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hALP, A Novel Transcriptional U Three Protein (t-UTP), Activates RNA Polymerase I Transcription by Binding and Acetylating the Upstream Binding Factor (UBF)*

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Transcription of ribosome RNA precursor (pre-rRNA) and pre-rRNA processing are coordinated by a subset of U three proteins (UTPs) known as transcriptional UTPs (t-UTPs), which participate in pre-rRNA transcription in addition to participation in 18 S rRNA processing. However, the mechanism by which t-UTPs function in pre-rRNA transcription remains undetermined. In the present study, we identified hALP, a histone acetyl-transferase as a novel t-UTP. We first showed that hALP is nucleolar, and is associated with U3 snoRNA and required for 18 S rRNA processing. Moreover, depletion of hALP resulted in a decreased level of 47 S pre-rRNA. Ectopic expression of hALP activated the rDNA promoter luciferase reporter and knockdown of hALP inhibited the reporter. In addition, hALP bound rDNA. Taken together these data identify hALP as a novel t-UTP. Immunoprecipitation and GST pulldown experiments showed that hALP binds the upstream binding factor (UBF) *in vivo* and *in vitro*. It is of importance that hALP acetylated UBF depending on HAT *in vivo*, and hALP but not hALP (ΔHAT) facilitated the nuclear translocation of the RNA polymerase I (Pol I)-associated factor 53 (PAF53) from the cytoplasm and promoted the association of UBF with PAF53. Thus, we provide a mechanism in which a novel t-UTP activates Pol I transcription by binding and acetylating UBF.

In eukaryotes the nucleolus is a compartment for ribosome biosynthesis, which includes transcription of the ribosomal RNA precursor (pre-rRNA),[4] processing of pre-rRNA, and sequential assembly of ribosomal large and small subunits.

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[4] The abbreviations used are: pre-rRNA, ribosomal RNA precursor; UBF, upstream binding factor; t-UTP, transcriptional UTP; snoRNA, small nucleolar RNA; ETS, external transcribed spacer; ITS, internal transcribed spacers; TAF, TBP-associated factor; TBP, TATA box-binding protein.

The large 47 S pre-rRNA containing the sequences for mature 18 S, 5.8 S, and 28 S rRNA, two external transcribed spacers (ETS), and two internal transcribed spacers (ITS) is transcribed by RNA polymerase (Pol) I from the ribosome gene (rDNA) in the nucleolus. After chemical modification including ribose methylation and pseudouridine conversion at numerous sites, the 47 S pre-rRNA is cleaved at specific sites to produce mature 18 S, 28 S, and 5.8 S rRNAs. The 18 S rRNA is incorporated into the ribosomal 40 S subunit, and the 28 S and 5.8 S rRNAs are incorporated into the ribosomal 60 S subunit with 5 S rRNA transcribed by polymerase III elsewhere.

Modifications and cleavages of pre-rRNA are directed by small nucleolar RNAs (snoRNAs) (1, 2). U3 snoRNA base pairs with sequences in the 5′ ETS and ITS-1 blanking 18 S rRNA (3) of the 47 S pre-rRNA and is required for 18 S rRNA processing. U3 snoRNA-associated proteins (UTPs) play essential roles in 40 S subunit biogenesis and are identified as small subunit (SSU) processome components as they are nucleolar, associated with U3 snoRNA and they are required for 18 S rRNA processing (4).

Pol I transcriptional activation involves association of the pre-initiation complex (PIC) with the rDNA promoter region followed by recruitment of other transcription factors and Pol I subunits. PIC contains the upstream binding factor (UBF) and the TATA box-binding protein (TBP) containing complex SL1 (5, 6). UBF is an HMG box-containing protein, which binds directly with rDNA and plays an important role in the recruitment of SL1 and Pol I to the rRNA promoter (7–9). SL1 is a multiprotein complex consisting of TBP and three distinct TBP-associated factors (TAFs) TAF$_I$110, TAF$_I$63, and TAF$_I$48 (10, 11). SL1 is required for accurate and promoter-specific Pol I transcription (6, 12, 13).

Because UBF is a key component of PIC in Pol I transcription, the activity of UBF is tightly regulated by its association with transcriptional factors and posttranslational self-modification. The function of UBF is impaired through interaction with inhibitory proteins of cell proliferation, such as Rb (14, 15), p130 (16), and p53 (17). Under different conditions of cell growth, UBF activity is mainly regulated by protein self-modifications such as phosphorylation and acetylation. UBF is phosphorylated at multiple sites in growing cells (18, 19), but is hypophosphorylated and transcriptionally inactive in quiescent cells (20, 21). UBF is phosphorylated by cyclinD1/CDK4

(19, 22), cyclinE/CDK2 (19), casein kinase II (23), ERK1/2 MAP-kinase (24), and the p70S6-kinase (25). Phosphorylation of UBF promotes interaction with Pol I and enhances the stability of UBF and SL1 complexes thereby activating Pol I transcription (18, 23, 25, 26). In addition, UBF activity is also regulated by acetylation (27–29). For instance, UBF is acetylated and activated by CBP/p300 while it is deacetylated and inhibited by Rb-HDAC. Acetylation of UBF facilitates transcription activation and derepression *in vitro* but does not change its DNA binding properties. It has been found that the RNA polymerase I associated factor 53 (PAF53) binds UBF and mediates the interaction between Pol I and UBF, and is involved in the formation of PIC at the rDNA promoter for the activation of rDNA transcription (30). It is of importance that acetylation of UBF mediates the translocation of PAF53 from the cytoplasm to the nucleus and thus mediates recruitment of Pol I to rDNA (31).

Recent studies have shown that Pol I transcription and pre-rRNA processing are coordinated in plants, yeast (32), and mammalian cells (33) by the SSU processome (34–37). The coordination of rDNA transcription and pre-rRNA processing is mediated mainly by UBF and a subset of UTPs which are known as transcriptional UTPs (t-UTPs) as they participate in Pol I transcription in addition to 18 S rRNA processing (37). The criteria for identification of a t-UTP is that it is required for Pol I transcription and binds rDNA, in addition to possessing the properties of a UTP. Up to this time, in yeast only seven UTPs including Utp4, Utp5, Utp8, Utp9, Utp10, Utp15, and Utp17 (37) and five human UTPs including UTP4, UTP5, UTP10, UTP15, and UTP17 have been identified as t-UTPs (38). However, the mechanisms by which t-UTPs function in Pol I transcription remain undetermined.

We previously identified 1A6/DRIM as human UTP20, which is required for 18 S rRNA processing (39). To further study the function of 1A6/DRIM in the SSU processome, we undertook isolation of 1A6/DRIM-interacting proteins using protein interaction assays with 1A6/DRIM, and KIAA1709 was found to be a 1A6/DRIM-interacting protein. Review of the pre-ribosomal network suggested that KIAA1709 (also known as KRE33) might be involved in early rRNA processing and 40 S subunit maturation (40). It was also reported that mutation of Kre33p specifically impaired export of the 40S subunit but not the 60 S subunit (41). More importantly, KRE33 is also known as hALP (human acetyltransferase-like protein) or NAT10 (N-acetyltransferase 10), which has been found to activate the hTERT promoter and acetylate free histones *in vitro* (42). Overexpression of hALP increases telomerase catalytic activity and induces telomere shortening (43). However, the function of hALP in the nucleolus remains unknown. In the present study, we identify hALP as a novel t-UTP. Further investigation demonstrated that hALP activates Pol I transcription by binding and acetylating UBF.

