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Investigation of functional roles of transcription termination factor-1 (TTF-I) in HIV-1 replication

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Transcription termination factor-1 (TTF-I) is an RNA polymerase 1-mediated transcription terminator and consisting of a C-terminal DNA-binding domain, central domain, and N-terminal regulatory domain. This protein binds to a so-called 'Sal box' composed of an 11-base pair motif. The interaction of TTF-I with the 'Sal box' is important for many cellular events, including efficient termination of RNA polymerase-1 activity involved in pre-rRNA synthesis and formation of a chromatin loop. To further understand the role of TTF-I in human immunodeficiency virus (HIV)-I virus production, we generated various TTF-I mutant forms. Through a series of studies of the over-expression of TTF-I and its derivatives along with co-transfection with either proviral DNA or HIV-I long terminal repeat (LTR)-driven reporter vectors, we determined that wild-type TTF-I downregulates HIV-I LTR activity and virus production, while the TTF-I Myb-like domain alone upregulated virus production, suggesting that wild-type TTF-I inhibits virus production and trans-activation of the LTR sequence; the Myb-like domain of TTF-I increased virus production and trans-activated LTR activity. [BMB Reports 2018; 51(7): 338-343]

INTRODUCTION

Transcription termination factor (TTF-I) is an RNA polymerase-1-mediated transcription terminator (1). It binds to a consensus terminator element composed of a so-called 'Sal box' 11-base pair motif (GGGTCGACCAG), which can be repeated up to 10 times downstream of the 3' end of the pre-rRNA sequence (2). Binding of TTF-I to the Sal box is required to stop the elongation reaction by RNA polymerase-1 and mediates termination of pre-rRNA synthesis that occurs predominantly at the first terminator element T_1 (3). In addition to these terminator elements, another TTF-I interacting element known as T_0 is typically located 170 base pairs upstream of the transcription start site (4). A previous study demonstrated that interacting of TTF-I and T_0 site could initiate efficient transcription and could recruit chromatin remodeling complexes for the expression regulation of rRNA genes (5, 6).

Oligomerization of TTF-I *in vitro* was shown to link two DNA fragments in *trans* (7). Thus, TTF-I could bind to T_0 and the downstream terminator sites T_1-T_{10} and consequently loop out of pre-rRNA coding region. This type of a chromatin looping is thought to facilitate and increase transcription initiation rate of the rRNA gene (8). Thus, TTF-I could contribute for transcription termination and efficient rDNA transcription initiation as well (7).

The modular structure of TTF-I consists of a C-terminal DNA-binding domain, central domain, and N-terminal regulatory domain (NRD). The C-terminal DNA-binding domain and central domain of TTF-I are required for transcription termination (9) and transcriptional activation on a nucleosomal rDNA template *in vitro* (10) as well as transcription initiation on chromatin (11). The DNA binding domain of TTF-I is highly conserved between human and mouse and is known to be highly homologous with the DNA binding domain of the proto-oncoprotein c-Myb and yeast transcription factor Reb1p (12-14). Mutations of the conserved tryptophan residues of c-Myb known to be important for DNA binding inhibited DNA binding activity of TTF-I (15). The central domain (430-445) of TTF-I has also been shown to be important for termination, because a deletion of the region affected termination, but not DNA binding activity (14).

A previous study demonstrated that the ability of full-length TTF-I to bind to the 'Sal box' was reduced compared with proteolytic derivatives of TTF-I (16). The DNA binding activity of recombinant full-length TTF-I is lower than that of N-terminal deletion mutants. This suggests that the N-terminus domain represses the specific DNA binding activity of TTF-I (16). In an *in vitro* transcription assay, both full-length TTF-I and N-terminal deletion mutants of TTF-I exhibited similar termination activities. This observation showed that the N-terminal domain (NRD) of TTF-I affected the DNA binding

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activity without affecting the transcription termination activities of TTF-I (14). This led to the suggestion that cellular protein(s) interact with the N-terminus of TTF-I and relieve its repressive effect (14).

A previous study of murine TTF-I revealed that the C-terminal DNA binding domain of mTTF-I is highly homologous with the c-Myb proto-oncogene protein and c-Myb protein markedly increased transactivation of the HIV-long-terminal repeat (LTR) promoter (17) and virus production (18). However, little is known about the functional roles of wild-type TTF-I.

Here, we examined the functional effects of wild-type TTF-I and TTF-I deletion mutants on HIV-I virus production and found that over-expression of wild-type TTF-I and TTF-I derivatives negatively affect HIV-I expression and LTR-driven reporter gene expression. Interestingly, however, we observed that the TTF-I 1-520 domain with or without HIV-I Tat protein trans-activates HIV-I LTR-mediated transcription and increases HIV-I virus production.

