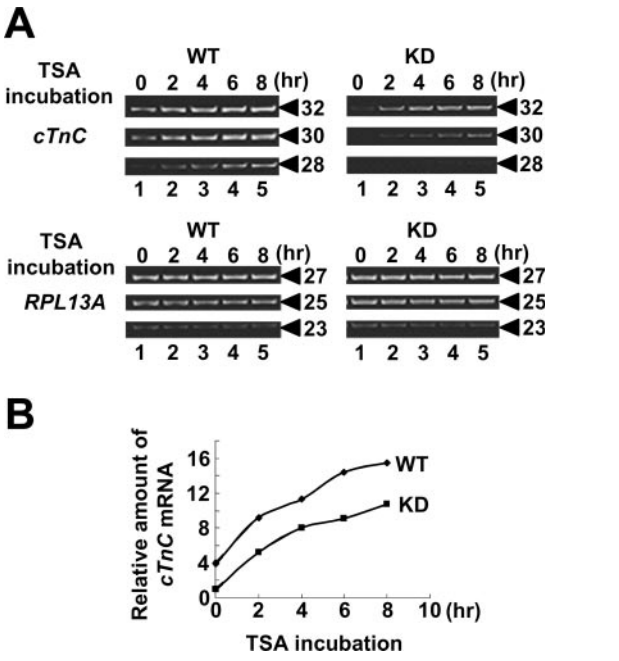

Figure 5. Time course-dependent increase of the level of the *cTnC* mRNA by TSA in WT and TAF-I KD cells. (**A**) WT and TAF-I KD cells were treated with 0.1 μM of TSA and collected cells at indicated time points. Total RNA was purified and analyzed by RT–PCR for semi-quantitative detection of transcripts from the *cTnC* and the *RPL13A* genes. PCR products amplified by 28, 30 and 32 PCR cycles for *cTnC*, and 23, 25 and 27 PCR cycles for *RPL13A*, respectively, were analyzed by a 6% polyacrylamide gel and visualized by staining with EtBr. (**B**) The band intensity of the *cTnC* was quantitatively measured with NIH image, and the relative average intensity for the *cTnC* is summarized, where the level of the *cTnC*-derived RT–PCR product in TAF-I KD at 0 h is set as 1.

cell line, we have demonstrated that the transcription of the model gene is stimulated by TAF-Iβ additively with histone acetylation. Since TAF-Iβ stimulated the model gene transcription independent of the level of histone acetylation, there is a high-possibility that the association of TAF-Iβ with the model gene is not affected by histone acetylation. To confirm this, we examined the amount of TAF-Iβ associated with the model gene promoter under various levels of histone acetylation. By using antibodies against TAF-Iβ and acetylated histone H3, we performed ChIP assays with CHO-*luc* cells that over-express TAF-Iβ WT and were treated with TSA (Figure 6). The ChIP assays showed that the amount of DNA co-immunoprecipitated with an antibody against the acetylated histone H3 increased gradually with the incubation periods in the presence of TSA (upper panel). This indicates that the histones associated with the model gene promoter are acetylated as a function of the incubation time with TSA. In contrast, the amount of DNA co-immunoprecipitated with an antibody against TAF-Iβ remained unaltered (lower panel). These results suggest that TAF-Iβ is associated with the model gene promoter independent of the histone acetylation level.

Histone tail and the acetylation-independent histone chaperone activity of TAF-I

Next, we examined whether acetylation of the histone N-terminal tail affects the histone binding activity of TAF-Iβ