EXPERIMENTAL PROCEDURES

Cell Culture and hALP siRNA—U2OS, H1299, and 293T cells were grown in DMEM medium supplemented with 10%

fetal bovine serum (FBS) according to the instructions provided by the American Type Culture Collection (ATCC). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C. For silencing hALP expression, siRNA (small interference RNA) targeting hALP (44): 5′-CAGCACCA-CUGCUGAGAAUAAGA-3′ was chemically synthesized together with an unrelated control siRNA (5′-ACUACCGUU-GUUAUAGGUG-3′) (Shanghai GenePharma Co., Ltd). These synthesized siRNAs were transfected into cells at a concentration of 100 nm with Lipofectamine 2000™ (Invitrogen) according to the manufacturer's instruction.

Plasmids and Antibodies—Plasmids coding hALP and UBF were generated by RT-PCR cloning and verified by DNA sequencing. A human rRNA promoter-luciferase reporter pHrD-IRES-Luc was constructed as previously described (45). Anti-hALP polyclonal antibody was kindly provided by Dr. Bo Zhang (Peking University Health Science Center), and anti-UBF, anti-topoisomerase I antibody, anti-GFP, anti-RhoA, and anti-β-actin antibodies were obtained from Santa Cruz. Anti-Flag antibody was from Sigma. Anti-PAF53 was from Biotechnology. Anti-acetyl-lysine antibody was from Upstate.

Metabolic Labeling and Analysis of Newly Synthesized rRNA—Pulse-chase labeling was performed as described previously (46). In brief, 72 h after transfection of chemically synthesized siRNA, U2OS cells were pulsed with L-[*methyl*-^{3}H]methionine (PerkinElmer Life Sciences). Cells were then chased in medium containing 15 μg/ml of non-radioactive methionine. Total RNA was isolated with Trizol reagent (Invitrogen). The [*methyl*-^{3}H]methionine-labeled RNAs were resolved on a 1% glyoxal-agarose gel (Ambion) and detected by fluorography.

Preparation of Cellular Extracts and Immunoprecipitation—To fractionate cytoplasmic and nuclear proteins, cells were washed with PBS and swollen in ice-cold hypotonic lysis buffer A (10 mM Tris-HCl pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 0.05% Triton X-100, 1 mM EGTA, and 1 mM DTT) in the presence of protease inhibitor mixture (Sigma). The nuclear pellet was collected by centrifugation and the supernatant was collected as cytoplasmic extract. Nuclear proteins were extracted in T buffer (20 mM HEPES pH7.9, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 10% glycerol with protease inhibitor mixture).

Immunoprecipitation was performed as described previously (47). Briefly, U2OS cell lysates were prepared and incubated with anti-hALP antibody or anti-acetyl-lysine antibody-coupled protein-A agarose beads (Amersham Biosciences). Precipitated proteins were analyzed by Western blotting and co-precipitated RNAs were isolated with Trizol reagent (Invitrogen).

Northern Blotting—Northern blotting for U3 snoRNA was performed as described previously (39). In brief, the RNA sample was resolved on a 7% polyacrylamide-8.3 M urea gel and blotted onto a Hybond membrane (Amersham Biosciences) by electroblotting (Bio-Rad). Blots were hybridized with a biotin-UTP-labeled U3 RNA probe. The hybridized U3 snoRNA was detected with a BrightStar® BioDetect™ Nonisotopic Detection kit (Ambion). For detection of 47 S rRNA, RNA was loaded onto 1% glyoxal-agarose (Northern-

Max®-Gly kit, Ambion) and blotted onto BrightStar®-PLUS positively charged Nylon membrane (Ambion). The DNA oligonucleotide sequence used to probe 47 S rRNA precursor is as follows (48): 5′- CCTCTCCAGCGACAGGTCGCCAG-AGGACAGCGTGTCAGCAATAACCCGGCGGCCAAAA-TG-3′. The DNA oligonucleotide was biotin-labeled with a Biotin 3′ End DNA Labeling kit (Pierce). Hybridization was performed as described previously (49). The hybridized 47 S rRNA was detected with the BrightStar® BioDetect™ Nonisotopic Detection kit (Ambion).

Immunoblotting—Proteins were separated on SDS-PAGE and transferred onto PVDF membranes (Amersham Biosciences). After blocking with 5% milk in PBS/T (0.5% Tween-20 in PBS buffer), blots were probed with corresponding antibodies. After washing with PBS/T, membranes were incubated with HRP-conjugated secondary antibodies. The immunocomplexes were detected with ECL Western blot Detection Reagent (GE Healthcare) before exposure to x-ray film.

GST Pulldown—GST-hALP and GST-UBF and their series deletion mutants were expressed in *E. coli*. Flag-UBF or Flag-hALP and its series deletion mutants were *in vitro* transcribed/translated with TNT®-coupled Reticulocyte Lysate Systems (Promega). GST-hALP bound Flag-UBF or GST-UBF bound Flag-hALP proteins were detected by Western blotting probed with anti-Flag antibody.

Immunofluorescence—Cells were plated on coverslips in 6-well plates 1 day before harvest. Double indirect immunofluorescence was performed with a monoclonal anti-UBF antibody and a polyclonal anti-hALP antibody. UBF-specific immunocomplex was detected with TRITC-conjugated goat anti-mouse IgG and hALP-specific immunocomplex was detected with FITC-conjugated goat anti-rabbit IgG. Immunofluorescence signals were recorded by confocal laser scanning microscopy (Leica TCS-ST2).

Chromatin Immunoprecipitation Assays (ChIP) and Re-ChIP—ChIP experiments were performed as described previously (50). Briefly, immunoprecipitation was performed with antibodies coupled to protein A/G-Sepharose after cells were fixed with 1% formaldehyde. Immunoprecipitated chromatin-derived DNA was analyzed by PCR with primer pairs specific for the ribosomal promoter as described previously (51) (forward primer: 5′-CGCTGCTCCCGCGTGTGTCC-3′; reverse primer: 5′-CAGCGACAGGTCGCCAGAGG-3′). The primer sequences used to amplify the U6 and GAPDH promoters were as described previously (52). The PCR products were resolved on agarose gel and visualized with ethidium bromide.

For Re-ChIP assays, reactions were immunoprecipitated with the anti-hALP antibody as previously described (50). Complexes were eluted by incubation in 10 mM DTT at 37 °C for 30 min and diluted 50 times with dilution buffer, followed by a second immunoprecipitation with either anti-UBF antibody or mouse IgG as described (52).

