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Investigation of function and regulation of the YB-1 cellular factor in HIV replication

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Y-box binding protein 1 (YB-1) is a member of the cold-shock domain (CSD) protein superfamily. It participates in a wide variety of cellular events, including transcription, RNA splicing, translation, DNA repair, drug resistance, and stress responses. We investigated putative functions of YB-1 in HIV-1 replication. Functional studies using overexpression or knockdown of YB-1 in conjunction with transfection of proviral DNA showed that YB-1 enhances virus production. We found YB-1 regulates HIV-1 production by stimulating viral transcription using HIV-1 LTR sequence U3RU5 with Luciferase assay. We also identified a specific region from amino acids 1 to 324 of YB-1 as necessary for the participation of the protein in the production of virions. [BMB Reports 2018; 51(6): 290-295]

INTRODUCTION

Y-box binding protein 1 (YB-1), a 36-kDa protein, is known primarily as a specific DNA binding protein and a member of the cold-shock domain (CSD) protein superfamily (1). The protein can be divided into three domains: an N-terminal domain consisting of an alanine/proline-rich region (A/P domain), the cold shock domain (CSD), and the large C-terminal domain (CTD) containing charge zipper motifs (2), which are alternating clusters of positively and negatively charged amino acid residues. The protein is known to be involved in many cellular DNA- and RNA-dependent events, which include transcription, regulation of mRNA stability, splicing, translation, and DNA repair (3).

YB-1 function also influences the expression of many viruses (4), both positively and negatively. For example, YB-1 reportedly has a positive effect on the replication of the adenovirus genome (5). However, it has also been shown that dengue virus production was greatly enhanced upon *YBX1* knockout, because the protein could bind to the 3′-UTR of dengue virus and inhibits its replication (6). In other cases, YB-1 could interact with the NS3/4A protease of hepatitis C virus to regulate viral RNA replication and particle production (7), and it was also shown to enhance influenza virus replication through an interaction with the vRNP (8). Other work has demonstrated that YB-1 increases the production of Murine leukemia virus-based vector by enhancing the intracellular stability of the viral RNAs, thus improving gRNA packaging (9). YB-1 may also interact with splicing factor SRp30C (SFRS9/SRSF9), which belongs to a conserved protein family involved in RNA splicing. The N-terminus of YB-1 co-localizes with SRp30C in the nucleus of mammalian cells (10).

Recently, it has been suggested that YB-1 enhances HIV-1 gene expression and viral production by stabilizing HIV-1 genomic RNA, preventing it from decaying (4). In the same study, YB-1 was shown to accomplish this by binding to stem-loop2 of HIV-1 5′-UTR (4). However, another study has shown that YB-1 could regulate HIV-1 transcription in association with Tat and TAR. In HIV-1-infected cells, YB-1 binds to TAR RNA, which is the binding target for Tat, an indispensable viral transcription factor encoded early in the HIV-1 life cycle (11). YB-1 also binds to Tat, and thus activates the HIV-1 LTR (−450/+80) (12). YB-1 interacts with the Tat protein and binds to TAR RNA, facilitating TAR-dependent Tat transactivation. Thus, YB-1 may enhance the life cycle of HIV-1 through several mechanisms, including RNA stabilization and transactivation.

To further understand the functions of YB-1 in HIV-1 replication, we undertook the present studies and found that YB-1 enhances HIV-1 production through transcriptional activation.

RESULTS AND DISCUSSION

Construction and expression of YB-1 and derivatives

YB-1 is composed of three domains: the alanine/proline-rich region (A/P domain), the cold shock domain (CSD), and the

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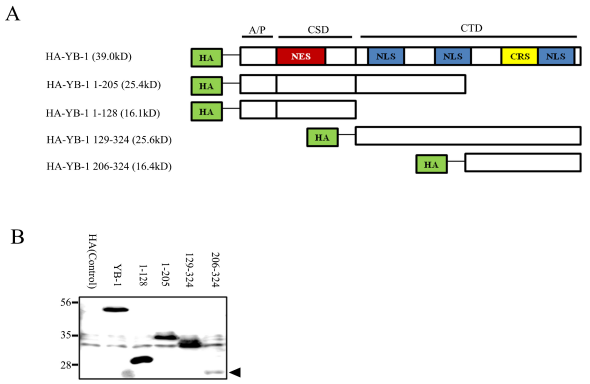

Fig. 1. The structure of YB1 and derivatives. (A) Scheme of YB-1 and its deletion mutants used in this study. NES: nuclear export signal; NLS: nuclear localization signal; CRS: cytoplasmic retention signal. (B) Protein expression of HA-YB-1. 293T cells were transfected with YB-1 and derivatives. Cell lysates were directly used for Western blotting using anti-HA antibody.