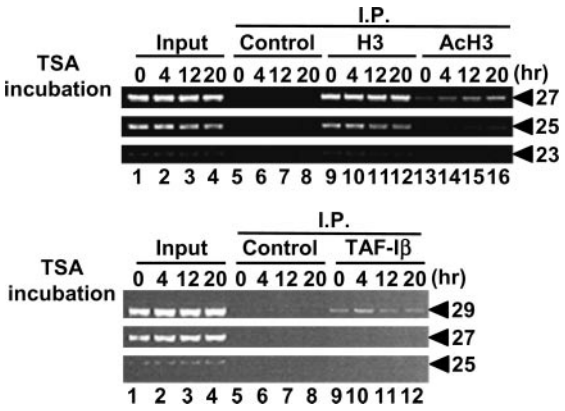

Figure 6. Effects of the histone acetylation level on associations of TAF-Iβ with the model gene chromatin. Cells were transfected with pCHA-TAF-Iβ and incubated for 48 h. Before collecting cells, cells were treated with 0.1 μM of TSA for 0, 4, 12 and 20 h. ChIP assays were performed with indicated antibodies. The HCMV minimal promoter region of the *luciferase* transgene was amplified by PCR using immunoprecipitated DNA as template. PCR products amplified by 23, 25, 27 and 29 PCR cycles (right of the panel) were analyzed by a 6% polyacrylamide gel and visualized by staining with EtBr. Input DNA was purified from 1/50 of cell lysates used for immunoprecipitation.

in *in vitro* immunoprecipitation assays (Figure 7B). To verify this, we prepared core histones (38) from mock-treated cells (N-core histones) and from sodium n-butyrate (NaBu)-treated cells (Ac-core histones) (Figure 7A). Sodium n-butylate is a potent inducer of erythroid differentiation and a well-known HDAC inhibitor (32). The core histones were co-immunoprecipitated with His-tagged TAF-Iβ under a physiological salt concentration, and the amount of co-immunoprecipitated core histone decreased as the salt concentration in the washing buffer increased (Figure 7B). The level of Ac-core histones co-immunoprecipitated with His-tagged TAF-Iβ was similar to that of the N-core histones, indicating that TAF-Iβ binds to the core histones independent of the histone acetylation level. Further, to confirm this, we prepared core histones devoid of the N-terminal tail [tail-less core histones (TL-core histones)] by partial digestion with trypsin (39). Immunoprecipitation assays demonstrated that TAF-Iβ binds to core histones, irrespective of the presence or absence of the N-terminal tail (Figure 7B). These results suggest that the histone N-terminal tail is not required for the binding of TAF-Iβ with the core histones. These *in vitro* observations were consistent with the results that TAF-Iβ is associated with the model gene promoter independent of the histone acetylation level (Figure 6).

Next, we examined the effect of the N-terminal tail of histones and its acetylation on the chromatin disassembly activity of TAF-Iβ. In Figure 2E, we showed that TAF-Iβ remodels the chromatin structure depending on its acidic cluster and dimer formation. The chromatin structure was reconstituted *in vitro* by using purified N-, Ac- or TL-core histones on a plasmid DNA (17), and the nucleosome structure was confirmed by MNase digestion (Figure 7C). Then, the reconstituted chromatin was subjected to nuclease sensitivity assays using the restriction endonuclease PvuII (Figure 7D and E). The nuclease sensitivity of the reconstituted chromatin was much lower than that of the naked DNA (Figure 7E, lanes 3 and 5), and it increased upon incubation with increasing amounts of TAF-Iβ (Figure 7E, lanes 5–7). The nuclease sensitivity of the chromatin reconstituted with Ac-core histones was slightly more sensitive than that of the other reconstituted chromatin structures (Figure 7E, lanes 5, 9 and 13). This is consistent with the previous finding that hyperacetylated chromatin forms an extended conformation as compared to the hypoacetylated chromatin (40). The nuclease sensitivity of the chromatin reconstituted with N-, Ac- and TL-core histones showed equal increase in a TAF-Iβ dose-dependent manner (Figure 7E, lanes 5–7, 9–11 and 13–15). These results indicate that TAF-Iβ remodels the chromatin structure irrespective of the histone tail and its acetylation.

DISCUSSION

Based on the result of the microarray analyses, we have demonstrated that TAF-I is involved in transcription stimulation and repression of gene sub-sets (Supplementary Table 1 and Figure 1B). Currently, we have not reached a consensus on the mechanism of gene down-regulation in TAF-I KD cells. However, it is noted that among the genes up-regulated by TAF-I KD, *TPD52L1*, *TFAP2C*, *DNAJC12* and *TFF1* genes are reported to be estrogen-responsive (1,41–43). The transcription of these genes was increased by estrogen in the estrogen receptor (ER)-positive breast cancer cells, such as MCF-7 cells. Hence, we propose that these estrogen-responsive genes are repressed by TAF-I possibly through its INHAT activity in the ER-negative cells such as HeLa cells.