RESULTS

hALP Is a SSU Processome Component—We first constructed plasmids coding GFP-hALP and a series of GFP-

hALP deletion mutants and transfected each of these plasmids into U2OS cells. The cellular localization of hALP was observed under fluorescence microscopy. As shown in Fig. 1*A* and previously described (44), GFP-hALP localized predominantly in the nucleolus. The GFP-hALP deletion mutants containing the C terminus including deletion mutants GFP-hALP-HAT+C, GFP-hALP-C, and GFP-hALP-ΔHAT localized in the nucleolus but the deletion mutant GFP-hALP-N localized in the cytoplasm indicating that the N terminus was not required for the nucleolar localization. However, compared with GFP-hALP-N, GFP-hALP-N+HAT localized in the nucleus suggesting that the HAT domain may mediate its localization in the nucleus. Expression of GFP-hALP and its deletion mutants were evaluated by Western blotting as shown in Fig. 1*A* (*lower right*).

To address whether hALP is required for rRNA processing, hALP-specific siRNA was transfected into U2OS cells and pulse-chase labeling was performed to analyze newly synthesized rRNA. Efficiency of hALP knockdown was determined by Western blotting using anti-hALP antibody (Fig. 1*B*, *left upper panel*). Densitometry scanning of hALP bands is shown in Fig. 1*B* (*right panel*). The pulse-chase results showed that the 18 S rRNA level clearly decreased at 15 min, 30 min, 45 min, and 1 h over the chase time in the hALP-deficient cells (Fig. 1*C*, *right panel*). Analysis of U3 snoRNA levels by Northern blotting showed that U3 snoRNA was not affected by knockdown of hALP (Fig. 1*B*, *left lower*). Taken together, these results demonstrate that depletion of hALP protein inhibits 18 S rRNA processing.

To determine whether hALP is associated with U3 snoRNA, immunoprecipitation was performed using anti-hALP antibody. Protein and RNA from the precipitates were analyzed in parallel. Protein extracted from one-half of the precipitate was subjected to Western blotting to analyze for hALP. RNA was extracted from the remaining half of the precipitate and used for Northern blotting to analyze for U3 snoRNA. The results show U3 snoRNA was present in the hALP-precipitate (Fig. 1*D*) demonstrating hALP was associated with U3 snoRNA. Taking these results together, we identify hALP as a SSU processome component.

hALP Activates Pol I Transcription—To determine whether hALP is required in Pol I transcription, 47 S rRNA was analyzed by Northern blotting when hALP was depleted by siRNA. The extreme 5′-end of the external transcribed sequence of the pre-rRNA, which is rapidly processed in the cell, was used as a probe for Northern blotting to determine the rate of rDNA transcription initiation as previously described (53). As shown in Fig. 2*A* (*left upper panel*), the 47 S rRNA level decreased in hALP-deficient cells. This experiment was repeated three times and the density of 47 S rRNA bands was scanned. The differences in the 47 S rRNA band densities were analyzed and showed that the 47 S rRNA level was significantly decreased compared with control siRNA-treated cells ($p < 0.05$, Fig. 2*A*, *left lower panel*). The efficiency of hALP knockdown by siRNA is shown in Fig. 2*A* (*right panels*). To further confirm the function of hALP in Pol I transcription, a human rRNA promoter luciferase reporter plasmid pHrD-IRES-Luc was constructed as described previ-
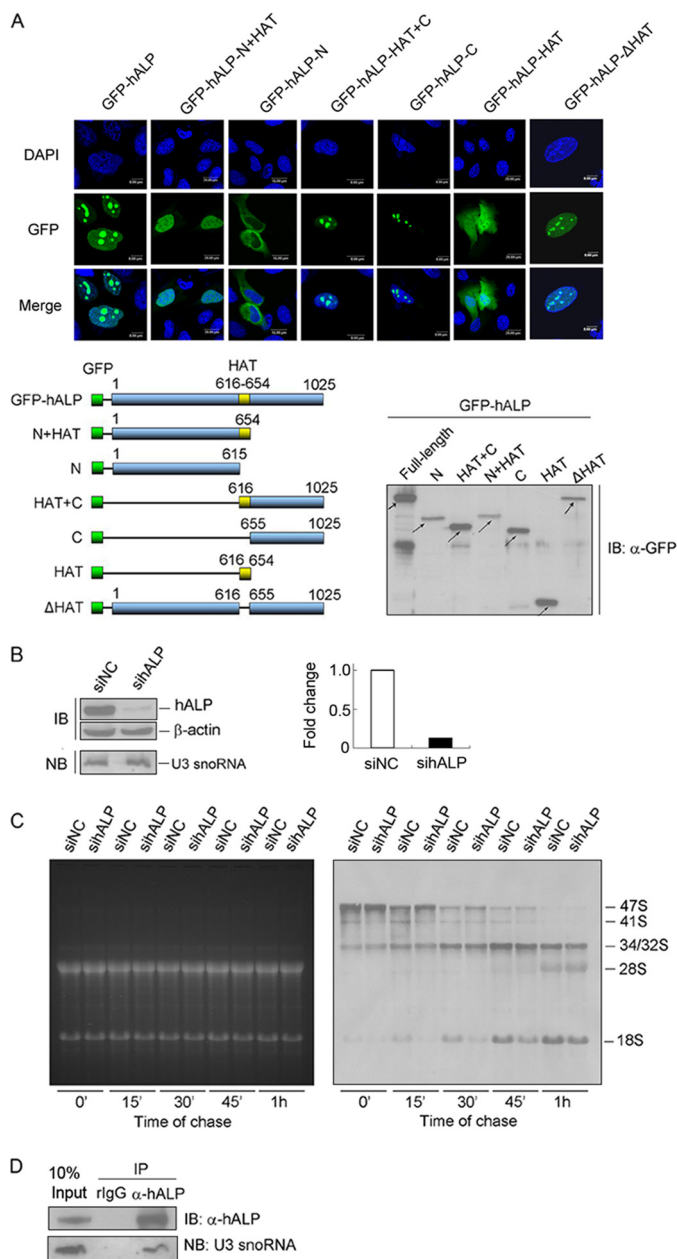
FIGURE 1. **hALP is an SSU processome component.** *A*, schematic structure of plasmids coding GFP-hALP and series deletion mutants (*lower left*). Plasmids coding GFP-hALP and its series deletion mutants were transfected into U2OS cells and cell lysates were prepared 24 h after transfection. Proteins from cell lysates were separated on SDS-PAGE and transferred onto a PVDF membrane. Blots were probed with anti-GFP antibody to detect expression of GFP-hALP proteins (*lower right*). The localization of GFP-hALP fusion proteins was viewed and photographed under confocal microscopy after transfections described above (*upper panel*). Nuclei were stained with DAPI. *B*, U2OS cells were transfected with a hALP-specific siRNA (*sihALP*) or a control siRNA (*siNC*). Cell lysates were prepared 72 h after transfection. Protein and RNA from cell lysates were analyzed in parallel. Proteins were subjected to Western blotting and probed with anti-hALP antibody. β-Actin was used as a loading control. RNA was separated on a 7% acrylamide gel and transferred onto a nylon membrane. The membrane was hybridized with Biotin-labeled U3 snoRNA-specific probe as described under "Experimental Procedures" (*left lower*). Densitometry scanning of hALP bands is shown (*right panel*). *C*, U2OS cells were transfected as described in *B*, and cells were pulsed with [³H]methionine 72 h after transfection. RNA was extracted from the cells at different time points as indicated at the bottom. RNA was resolved on 1% agarose gels and stained with EB and photographed (*left panel*) and newly synthesized rRNA was detected by autoradiography (*right panel*). *D*, immunoprecipitation was performed with anti-hALP antibody on U2OS cell lysates. Pre-immune rabbit IgG (*rIgG*) was used as a control.