RESULTS

Construction of full-length TTF-I and its derivatives

To further understand the functions of hTTF-I in the HIV-I life cycle, we constructed a number of TTF-I derivatives as depicted in Fig. 1A. The constructs of hTTF-I full-length (FL) as well as its derivatives were transfected into 293T cells to confirm its protein expression along with an internal control vector, pDS_Red expression N1, which expressed red fluorescent protein. The resulting cells were harvested and analyzed by western blotting (Fig. 1B). TTF-I FL and its derivatives were all expressed as their expected sizes but to different degrees, with the TTF-I 224-905 (ΔNRD) construct showing the highest expression.

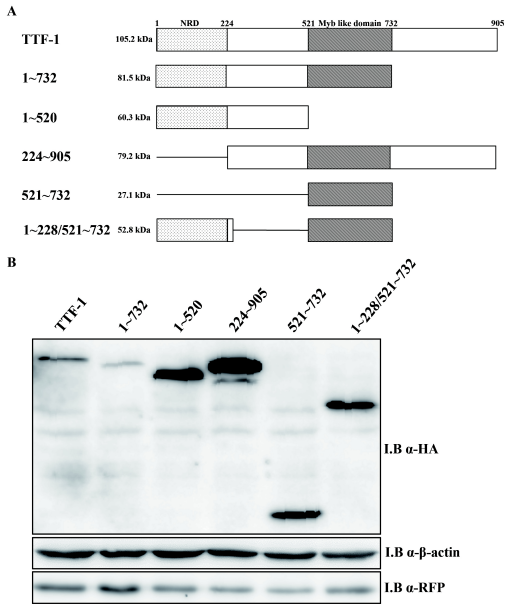

Fig. 1. Construction of TTF-I full-length and derivatives. (A) cloning scheme of pCMV_HA/TTF1 wild-type and derivatives (1-732, 1-520, 224-905, 521-732, 1-228_521-732). (B) protein expression of TTF1 derivatives is verified. 293T cell were transfected wtTTF1 and derivatives and cell lysate analysis on immunoblot by using α-TTF1 (wtTTF-I, 1-732) or α-HA (1-520, 224-905, 521-732, 1-228_521-732) anti-body.

Localization of full-length TTF-I and its derivatives

It was reported previously that murine TTF-I localizes in the nucleolus, and the N-terminal regulation domain (NRD) of murine TTF-I contains the nucleolus localization sequence (NoLS) (14, 19). To investigate the localization of wild-type hTTF-I and its derivatives, HeLa cells were transfected with all prepared constructs and their localization was evaluated using an HA-tag antibody in immunostaining studies (Fig. 2). The results showed that hTTF-I wild-type, 1-732, 1-520, and 1-228/521-732 were localized in the nucleolus. In contrast, hTTF-I 224-905 and 521-732 derivatives were localized to the nucleus. These results are in good agreement with a previous study showing that NRD of hTTF-I has nucleolus localization activity because of its NoLS.

Effect of hTTF-I wild-type and mutants on HIV-I LTR promoter activity

Next, to further evaluate whether hTTF-I affects *trans*-activation of HIV-I LTR-driven promoter activity, we employed HIV-I LTR promoter-driven EGFP reporter gene expression, pGL/U3RU5/EGFP. Expression constructs of hTTF-I wild-type and its derivatives were transfected respectively into 293T cells along with a pGL/U3RU5/EGFP and pDS_Red as a transfection efficiency control vector. The effect of hTTF-I wild-type and mutants on EGFP expression was evaluated by fluorescence

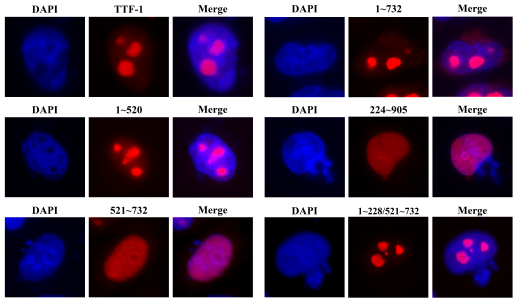

Fig. 2. Localization of TTF-1 full-length and derivatives. hTTF-I full length and hTTF-I derivatives (1-732, 1-520, 224-905, 521-732, and 1-228_521-732) were expressed in Hela cell. 24 hours later, cells were stained in 100 ng/ml DAPI for nuclei, anti-HA or TTF-I antibody for HA tagged TTF-I derivatives protein (Rhodamine, Red).

microscopy after co-transfection (Fig. 3A). We found that hTTF-I wild-type and 1-732 rather weakly inhibited EGFP expression compared to the control vector. In contrast, hTTF-I 521-732 slightly increased LTR activity by 1.5-fold (Fig. 3B). The result was verified by western blot analysis in the reporter gene assay. Examination of the effect of these constructs in the presence of Tat protein revealed that hTTF-I FL and 1-732 with Tat protein present showed similar results as in the absence of Tat. However, TTF-I 521-732 showed further increased expression of EGFP in the presence of Tat (Fig. 3C).