TAF-Iβ stimulated transcription additively with histone acetylation induced by the low but not the high-concentration of HDAC inhibitors (Figure 4). Since the order of acetylation of the various lysine residues in histones is not random (44), it is possible that the initially acetylated sites make the nucleosomal DNA more accessible to the remodeling factors by facilitating a conformational transition of the nucleosome. Consistent with this finding, it has been reported that the low concentration of HDAC inhibitor causes local structural changes of the nucleosomes and enhances the basal transcription from the MMTV promoter independent of a hormone ligand, while the high-concentrations of inhibitors inhibits the transcription (45). We demonstrated that TAF-Iβ targets the C-terminal histone fold domain rather than the N-terminal histone tail of the histones in nucleosomes (Figure 7B and E). It is possible that TAF-Iβ contacts the DNA-wrapped core region of this transcription-competent chromatin and loosens the histone fold–DNA interaction independent of histone acetylation, thereby promoting the formation of the basal transcription machinery or attenuation of resumption of the competent chromatin to the repressed chromatin structure. Once genes are sufficiently activated by forming the loosened nucleosome structure, TAF-I may not be required. We found that the MNase sensitivity is greatly increased at the model gene promoter region in the CHO-*luc* cells after incubation with 1 mM of TSA for 24 h (data not shown). It is possible that histones are highly acetylated and most of the histones might be removed from the promoter region, so that a large amount of histone chaperone is not required. Although we have focused on the transcription stimulatory