ously (45). A plasmid coding hALP was cotransfected with pHrD-IRES-Luc into U2OS cells and luciferase activity was measured 24 h post-transfection. The DNA content was kept constant in each transfection experiment. As shown in Fig. 2*B*, hALP activated the rDNA promoter luciferase reporter in a dose-dependent manner while the vector plasmid did not show any effect. rDNA promoter luciferase reporter activity was also determined after endogenous hALP was silenced by siRNA. The results showed that knockdown of hALP inhibited the rDNA promoter luciferase activity without affecting the expression level of the Pol I transcription factor UBF (Fig. 2*C*). Thus, we demonstrate that hALP activates Pol I transcription.

hALP Binds rDNA—To determine if hALP binds rDNA *in vivo*, ChIP experiments were carried out with anti-hALP antibody. A fragment of DNA from the rDNA promoter region (51) was amplified by PCR with DNA derived from the immunoprecipitates. Fig. 3*A* shows that hALP binds rDNA *in vivo*. To determine whether hALP is associated with UBF at the rDNA, a re-ChIP experiment was performed. ChIP assay was first performed with hALP-specific antibody, and the precipitates were then subjected to re-ChIP using anti-UBF antibody. As shown in Fig. 3*B*, the rDNA fragment was present in the re-ChIP precipitate whereas the genomic DNA from the promoters of U6 and GAPDH genes was not enriched in the precipitates, demonstrating hALP is specifically associated with UBF at the rDNA promoter *in vivo*.

hALP Binds UBF in Vivo and in Vitro—To further investigate whether hALP binds UBF *in vivo*, immunoprecipitation was performed with anti-hALP antibody on U2OS cell lysates and the existence of UBF in the hALP-specific immunoprecipitate was identified by Western blotting. Fig. 4*A* shows that UBF was present in the hALP-specific immunocomplexes demonstrating that hALP is associated with UBF *in vivo*. The interaction between hALP and UBF was further verified by indirect immunofluorescence staining, which showed that hALP colocalized with UBF in the nucleolus (Fig. 4*B*).

We then asked if hALP interacts with UBF *in vitro*. To this end, GST pulldown experiments were performed with *Escherichia coli* expressed series deletion mutants of GST-hALP fusion proteins and *in vitro* transcribed/translated Flag-UBF. The GST-hALP associated Flag-UBF was identified by Western blotting using anti-Flag antibody. As shown in Fig. 4*C*, both the N terminus (hALP-N) and the C terminus of hALP (hALP-C) interacted with UBF. To map the hALP-interacting domain in UBF, GST pulldown experiments were performed with series deletion mutants of GST-UBF fusion proteins and *in vitro* transcribed/translated Flag-hALP proteins. Fig. 4*D* shows that hALP interacted with Box1, Box2, Box4 + 5 and Box6+tail of UBF but not with Box3. The N terminus of hALP (hALP-N) interacted with Box4 + 5 and Box6+tail, and

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Proteins from one half of the immunoprecipitates were separated on SDS-PAGE and transferred onto PVDF membrane. Blots were probed with anti-hALP antibody (*upper panel*). RNA was extracted from the remaining half of the immunoprecipitates and subjected to Northern blotting probed with Biotin-labeled U3 snoRNA (*lower panel*). Ten percent of the protein or RNA was loaded as an input control.
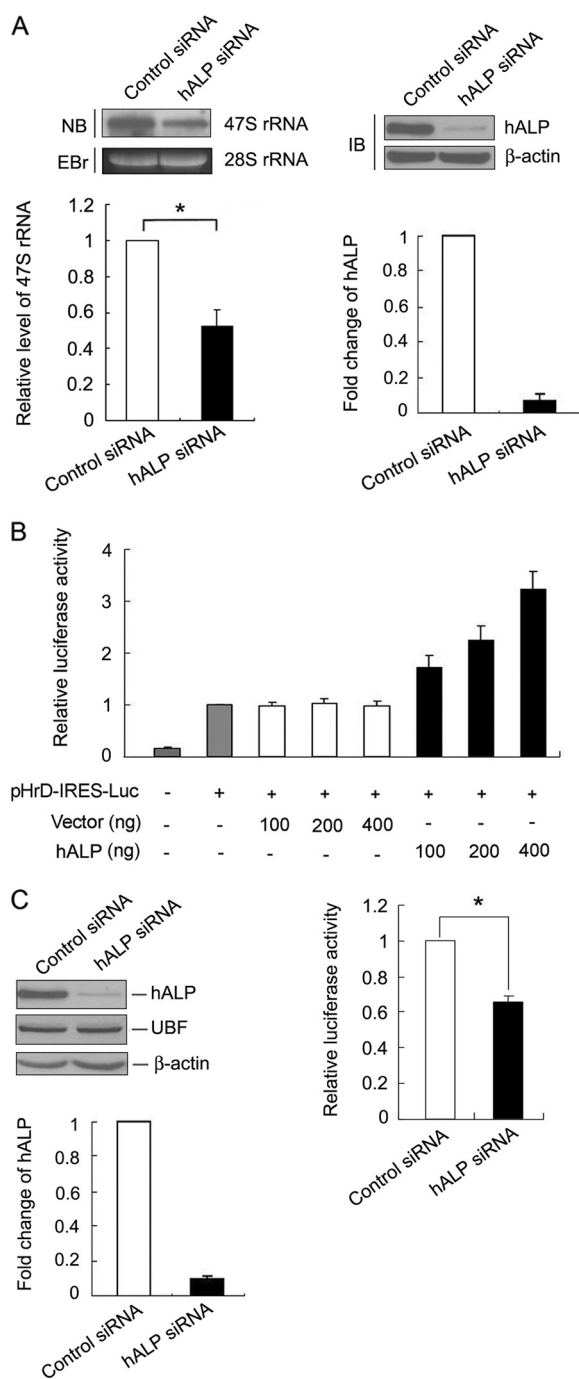
A