To further verify the results of the EGFP reporter gene assay, we also employed another reporter vector system containing the Firefly luciferase (Fluc) gene rather than the EGFP gene. For the luciferase assay, hTTF-I wild-type and its derivatives were transfected again into 293T cells along with pGL/U3RU5/Fluc and pDS_Red, and luciferase activity was measured using a fluorescence meter (Fig. 3D). We observed weak inhibition of luciferase activity when TTF-I wild-type or 1-732 alone was expressed, whereas the activity of TTF-I 521-732 in the presence of Tat resulted in increased luciferase activity, which was confirmed by western blot analysis (Fig. 3E). These results indicate that the hTTF-I 521-732 domain does not function as a transcriptional activator. However, its activity was weakly enhanced in the presence of Tat protein in the cell.

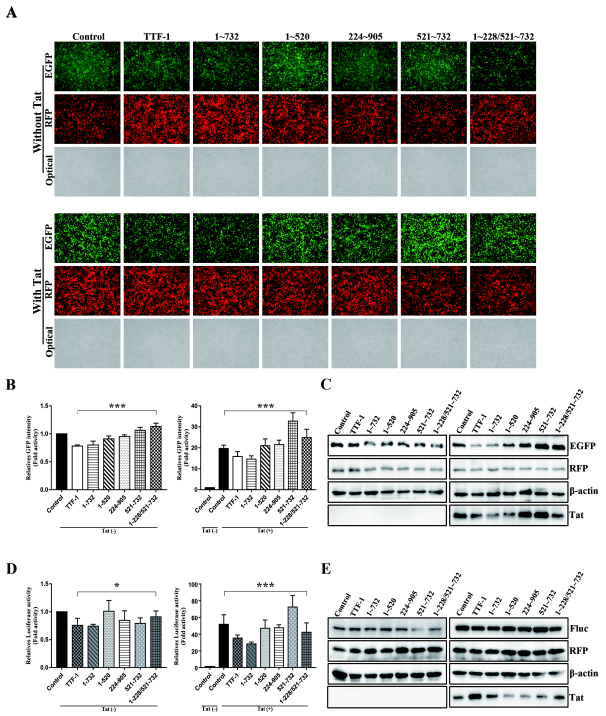

Fig. 3. Effect of hTTF-I full-length and derivatives on HIV-I LTR promoter. Trans-activation of HIV-I LTR driven reporter vector construct in 293T cell by TTF-I or derivatives. 293T cell were co-transfected reporter gene (pGL3/HIV1/Fluc or pGL3/HIV1/EGFP), RFP expression vector (pDS_Red expression N1, internal control) and HIV-I_Tat for LTR activity assay. (A) Fluorescent inverted microscope (B) LTR assay, cell lysates were measured GFP intensity and normalized by RFP intensity. (C) HIV-I LTR promoter activity was analyzed on immunoblots by using α-GFP and α-RFP antibody. (D) luciferase assay, cell lysates were measured firefly luciferase activity and RFP intensity. luciferase activity normalized by RFP intensity. (E) Luciferase activity was analyzed on immunoblots by using α-Firefly and α-RFP antibody. Statistical significance was determined by one-way analysis of variance (ANOVA); ***P < 0.001 and *P < 0.05.

Functional role of hTTF-I and its derivatives in HIV-I virus production

To evaluate the functional role of hTTF-I in the HIV-I life cycle, we used the pNL4-3/EGFP plasmid containing the EGFP gene rather than the HIV-I Nef (negative regulatory factor) gene (20). 293T cells were transfected with hTTF-I wild-type and its derivatives along with pNL4-3/EGFP and pDS_Red plasmid, a transfection efficiency control. At 48 h post-transfection, we evaluated EGFP expression by fluorescence microscopy (Fig. 4A) and the cell medium was harvested to analyze virus production. We observed that TTF-I wild-type as well as 1-732 inhibited the expression of EGFP, while TTF-I 521-732 increased EGFP protein levels (Fig. 4B). To determine whether hTTF-I wild-type and its derivatives affect the production of viral proteins, cell lysates and cell medium were analyzed by western blotting (Fig. 4C). Western blot analysis showed that efficient production of viral proteins p24 and p55 occurred when TTF-I 521-732 was expressed, which is consistent with the LTR-reporter gene assay results, whereas both full length TTF-I and 1-732 decreased production of viral p55 as determined by western blotting. The p24 level in the virus mixture was determined by enzyme-linked immunosorbent assay (Fig. 4D).