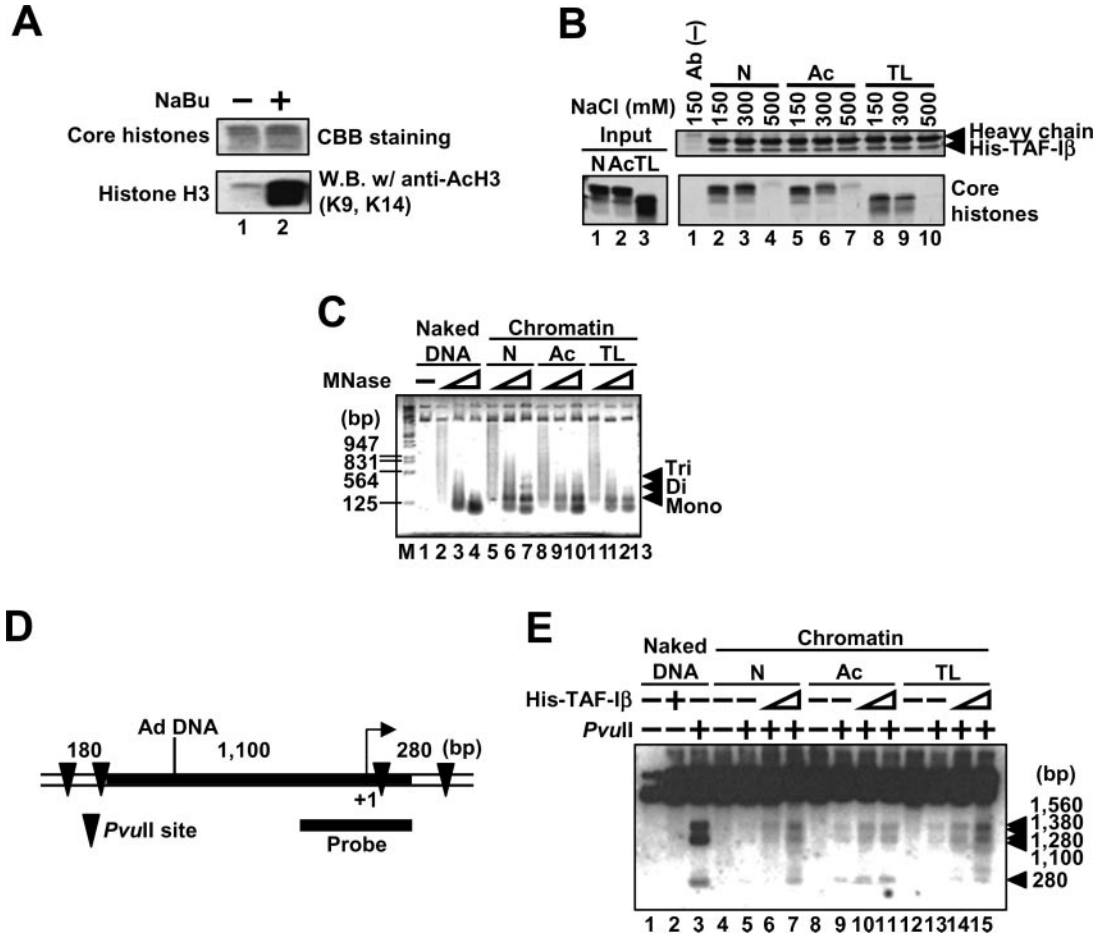

Figure 7. TAF-Iβ binds to core histones and remodels the chromatin structure independent of acetylation and the N-terminal tail of histones. (**A**) Preparation of hyperacetylated core histones. Core histones were purified HeLa cells incubated in the absence (lane 1) or presence of 10 mM of NaBu (lane 2). Core histones were separated through a 15% SDS–PAGE and visualized by staining with CBB (upper panel). The histone acetylation level was examined by western blotting using anti-acetylated histone H3 antibody (lower panel). (**B**) Immunoprecipitation assays. One microgram of core histones prepared from mock-treated cells (N, lanes 1–4), hyperacetylated core histones prepared from NaBu-treated cells (Ac, lanes 5–7), and tail-less core histones (TL, lanes 8–10) were mixed with bacterially expressed hexa-histidine (His)-tagged TAF-Iβ (1 μg) in IP buffer [50 mM Tris–HCl (pH 7.9), 1 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethlysulfonyl fluoride (PMSF) and 10 mM NaBu] containing 150 mM NaCl and incubated at 4°C for 2 h. Protein complexes were immunoprecipitated without (lane 1) or with (lanes 2–10) the anti-TAF-Iβ antibody. Proteins bound to protein A-sepharose beads were separated through a 15% SDS–PAGE and visualized by silver staining. Three hundreds nanograms of each core histone was loaded as Input (left panel). NaCl concentrations in the washing buffer are indicated at the top of the lanes. (**C**) MNase digestion of reconstituted chromatin. The pUC119-ML2 DNA was assembled into chromatin using core histones prepared from mock-treated cells (N), hyperacetylated core histones prepared from NaBu-treated cells (Ac), and tail-less core histones (TL) by the salt dialysis method, respectively. Naked DNA (lanes 1–4) and reconstituted chromatin with core histones prepared from mock-treated cells (N-chromatin, lanes 5–7), and hyperacetylated core histones prepared from NaBu-treated cells (Ac-chromatin, lanes 8–10), or tail-less core histones (TL-chromatin, lanes 11–13) were partially digested without (lane 1) or with 0.01 U (lanes 2, 5, 8 and 11), 0.04 U (lanes 3, 6, 9 and 12), and 0.1 U (lanes 4, 7, 10 and 13) of MNase. Purified DNAs was separated on 1.4% agarose gel electrophoresis and visualized by SYBR Gold staining. Lane M shows DNA size markers. (**D**) The diagram of the restriction sites of the Ad ML promoter region on pUC119-ML2 plasmid DNA. The arrow shows the transcription initiation site of the ML gene. The arrowheads show the sites of PvuII. The filled region shows the Ad DNA on pUC119 vector. Numbers represent the size of each DNA fragment generated by PvuII. The probe for Southern blotting is indicated. (**E**) Restriction endonuclease sensitivity assays. Fifty nanogram of naked DNA (lanes 1–3) and reconstituted chromatins (50 ng DNA, N-chromatin; lanes 4–7, Ac-chromatin; lanes 8–11, and TL-chromatin; lanes 12–15) were incubated without (lanes 1, 3–5, 8–9 and 12–13) or with His-tagged TAF-Iβ (0.5 μg for lanes 6, 10 and 14, and 1.5 μg for lanes 2, 7, 11 and 15) at 30°C for 30 min. The mixture was subjected to partial digestion without (lanes 1–2, 4, 8 and 12) or with PvuII (1 U, lanes 3, 5–7, 9–11 and 13–15) at 37°C for 5 min. DNAs were purified, separated by electrophoresis on a 1% agarose gel, and subjected to Southern blotting followed by autoradiography. The probe for Southern blotting is shown in Figure 6D.