B

C

FIGURE 2. **hALP activates 47 S rRNA transcription.** *A*, hALP-specific siRNA was transfected into 293T cells. RNA was extracted 72 h after transfection and resolved on a 1% glyoxal-agarose gel and transferred onto a positively charged nylon membrane after staining with EB and photographing. Blots were probed with a biotin-labeled DNA oligonucleotide, which hybridizes with the extreme 5′-end of the external transcribed sequence (5′-ETS) of the pre-rRNA as described under "Experimental Procedures" (*left upper*). The 28 S rRNA stained with EB was used as a loading control. A summary of densitometry scanning of the 47 S rRNA bands from three independent experiments is shown (*left lower*). Cell lysates were simultaneously prepared 72 h after transfection of siRNAs, and proteins from the cell lysates were subjected to Western blotting and probed with anti-hALP. β-Actin was used as a loading control (*upper right*). Densitometry scanning of hALP bands from three independent experiments is shown (*lower right*). *B*, pHrD-IRES-Luc plasmid was co-transfected with increasing amount of hALP plasmid or vector plasmid into 293T cells. The amount of transfected DNA was kept constant by compensating with a carrier plasmid in each transfection. Luciferase activity was measured 24 h after transfection. The results shown are summarized from three independent experiments in duplicate. Bars
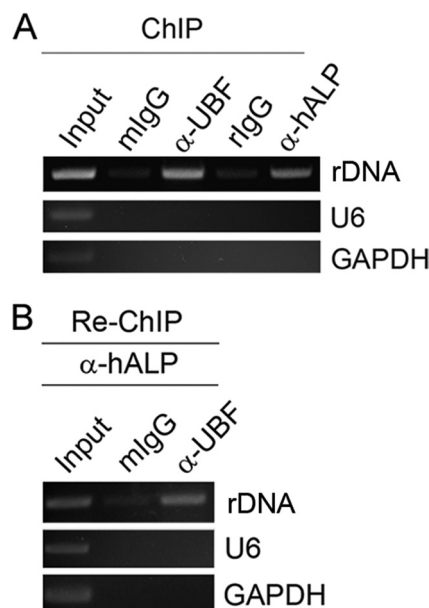
A

B

FIGURE 3. **hALP binds rDNA *in vivo*.** *A*, ChIP assay was performed with antibodies directed against either hALP or UBF. Mouse IgG or rabbit IgG (mIgG or rIgG) were used as controls for the ChIP assay. DNA was extracted from the precipitates and a DNA fragment from the rDNA promoter sequence was amplified by PCR. The PCR products were resolved on agarose gels and stained with EB. DNA fragments from either U6 or GAPDH were also amplified as controls. *B*, re-ChIP experiments were performed to confirm that hALP binds UBF at the rDNA promoter. First a ChIP experiment was performed with anti-hALP antibody. Re-ChIP was performed using anti-UBF antibody. Mouse IgG (mIgG) was used as an antibody control. A DNA fragment from the rDNA promoter sequence was amplified by PCR. The PCR products were resolved on agarose gels and stained with EB. DNA fragments from either the U6 or GAPDH promoter were also amplified as controls.

the C terminus of hALP (hALP-C) interacted with Box1 and Box2 of UBF.

hALP Acetylation of UBF Depends on HAT in Vivo—To determine whether hALP acetylates UBF *in vivo*, hALP was depleted by siRNA in H1299 cells and *in vivo* acetylation experiments were performed as described (31). Immunoprecipitation was performed with an anti-acetyl-lysine antibody and proteins from immunoprecipitates were subjected to Western blotting and probed with anti-UBF antibody. Fig. 5*A* shows that knockdown of hALP resulted in decreased acetylation of UBF without affecting the total protein level of UBF. A summary of the densitometry scanning of acetylated UBF bands from three independent experiments is shown in Fig. 5*A* (*right panel*). The results showed that knockdown of hALP significantly inhibited UBF acetylation compared with that in the control siRNA-treated cells (*p* < 0.01). Accordingly, Flag-hALP or Flag-hALP (ΔHAT) was transfected into H1299 cells

represent mean ± S.D. (*n* = 6). *C*, hALP-specific siRNA was transfected into 293T cells. Seventy-two hours after transfection of siRNA, pHrD-IRES-Luc plasmid was transfected into the cells. Luciferase activity was measured 24 h after transfection of pHrD-IRES-Luc. The fold change of Luciferase activity which is shown is summarized from three independent experiments in duplicate (*right panel*). Bars represent mean ± S.D. (*n* = 6). Proteins extracted from the above transfections were subjected to Western blotting for detection of hALP and UBF. β-Actin was used as a loading control (*left upper*). Densitometry scanning of hALP bands in three independent experiments is summarized and shown (*left lower*). Statistical analyses were performed with the two-tailed unpaired *t* test. * represents *p* < 0.05.
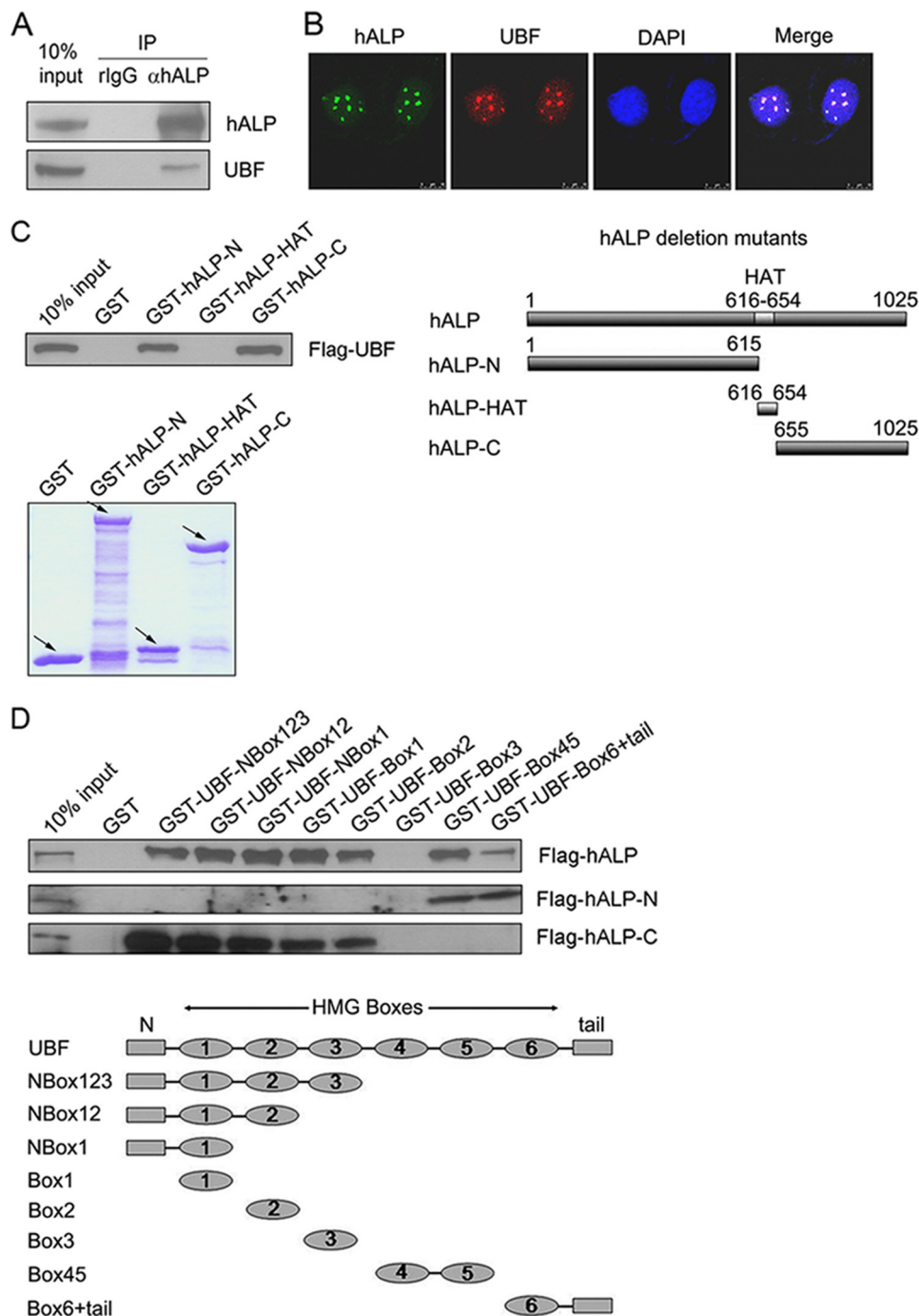
FIGURE 4. **hALP binds UBF *in vivo* and *in vitro*.** *A*, immunoprecipitation was performed with anti-hALP antibody on whole cell lysates extracted from U2OS cells. Proteins from hALP-specific immunoprecipitates were separated on SDS-PAGE and blotted onto PVDF membranes. The upper part of the blot was probed with anti-hALP antibody, and the lower part of the same blot was hybridized with anti-UBF antibody. *B*, U2OS cells were seeded on coverslips and indirect immunofluorescence was performed using polyclonal anti-hALP antibody and monoclonal anti-UBF antibody. hALP and UBF immunocomplexes were detected with FITC-conjugated goat anti-rabbit IgG and TRITC-conjugated goat anti-mouse IgG, respectively. Nuclei were stained with DAPI. Fluorescence images were photographed under confocal microscopy. *C*, GST pulldown experiments were performed with *E. coli* expressed series deletion mutants of GST-hALP fusion proteins and *in vitro* transcribed/translated Flag-UBF. GST-hALP associated UBF was detected by Western blotting using anti-Flag antibody (*left upper*). A schematic presentation of plasmids coding series deletion mutants of GST-hALP fusion proteins is shown (*right panel*). GST-hALP deletion mutant fusion proteins used in GST pulldown experiments was stained with Coomassie Bright Blue (*left lower*). *D*, GST pulldown experiments were performed with *E. coli* expressed series deletion mutants of GST-UBF fusion proteins and *in vitro* transcribed/translated Flag-hALP proteins. GST-UBF-associated hALP proteins were detected by Western blotting using anti-Flag antibody (*upper panels*). A schematic presentation of plasmids coding series deletion mutants of GST-UBF fusion proteins is shown in the *lower panel*.