The results of the LTR assay and proviral DNA transfection indicated that TTF-I wild-type and 1-732 weakly inhibited both LTR-mediated transcription activity and production of HIV-I virus, while the TTF-I 521-732 domain increased LTR activity, which was further augmented in the presence of Tat protein and thus led to activation of virus production.

DISCUSSION

Studies of the functional roles of hTTF-I in HIV have been very limited. A previous study demonstrated that the murine TTF-I Myb-like domain is highly homologous to c-Myb protein (15). In addition, it has been reported that the proto-oncoprotein c-Myb binds the viral LTR sequence and trans-activates the HIV LTR sequence (17). Additionally, c-Myb protein influences HIV-I gene expression and virus production (18). In this study, we investigated the effect of hTTF-I on the HIV-I LTR promoter and virus production.

First, our study revealed the localization activity of hTTF-IFL

and its derivatives for the first time in human cells. A previous study reported that the N-terminal domain (121-210) of murine TTF-I has an NoLS and ARF tumor suppressor binding site (19), but the localization of hTTF-I was unknown. We demonstrated that the N-terminal domain (1-223) of hTTF-I contributed to nucleolar localization of hTTF-I (Fig. 2). These results are in close agreement with those of previous studies.

Based on the HIV-I LTR promoter assay, we found that hTTF-I FL and 1-732 weakly reduced trans-activation of the LTR sequence, regardless of the presence of HIV-I Tat; other derivatives had no effect on trans-activation of the LTR sequence in the absence of Tat protein. However, hTTF-I 521-732 with Tat trans-activated the LTR sequence (Fig. 3), as hTTF-I 521-732 has striking homology with the DNA-binding domain of c-Myb protein. It was previously reported that c-Myb protein markedly increased trans-activation of the HIV-I LTR sequence (17). Our results differ from those of Dasgupta *et al*. who studied the effect of original c-Myb protein on the LTR promoter, while hTTF-I 521-732 showed homology with only the DNA-binding domain of Myb protein. Thus, hTTF-I 521-732 has lower trans-activation activity than the original c-Myb protein. Thus, our results suggest that hTTF-I FL, 1-732, and 521-732 could regulate trans-activation of LTR sequence.

The ability of hTTF-I FL and its derivatives to regulate trans-activation of the HIV-I LTR promoter suggests that it also regulates virus production. To investigate whether hTTF-I FL and its derivatives affect the virus production of HIV-I, we co-transfected hTTF-I derivatives along with the pNL4-3/EGFP pro-viral vector. The expression of hTTF-I FL and 1-732 weakly inhibited virus production, while hTTF-I 521-732 had the opposite effect. This data was verified by measuring the p24 protein level by enzyme-linked immunosorbent assay of the media (Fig. 4). The results of hTTF-I 521-732 are consistent with the data, suggesting that c-Myb protein increased HIV-I gene expression and virus production (18). However, hTTF-I 1-732 exhibited inhibitory activity, overcoming the effect of TTF-I 521-732.

Our data clearly show that hTTF-I localizes in the nucleolus and N-terminus (1-224) of hTTF-I which include NoLS. In addition, we demonstrated that the expression of hTTF-I 521-732 increased HIV-I production by regulating transactivation of the LTR promoter. However, wild-type hTTF-I FL and domain 1-732 inhibited HIV-I virus production. It remains unclear how and what part of the hTTF-I FL decreased transactivation of HIV-I LTR and ultimately virus production. Further studies are required to identify the domain that down-regulates virus production in the domain of hTTF-I.