large C-terminal domain (CTD). To investigate in detail the function and effect of YB-1 on HIV-1 expression and production, we first made YB-1 and its various derivatives, all tagged with HA at the N-terminus, as depicted schematically in Fig. 1. The set of derivatives included 1) positions 1-205 with a half of C-terminal deletion, 2) 1-128 with a complete C-terminal deletion, 3) 129-324 having an N-terminal deletion, and 4) 206-324 having only half of the C-terminal region (Fig. 1A). We then examined the expression of these YB-1 derivatives by SDS-PAGE and western blot analysis. YB-1 is a 36.0-kDa protein, but, as previously observed (13-15), it showed an abnormal mobility in SDS-PAGE, as a 50 kDa-protein. All other YB-1 derivatives showed quite a good level of expression, except the one consisting only of positions 206-324, which showed quite a weak level of expression (Fig. 1B).

Effect of YB-1 on virus production of HIV-1

It is known that YB-1 enhances viral reproduction in many different types of viruses, including HIV-1 (5, 6, 16, 17). To further understand the mechanism by which YB-1 enhances production of HIV-1, especially with regard to viral gene expression, increasing doses of YB-1 were co-transfected with

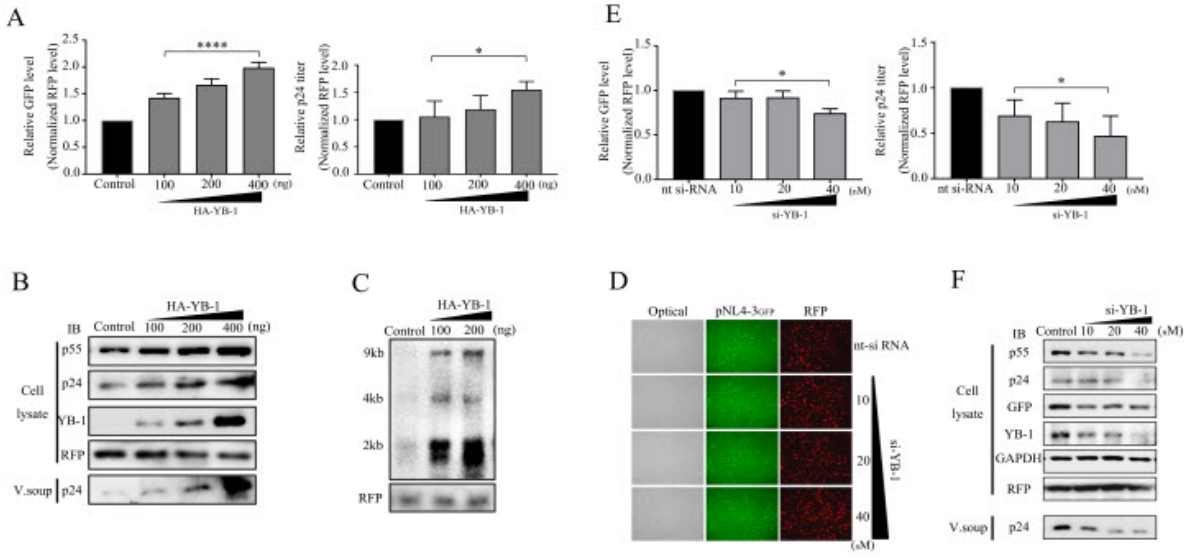

Fig. 2. Effect of YB1 on virus production of HIV-1. 293T cells co-transfected proviral DNA, pNL4-3$_{GFP}$ with control plasmid or pCMV-HA-YB-1 and RFP expression plasmid. (A) The viral expression level of pNL4-3$_{GFP}$ was quantified as the ratio of relative GFP signal normalized by RFP level (Left). Virus titer of viral soup was quantified by p24^{Gag} ELISA and normalized by RFP level (Right). Fold changes were determined compared to control samples. (B) Western blot results of cell Lysate and viral soup. (C) Northern blot results of RNA. (D-F) Effect of knock-down of YB-1 on HIV-1 virus production. 293T cells co-transfected pNL4-3$_{GFP}$ with control non-targeting siRNA (ntsiRNA) or si-YB-1 and RFP expression plasmid (D) Fluorescent microscope image. (E) GFP signal normalized by the levels of RFP (Left). Virus titer of viral soup was quantified by p24^{Gag} ELISA and normalized by RFP level (Right). Result are mean ± SD over three experiments. Statistical significance was performed using one-way ANOVA (****P < 0.0001, *P < 0.0332). (F) Western blot results of Cell lysate and viral soup.