and acetylation of UBF was analyzed by immunoprecipitation with anti-acetyl-lysine antibody followed by immunoblotting and probing with anti-UBF antibody. The results showed that ectopic expression of Flag-hALP promoted UBF acetylation

(Fig. 5*B*, *left panel*) whereas Flag-hALP (ΔHAT) failed to do so (Fig. 5*C*, *left panel*). The experiments in Fig. 5, *B* and *C* were repeated three times. Densitometry scanning of acetylated UBF bands from three independent experiments is sum-
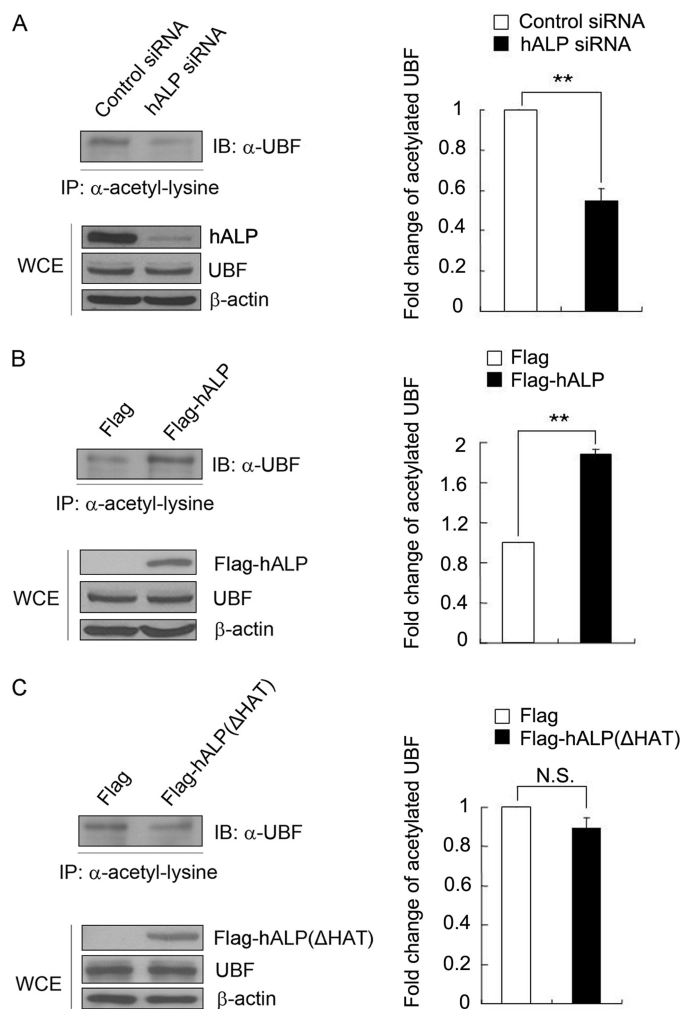
FIGURE 5. **hALP acetylated UBF depending on HAT *in vivo*.** *A*, hALP-specific siRNA was transfected into H1299 cells. Cell lysates were prepared 72 h after transfection. Immunoprecipitation was performed with anti-acetyl-lysine antibody on the cell lysates. Proteins from the immunoprecipitates were separated on SDS-PAGE and transferred onto a PVDF membrane. Blots were probed with anti-UBF antibody (*left upper*). Proteins extracted directly from whole cell extracts (*WCE*) were subjected to Western blotting to detect protein levels of hALP and UBF. β-Actin was used as a loading control (*left lower*). Densitometry scanning of acetylated UBF bands from three independent experiments is summarized in the *right panel*. *B*, plasmid coding Flag-hALP or Flag vector was transfected into H1299 cells and cell lysates were prepared 24 h after transfection. Immunoprecipitation was performed with anti-acetyl-lysine antibody on the cell lysates. Proteins from the immunoprecipitates were subjected to Western blotting for detection of UBF (*left upper*). Proteins directly extracted from the transfected cells were subjected to Western blotting probed with anti-Flag or anti-UBF antibodies (*left lower*). β-Actin was used as a loading control. Densitometry scanning of acetylated UBF bands from three independent experiments is summarized in the *right panel*. *C*, plasmid coding either Flag-hALP (ΔHAT) or Flag vector was transfected into H1299 cells, and cell lysates were prepared 24 h after transfection. Immunoprecipitation was performed with anti-acetyl-lysine antibody on the cell lysates. Proteins from the immunoprecipitates were subjected to Western blotting for detection of UBF (*left upper*). Proteins extracted directly from the transfected cells were subjected to Western blotting and probed with anti-Flag or anti-UBF antibodies (*left lower*). β-Actin was used as a loading control. Densitometry scanning of acetylated UBF bands from three independent experiments is summarized in the *right panel*. Statistical analyses were performed with the two-tailed unpaired *t* test. ** represents $p < 0.01$. *N.S.*, no significance.

marized and shown in the *right panels* of Fig. 5, *B* and *C*. The results show that ectopic expression of Flag-hALP but not Flag-hALP (ΔHAT) significantly enhances acetylation of UBF.