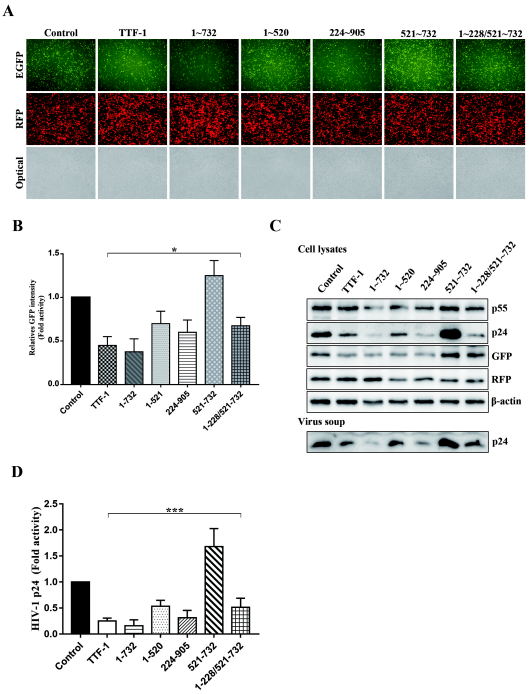

Fig. 4. Functional role of wtTTF-I and derivatives in HIV-I virus production. 293T cell co-transfected TTF-I wild-type or derivatives and pNL4-3/EGFP provirus vector. The fluorescent intensities are measured by fluorometer and GFP intensity was normalized by RFP intensity. (A) Fluorescent inverted microscope (B) Virus protein $p24^{Gag}$ level analyzed on immunoblots, by α-$p24^{Gag}$ antibody. (C) The fluorescent intensities are measured by fluorometer and GFP intensity was normalized by RFP intensity. (D) Virus titer of viral soup was determined by $p24^{Gag}$ ELISA then normalized by RFP level, % of internal control (level of RFP). Statistical significance was determined by one-way analysis of variance (ANOVA); ***P < 0.001 and *P < 0.05.

MATERIALS AND METHODS

Cell culture and transfection

Hek 293T cells and HeLa cells were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (Tissue Culture Biologicals, Long Beach, CA, USA) and 1% penicillin/streptomycin (GIBCO, Grand Island, NY, USA) in 5% CO_2 at 37°C. Cells were transfected TTF-I wild-type and its derivatives with reporter vectors using JetPEI reagent (Polyplus-transfection, San Marcos, CA, USA) as per the manufacturer's instructions.

Western blotting

For western blot analyses, we separated the proteins by either 12% or 13.5% SDS-PAGE followed by electroblotting onto a polyvinylidene difluoride *membrane* (Merck Millipore, Billerica, MA, USA), followed by probing over night at 4°C or 2 h at room temperature with specific antibodies. After three washes with 1% phosphate-buffered saline containing Tween 20 (PBS-T), the membranes were incubated with the corresponding secondary antibodies. The signals were visualized with an Enhanced Chemiluminescence System LAS 4000 (Fuji Film,

Tokyo, Japan) as per the manufacturer's instructions.

Immunofluorescence (IF) analysis
For immunofluorescence, HeLa cells were seeded onto coverslips (Paul Marienfeld GmbH & Co., Königshofen, Germany) at 5×10^4 cells/well in 24-well tissue culture plates. The next day, cells were transfected with TTF-I FL and its derivatives, using jetPEI as per the manufacturer's protocol (Polyplus-transfection). After 24 h, the cells were washed with PBS and fixed 4% paraformaldehyde, permeabilized for 20 min, blocked in 0.1% Triton X-100, 2% bovine serum albumin, and 5% normal horse serum for 30 min, and incubated with primary antibody in 1% bovine serum albumin in PBS-T for 2 h at RT. Cells were washed three times with PBS-T (0.1% Tween-20) for 10 min and incubated with fluorescent-conjugated secondary antibody in the dark for 1 h, followed by three washes in PBS-T for 10 min each. Cells were counterstained with DAPI (1 μg/ml in PBS-T). After mounting in a 10-μl drop of Vectashield h-1000 solution (Vector Laboratories Inc., Burlingame, CA, USA), samples were sealed with nail polish.

LTR based reporter gene assay
For reporter gene assay, HEK 293T cells were transfected U3RU5-Fluc or U3RU5-EGFP with TTF-I wild-type and its derivatives. Cells were harvested and lysed after 24 hour incubation by Radioimmunoprecipitation assay (RIPA) buffer or Passive Lysis Buffer (Promega Corp.) as per the manufacturer's instruction. The luciferase activity or EGFP signal were measured by Fluorometer (Tecan Corp.) Data were normalized by RFP level.

HIV-1 p24 ELISA assay
Cells were transfected TTF-I wild-type and its derivatives with pNL4-3_EGFP using JetPEI reagent (Polyplus-transfection, San Marcos, CA, USA) according to the manufacturer's instructions. For ELISA assay, supernatant was harvested at 48 h post-transfection and the quantity of virus of each sample were assessed using HIV-1 p24 ELISA kit (Xpressbio, Frederick MD, USA) as recommend by manufacturer's instructions and was normalized by RFP level.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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