a proviral DNA pNL4-3$_{GFP}$ vector in 293T cells. At 48 hours post-transfection, we analyzed the effect of YB-1 in HIV-1 production by measuring the expression of GFP and the quantity of viral p55 and p24 proteins. Cell lysate was separated by SDS-PAGE, and p55 and p24 were visualized with the anti-p55 antibody. The concentration of p24 in the viral supernatant was measured by an ELISA assay. GFP expression scaled up to two-fold with increasing concentration of YB-1 (Fig. 2A), while the p24 titer of viral supernatant scaled up to 1.6-fold with YB-1 (Fig. 2A). These results were confirmed by western blot analysis of the data as shown in Fig. 2B. The results shown in Fig. 2B confirm that YB-1 increases the quantity of p24 and p55 in the cell, as well as the concentration of p24 in the viral supernatant. This result is consistent with a previous report that YB-1 could promote HIV-1 production, which was analyzed similarly by examining with the pNL4-3$_{luc}$ expressing a luciferase reporter gene (4).

We then confirmed the effects of YB-1 on the RNA expression of HIV-1 by northern blot (Fig. 2C). Collectively, the level of HIV-1 RNA scaled positively with the concentration of YB-1. Next, we performed YB-1 knockdown using siRNA (Fig. 2D-F). When we reduced the expression of YB-1, virus production (as indicated by GFP expression and the concentration of p24 in the viral supernatent.) also decreased (Fig. 2F). Western blot analysis confirmed these results (Fig. 2F). In conclusion, these results confirm that YB-1 enhances HIV-1 production by increasing the RNA expression level, with knockdown of YB-1 expression having the opposite effect.

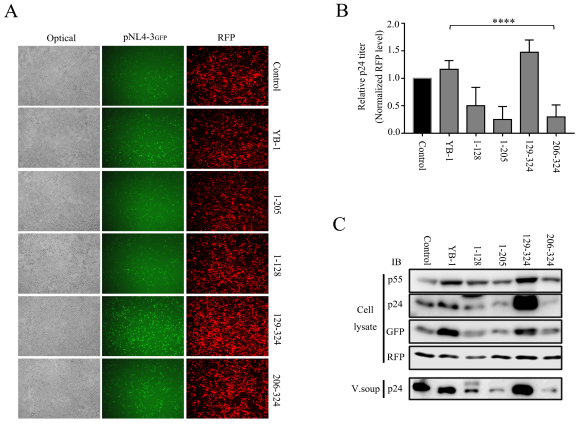

Fig. 3. Effect of YB1 and derivatives on virus production of HIV-1. 293T cells co-transfected pNL4-3$_{GFP}$ with control plasmid or HA-YB-1 or mutants and RFP expression plasmid. (A) Fluorescent microscope image. (B) Virus titer of viral soup was quantified by p24^{Gag} ELISA and normalized by RFP level. Statistical significance was performed using one-way ANOVA (****P < 0.0001). (C) Western blot results of cell lysate and viral soup.

Effect of YB-1 derivatives on the production of HIV-1

Having confirmed that HIV-1 production increases with the higher concentration of YB-1 (Fig. 2), we mapped the region of YB-1 responsible for this effect on HIV-1 production. Following the same experimental approach, YB-1 and its derivatives were co-transfected with pNL4-3GFP in 293T cells. At 48 hours post-transfection, we analyzed the effect of YB-1 on HIV-1 production by measuring GFP, p55, and p24 levels, both intracellularly and in the media. Relative GFP signal and p24 titer were normalized by RFP level for equivalent transfection efficiency. Expression of GFP and RFP was determined with an Inverted Fluorescence Microscope and measured by Fluorometer (Fig. 3A). The relative p24 titer of viral supernatant only increased with transfection of full length YB-1 or the 129-324 region of YB-1 (Fig. 3B). These results were confirmed by western blot analysis showing that full-length YB-1 and residues 129-324 increased the quantity of GFP in the cells and the quantity of p24 protein in the cells and media. These results suggest that residues 129-324 of YB-1 play an important role in the enhancement of HIV-1 production.