These results demonstrate that hALP acetylation of UBF depends on HAT.

hALP Promotes Nuclear Translocation of PAF53 from the Cytoplasm and Recruitment of PAF53 to UBF Depending on HAT—To investigate the consequences of hALP-mediated UBF acetylation, we first analyzed nuclear translocation of PAF53 when hALP was silenced. As shown in Fig. 6*A* (*left panel*), the nuclear PAF53 level decreased in hALP deficient cells and the cytoplasmic PAF53 level increased accordingly. This experiment was repeated three times and densitometry scanning of nuclear and cytoplasmic PAF53 bands is summarized and shown in Fig. 6*A* (*right panel*). The results show that knockdown of hALP significantly inhibited PAF53 translocation from the cytoplasm to the nucleus ($p < 0.05$). To confirm this phenomenon, Flag-hALP was transfected into H1299 cells and both cytoplasmic and nuclear PAF53 were evaluated with Western blotting on cell fractions. Fig. 6*B* shows that ectopic expression of Flag-hALP resulted in an increased level of nuclear PAF53 and a decreased cytoplasmic level of PAF53. This experiment was repeated three times and the densitometry scanning of nuclear and cytoplasmic PAF53 bands is summarized and shown in the *right panel* of Fig. 6*B*. These data show that ectopic expression of hALP significantly enhanced nuclear translocation from the cytoplasm ($p < 0.05$). It was notable that UBF levels were not affected by changes in hALP expression. To further determine if this effect is dependent on the hALP-mediated UBF acetylation. The HAT-deleted hALP deletion mutant Flag-hALP (ΔHAT) was transfected into H1299 cells and translocation of PAF53 was analyzed. As shown in Fig. 6*C* (*left panel*), neither cytoplasmic PAF53 nor nuclear PAF53 was altered by expression of Flag-hALP (ΔHAT), indicating that Flag-hALP (ΔHAT) failed to induce nuclear translocation of PAF53. This experiment was repeated three times and densitometry scanning of nuclear and cytoplasmic PAF53 bands is summarized and shown in the right panel of Fig. 6*C* ($p > 0.05$). These results collectively demonstrate that hALP promotes nuclear translocation of PAF53 from the cytoplasm depending on HAT. Thus, we conclude that hALP affects nuclear translocation of PAF53 by acetylating UBF.

It has been reported that acetylation of UBF promotes the interaction of UBF with PAF53 (31). To this end, we examined the interaction of UBF and PAF53 before and after knockdown of hALP in H1299 cells. The results show that when hALP was silenced by siRNA, total UBF and PAF53 levels were not affected, however the association between UBF and PAF53 was dramatically inhibited (Fig. 6*D*, *left panel*). Densitometry scanning of UBF-associated PAF53 bands from three independent experiments is summarized and shown in Fig. 6*D* (*right panel*). It shows that depletion of hALP significantly disrupted the interaction between UBF and PAF53 ($p < 0.01$). Taken together, these findings demonstrated that hALP acetylated UBF and consequently facilitated association of UBF and PAF53 to activate Pol I transcription.

DISCUSSION

UTPs have been found to be essential components of the SSU processome (36) and t-UTPs play important roles in the
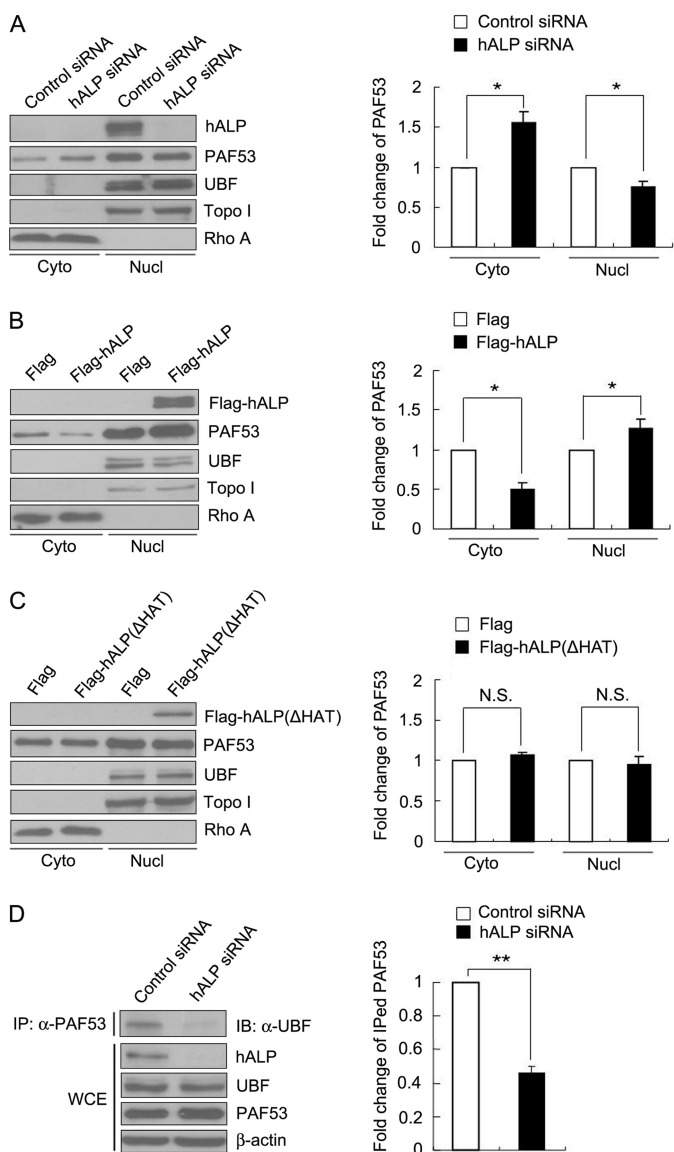
FIGURE 6. **hALP promoted nuclear translocation of PAF53 from the cytoplasm and enhanced the association of UBF with PAF53.** *A*, hALP-specific siRNA was transfected into H1299 cells. Cytoplasmic and nuclear fractions were prepared 72 h after transfection as described under "Experimental Procedures." Proteins from the cellular fractions were separated on a SDS-PAGE and transferred onto a PVDF membrane. Blots were probed with indicated antibodies. Nuclear protein topoisomerase I (*Topo I*) and cytoplasmic protein Rho A were used as nuclear and cytoplasmic markers respectively to verify correct cellular fractionation. Cytoplasmic and nuclear fractions are represented by *Cyto* and *Nucl*, respectively. A summary of densitometry scanning of PAF53 bands from three independent experiments is shown (*right panel*). *B*, plasmid coding Flag-hALP or Flag vector was transfected into H1299 cells. Cytoplasmic and nuclear cellular fractions were prepared 24 h after transfection. Proteins from the cellular fractions were separated on SDS-PAGE and transferred onto a PVDF membrane. Western blotting was performed with indicated antibodies as described in *A*. A summary of densitometry scanning of PAF53 bands from three independent experiments is shown (*right panel*). *C*, plasmid coding Flag-hALP (ΔHAT) or Flag vector was transfected into H1299 cells. Cytoplasmic and nuclear cellular fractions were prepared 24 h after transfection. Proteins from the cellular fractions were subjected to Western blotting performed with indicated antibodies as described in *A*. A summary of densitometry scanning of PAF53 bands from three independent experiments is shown (*right panel*). *D*, hALP-specific siRNA was transfected into H1299 cells. Whole cell extracts were prepared 72 h after transfection. Immunoprecipitation was performed with anti-PAF53 antibody on the cell lysates. Proteins from immunoprecipitation were separated on SDS-PAGE and transferred onto a PVDF membrane. Blots were hybridized with anti-UBF antibody (*left upper*). Proteins from