function of TAF-I in this study, it is possible that our finding on the TAF-I-mediated mechanism may be applicable to transcription inhibition as well. Remodeling of the chromatin structure by TAF-I might promote the binding of a repressor(s) to the DNA, thereby repressing the transcription independent of its INHAT function.

Why TAF-I does not play a role similar to that of INHAT at the *cTnC* gene and the model gene promoters used in this study remains to be determined. A current model of the INHAT function indicates that TAF-I binds stably to nucleosomes and represses the access of HATs by masking the N-terminal histone tail (19). However, we could not detect stable TAF-I-nucleosome complexes *in vitro* and *in vivo* (data not shown). We found that only chromatin from cells cross-linked with formaldehyde, but not from naive cells, co-immunoprecipitated with TAF-Iβ (Figures 1D and 3B, and data not shown). These results indicate the possibility that association of TAF-I with chromatin is weak and/or

transient *in vivo* because TAF-I is a member of chaperones. Although TAF-I and chromatin cannot form a stable complex, a transient contact of TAF-I with the nucleosomes may be sufficient to remodel the chromatin structure (Figures 2E and 7E). However, this could be less effective in remodeling and is not a gene-specific process. In fact, the molecular number of the TAF-Iβ dimer required for remodeling was 5 to 15 times more than that of histones in our *in vitro* chromatin remodeling assays (Figures 2E and 7E). It is possible that TAF-I alone cannot function efficiently as a transcription stimulatory factor or INHAT *in vivo*. We hypothesize that other cellular factors/cofactors would support the gene-specific function of TAF-I for recruiting TAF-I on a specific gene locus and/or modulating the function of TAF-I. Although TAF-IβΔC3 could be associated with the model gene promoter region, it was found to be incapable of stimulating transcription *in vivo* (Figures 2C and 3D) and remodeling the chromatin structure *in vitro* (Figure 2E). These suggest that recruiting TAF-I on the specific chromatin region could be dependent on proper dimer formation and/or the resultant exposure of the hydrophilic surface by dimer formation (10) rather than the interaction between histones and the acidic cluster of TAF-I. Further, it is interesting to hypothesize that the C-terminal acidic cluster is a functional domain of TAF-Iβ for the chromatin remodeling, and the N-terminal region containing the coiled-coil dimerization region with the hydrophilic surface is a binding domain for the gene-specific factor to recruit TAF-I on the chromatin. For example, THAP7, a recently identified TAF-I interacting protein, recruits TAF-Iβ to a specific promoter and down-regulates the transcriptional activity of the retinoic acid receptor and ER (46). It is worthwhile to note that 120–225 amino acid residues of TAF-Iβ is essential for the binding to THAP7, and the N-terminal region containing the coiled-coil region greatly enhances this interaction, while the C-terminal acidic cluster is dispensable (46). Fe65 behaves like an adaptor protein that assembles TAF-I and Tip60 on the *KAI1* gene chromatin, and consequently stimulates its transcription (23). We demonstrated that TAF-I may specifically targets MLL on certain gene loci (22). In this sense, systematic identification of genes regulated by TAF-I and genomic regions associated with TAF-I could be useful to obtain a cue. We propose that transcriptome and interactome analyses can greatly aid in the investigation and classification of the gene regulatory roles for not only TAF-I and but also other histone chaperones.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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Conflict of interest statement. None declared.

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