It is known that YB-1 has one nuclear export signal (NES; 52-101), three nuclear localization signals (NLS; 149-156, 185-194, 276-292), and one retention signal (CRS; 247-267), regulating the localization of YB-1 (18). Most of the YB-1 is located in the cytoplasm (10, 19), but it can shuttle between the nucleus and the cytoplasm. YB-1 129-324 has three NLSs, localizing it in the nucleus (18, 20). These results suggest that

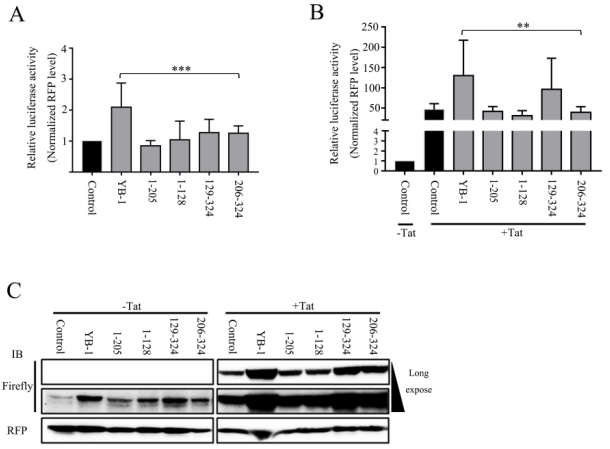

Fig. 4. Effect of YB-1 and mutants on Tat activation of the HIV-1 LTR sequence U3RU5. 200 ng of Luciferase reporter construct containing the LTR sequence U3RU5 was transfected into 293 T cells either alone (A) or together with 200 ng of Tat (B) and YB-1 or mutants expression plasmids. Luciferase activity was normalized by RFP level. Fold changes were determined compared to the basal transcription activity of control sample. Data presented are means + SD of three independent measurements. Statistical significance was performed using one-way ANOVA (***P < 0.0002, **P < 0.0021). (C) Western blot results of cell lysate.

the 129-324 region of YB-1 is a major enhancer of HIV-1 production and, given that 129-324 is localized in the nucleus especially strongly, we infer that YB-1 localized in the nucleus participate in increasing HIV-1 production.

Effect of YB-1 on Tat-induced transcriptional activation of the HIV-1 LTR sequence U3RU5

Previous work with human astrocytic U-87MG cells showed that YB-1 enhances viral Tat protein-induced transcriptional activation of HIV-1 U3R by binding with Tat and TAR RNA (11, 12). We confirmed that YB-1 increases RNA expression of HIV-1 (Fig. 2C). In order to understand further functional roles of YB-1 in this process, we tested whether YB-1 and reduced YB-1 derivatives could promote trans-activation with Tat protein on HIV-1 U3RU5-driven reporter gene assays (Fig. 4) and, if so, what region(s) of YB-1 were involved in this Tat-induced activation.

Transcriptional activation of a reporter gene that contains a firefly gene was confirmed by co-transfection in presence of YB-1 or a control vector. Each derivative of YB-1 was also examined for basal and Tat-mediated transcriptional activation of the U3RU5 promoter. The data showed that YB-1 and derivatives have various effects on trans-activation of U3RU5. YB-1 increased basal trans-activity about 1.6 fold. Other derivatives also increased the level of basal transcriptional activation, except the 1-205 domain of YB-1. In Tat-mediated transcription, YB-1 and the 129-324 derivative were able to increase the level of transcriptional activation of the U3RU5 up to 130.2- and 96.6-fold, but other derivatives had no effect relative to a control vector.

In this study, we used the EGFP reporter gene system (data not shown) and, as a corollary, we found no difference in the result between EGFP and the luciferase reporter gene. This implies that the luciferase reporter system does not affect translation activity.

The results demonstrate that YB-1 has a functional role in regulating HIV-1 expression and production through transcriptional activation (Fig. 2C). So, we suggest that YB-1 is required in the processing of HIV-1 transcription and thus increases HIV-1 production, as it stimulates Tat-induced transcription of viral genes. Specially, the 129-324 region of YB-1 is a major enhancer of HIV-1 production through this mechanism. In the LTR assay using U3RU5, we obtained a different pattern of data between basal and Tat-mediated transcription of the U3RU5 promoter. The 1-205 and 206-324 regions increase the basal transcriptional level, but the Tat-mediated transcriptional activation was not demonstrated. This is further evidence that YB-1 interacts with Tat through the 125-318 region (11). The 1-205 and 206-324 regions are most likely missing the residues that interact with Tat. The 1-128 region also does not contain the specific region that interacts with Tat; therefore, this mutant did not show an increase in Tat-induced activation. Our study indicates that YB-1 increases the production of HIV-1, at least partially, through enhancement of Tat-induced transcription of the U3RU5 promoter, and that at least residues 129-324 of YB-1 are required for this process.