coordination of rDNA transcription and pre-rRNA processing (37). Up to the present, only five t-UTPs including UTP4, UTP5, UTP10, UTP15, and UTP17 have been identified as human t-UTPs. However, the mechanisms by which t-UTPs activate pol I transcription remain undetermined.

In the present study, we showed that hALP is nucleolar and is associated with U3 snoRNA, and depletion of hALP results in inhibition of 18 S rRNA processing. These findings identify hALP as a UTP. We also showed that knockdown of hALP resulted in a decrease in 47 S rRNA levels, and ectopic expression of hALP activated rDNA promoter luciferase activity and silencing of hALP inhibited luciferase activity demonstrating that hALP activates Pol I transcription. In addition, hALP binds rDNA and UBF. Collectively, these findings identified hALP as a novel human t-UTP. We then set out to investigate the mechanisms by which hALP activates Pol I transcription.

UBF is an essential component of PIC for transcription activated by Pol I. The activity of UBF in Pol I transcription has been demonstrated to be regulated by phosphorylation and acetylation. Important regulators of proliferation function in Pol I transcription by interrupting the interaction of SL1 with UBF or/and affecting modifications of UBF. For example, p53 represses Pol I transcription by preventing interaction between UBF and SL1 (17). Rb interacts with UBF and recruits HDAC to UBF to repress UBF acetylation (14, 15, 27).

In the present study, *in vivo* binding of hALP with UBF was identified by immunoprecipitation and immunofluorescence and *in vitro* interaction between hALP and UBF was demonstrated by GST pulldown assays. In addition, GST pulldown experiments demonstrated that the N terminus of hALP interacts with HMG Box45 and Box6+tail of UBF, whereas the C terminus of hALP interacts with HMG Box1 and 2 of UBF *in vitro*. Previous investigation has found that both Rb and CBP interact with HMG Box1 and 2 of UBF and formation of UBF-CBP complexes activate transcription of Pol I (15, 27). UBF is efficiently acetylated by CBP at HMG Box1, Box2, and Box45+Tail (27). Excess Rb prevents UBF-CBP complex formation and deacetylates UBF by recruiting HDAC and thus results in its inactivation. Thus, CBP and Rb-HDAC competitively regulate UBF acetylation states and ribosomal transcription in a "flip-flop" manner (27). In the present study, the biological function of the interaction of hALP with UBF was demonstrated by the results that knockdown of hALP inhibited UBF acetylation and ectopic expression of hALP enhanced UBF acetylation. We further demonstrated that hALP-mediated UBF acetylation is HAT-dependent. Thus, we identified a novel human t-UTP which binds and acetylates UBF. We will further investigate which HMG boxes of UBF are acetylated by hALP and investigate whether hALP functions synergistically with CBP in the acetylation of UBF.

whole cell extracts (WCE) were separated on SDS-PAGE and transferred onto a PVDF membrane. Blots were probed with antibodies directed against hALP, UBF, or PAF53. β-Actin was used as a loading control. A summary of densitometry scanning of PAF53-associated UBF bands from three independent experiments is shown (*right panel*). Statistical analyses were performed with the two-tailed unpaired *t* test. * represents $p < 0.05$; ** represents $p < 0.01$; *N.S.*, no significance.

It has been found that acetylation of UBF does not change its rDNA binding property but does enhance the nuclear translocation of PAF53 and promotes the interaction of UBF with PAF53. In the present study, we demonstrated that ectopic expression of hALP enhanced nuclear translocation of PAF53 from the cytoplasm and depletion of hALP inhibited translocation. In addition, hALP (ΔHAT) which failed to acetylate UBF, lost the ability to promote PAF53 nuclear translocation. In addition, hALP-mediated UBF acetylation facilitated interaction of UBF with PAF53. In summary, we provide a mechanism in this study in which a novel t-UTP, hALP recruits PAF53 to UBF by binding and acetylating UBF.

TAF$_I$63 which is a subunit of the SL1 factor, was found to be acetylated by PCAF, and its acetylation enhanced binding to the rDNA promoter (54). It would therefore be of interest to investigate whether hALP also acetylates SL1 factor in Pol I transcription. Because t-UTPs function in a protein complex and UBF activity is also regulated by phosphorylation, we will next investigate whether t-UTPs also recruit protein kinases or phosphatases to UBF to affect phosphorylation of UBF.

The nucleolus is an interphase cellular organelle, which is converted into the nucleolar organization region (NOR) on 5 specific chromosomes during mitosis. In late stage of mitosis, chromosomes are divided into two daughter cells and de-condense into chromatin, while the nucleolus reforms from specific regions of five relaxed chromosome to prepare to produce ribosomes for protein synthesis and cells complete cytokinesis. It has previously been found that hALP is associated with the nuclear membrane during late stage of mitosis by associating with another nuclear membrane protein hSUN1 and hALP acetylates histones for chromosome de-condensation (55, 56). It has also been found that depletion of hALP causes defects in nucleolar reformation and cytokinesis leading to G2/M cell cycle arrest and delay of mitotic exit (57). Our data demonstrate that hALP functions as a novel t-UTP which is required for Pol I transcription and 18 S rRNA processing. Taken together, hALP plays essential roles in cell division mainly in late stage of mitosis including nuclear membrane formation, chromosome de-condensation, nucleolus reformation, rDNA transcription and early rRNA processing. In addition, hALP has previously been found to be overexpressed in some types of human tumors (44), which suggests the function of hALP in human tumorigenesis needs further investigation.

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