Mu X. *et al*. (4) reported that YB-1 could not stimulate activation of HIV-1 U3RU5 even though they used the same form of DNA and same cell line as our experiment. Thus, Mu X. *et al*. shown that YB-1 enhances HIV-1 production by increasing the stability of the RNA only. However, as shown in our data and by Sayawa *et al*. (12) , many evidences support that YB-1 enhances viral transcription of HIV-1 and Tat activation. In addition, Ansary *et al*. (11) reported the interaction between Tat and YB-1. U3RU5 and YB-1 was transfected as 1:1 ratio in the report of Sawaya *et al*. And we found that the increase was more pronounced at the ratio of U3RU5 to YB-1 was 1: 4, and all experiments were carried out under the same conditions. Mu X. *et al*. did not pay an attention to transcription of HIV-1, and it appears thus that they did not seem to focus on the concentration of U3RU5 and YB-1. Therefore, the disagreement between our data and those previously reported by others suggest that different concentration condition of U3RU5 and YB-1. But because of Mu *et al*. data about HIV-1 RNA stabilizing of YB-1, it cannot be assured that YB-1 increases HIV-1 production only by transcription activation. In this study, we confirmed the increase of viral transcription as one of the mechanisms that enhancing HIV-1 production of YB-1 in 293T cell line. And we also discovered that at least residues 129-324 of YB-1 are required for this process. It remains to be a challenge to find out what other mechanisms which regulate HIV-1 production are unfolding and what synergistic effects of these functions are to be. And YB-1 also interacts with many other proteins (21), possibly including other factors in the regulation of HIV-1 production, so confounding effects are still possible. Therefore, the associations between YB-1, HIV-1, and other factors also require further study.

MATERIALS AND METHODS

Cell culture and transfection

293T are isolated from human embryonic kidneys (HEK) obtained from the ATCC. In general, cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc.). Transfection of 293T cells using jet-PEI (Polyplus-transfection Co.). Transfection for non-targeting siRNA (on-target plus negative controls, Thermo Fisher Scientific Co.) and si-YB-1 (on-traget plus, Thermo Fisher Scientific Co.) of 293T cells were using Lipofector-EZ (Aptabio Therapeutics Inc.). pDsRed-Express-N1 vector which is RFP expression vector was used as internal control in all transfections.

Construction of plasmids

HA-YB-1 and derivative vectors were created using appropriate DNA restriction enzymes, or PCR amplification

with pCMV6-XL5-YB-1 as a template. Full-length YB-1 and all derivatives were cloned in frame with the pCMV-HA vector. Plasmids used in this study to encode YB-1 and its derivatives, as well as the primers used to construct the plasmids, are listed in Supplement Table 1.

HIV-1-producing plasmid pNL4-3 is the structure in which Nef was replaced with GFP. This plasmid has been described in Kim *et al*. (22). U3RU5 assay transfection reporter vectors used in this study have been described in Jeong *et al*. (23).

Western blot & northern blot analysis

For western blot analysis, transfected cells were lysed using *RIPA lysis* buffer (25 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% TritonX-100, pH 7.4) *with* protein Inhibitors cocktail (Roche Molecular Systems Inc.) and phenylmethylsulfonyl fluoride (PMSF). Cell lysates were boiled in SDS sample buffer for 5 min. Each sample was separated by SDS-PAGE and transferred to a PVDF membrane (Merck Millipore Ltd.). The membranes were blocked with 5% skim milk in phosphate-buffered saline containing 0.1% Tween-20 (PBST) and incubated with the appropriate first antibody overnight at 4°C. The membrane was further incubated with the appropriate secondary antibody. After 3 washes with TBS-T at room temperature, the protein band was visualized with EZ West Lumi Plus (ECL) substrates (ATTO Technology Inc.).

For Northern blot analysis, total cell RNA was isolated 48 h post-transfection using RNAiso Plus (TaKaRa bio Inc.) according to the manufacturer's instructions. RNA levels were measured by northern blotting using DIG Northern Starter Kit (Roche Molecular Systems Inc.) according to the manufacturer's instructions.

Luciferase assay

Cells transfected with U3RU5-luc, RFP expression plasmid, and factor plasmid were lysed by Passive Lysis Buffer (Promega Corp.) according to the manufacturer's instructions. The luciferase activity level was normalized by the RFP level. RFP level measured by a Fluorometer.

Antibodies

Anti-HA monoclonal antibody (Genescript biotech Co.), Anti-p55 polyclonal antibody (Thermo Fisher Scientific Co.), Anti-p24 monoclonal antibody (Abcam plc.), Anti-RFP antibody polyclonal (TaKaRa bio Inc.), Anti-GFP antibody monoclonal (Clontech Laboratories Co.) and Anti-Firefly antibody monoclonal (Calbiochem®) are commercially available.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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