Acetylation of UBF changes during the cell cycle and regulates the interaction of UBF with RNA polymerase I

Joachim Meraner, Markus Lechner, Adele Loidl, Maria Goralik-Schramel, Renate Voit[1], Ingrid Grummt[1] and Peter Loidl*

Division of Molecular Biology, Biocenter, Innsbruck Medical University, A-6020 Innsbruck, Austria and [1]Division of Molecular Biology of the Cell II, German Cancer Research Center, D-69120 Heidelberg, Germany

ABSTRACT

The upstream binding factor UBF, an activator of RNA polymerase I transcription, is posttranslationally modified by phosphorylation and acetylation. We found that in NIH3T3 cells, UBF is acetylated in S-phase but not in G_1-phase. To assess the role of acetylation in regulation of UBF activity, we have established an NIH3T3 cell line that inducibly over-expresses HDAC1. Both *in vivo* and *in vitro*, HDAC1 efficiently hypoacetylates UBF. Immunoprecipitation with antibodies against the Pol I-associated factor PAF53 co-precipitated UBF in mock cells but not in cells overexpressing HDAC1. Pull-down experiments showed that acetylation of UBF augments the interaction with Pol I. Consistent with acetylation of UBF being important for association of PAF53 and recruitment of Pol I, the level of Pol I associated with rDNA and pre-rRNA synthesis were reduced in cells overexpressing HDAC1. The results suggest that acetylation and deacetylation of UBF regulate rRNA synthesis during cell cycle progression.

INTRODUCTION

Cells contain multiple copies of the genes encoding ribosomal RNA, ranging from several hundred in most mammals to several thousand copies in some plants. Roughly 50% of a cell's transcriptional activity is due to the synthesis of rRNA (1,2). About half of the rRNA genes are active, while the other half is silent. There is increasing evidence that rRNA synthesis is regulated by varying the transcription rate of potentially active genes rather than by varying the ratio of active to inactive genes (3,4). As changes in rDNA transcription affect cell growth and proliferation, rDNA transcription has to be stringently regulated in response to divergent conditions, ranging from high proliferation rates to conditions of restricted growth under nutrient depletion, from early embryonic development to stages of cellular senescence (5,6). Moreover, rDNA transcription in mammalian cells fluctuates during the cell cycle, being low in early G_1-phase, reaching highest levels in S- and G_2-phase, and being shut off in mitosis (5,7). Important regulators of proliferation, such as the c-myc protein and the tumour suppressors pRb and p53, control the readout of rRNA genes (5,8).

One of the key components of the RNA polymerase I transcription initiation complex is the upstream binding factor UBF, whose activity is regulated by posttranslational modifications. In growing cells, UBF is phosphorylated at multiple sites, and phosphorylation regulates its interaction with SL1 and enhances the association of UBF with Pol I (9,10). In quiescent cells, on the other hand, UBF is hypophosphorylated and transcriptionally inactive (11,12). In addition to phosphorylation, acetylation of UBF plays an important role in the control of rDNA transcription (13,14). Acetylation and deacetylation regulate UBF activity without affecting its DNA binding properties. CBP-dependent acetylation of UBF has been shown to facilitate transcription activation, in part by counteracting the repressive effect of the tumour suppressors pRb and p130 (15–19). A 'flip-flop' model has been suggested for the regulation of rDNA transcription through acetylation of UBF by CBP and deacetylation by HDAC (14). According to this model, association with and acetylation by CBP activates Pol I transcription. Excess pRb prevents the formation of the UBF–CBP complex and, by recruiting HDAC, catalyzes UBF deacetylation

and Pol I inactivation. Though this is an attractive model, the mechanism by which UBF acetylation affects Pol I transcription remains unknown.

To elucidate the effect of acetylation and deacetylation of UBF on RNA polymerase I recruitment and rDNA transcription, we generated a cell line that overexpresses flag-tagged HDAC1 under the control of an inducible promoter. Using this cell line, we demonstrate that UBF is deacetylated upon induction of HDAC1, whereas pRb remains acetylated. Deacetylated UBF does not interact with PAF53, a subunit of Pol I, suggesting that acetylation of UBF promotes the association of Pol I with the rDNA promoter. As a consequence, the assembly of productive Pol I transcription complexes is impaired, leading to a decrease in pre-rRNA synthesis. The finding that association of acetylated UBF and PAF53 in NIH3T3 cells occurs exclusively during S- and G_2-phase underscores the importance of UBF acetylation for cell cycle-dependent regulation of rRNA synthesis.

MATERIALS AND METHODS

Cell culture and synchronization

NIH3T3 cells (DSMZ ACC59) were cultivated in DMEM/Ham′s F12-K medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 0.1 mg/ml penicillin-streptomycin. Cell cycle durations of exponentially growing cells were wild type, 15.5 h; HDAC1-flag (clone 1D), 22.5 h; mock control, 21.7 h. For synchronization cells were grown in medium with 0.5% FCS for 48 h and induced for proliferation by adding of fresh medium containing 10% FCS. Cell cycle-specific stages were obtained by harvesting cells at 0 h (G_0), 10 h (G_1), 16 h (S) and 20 h (G_2), respectively.

Transfection of cells

To generate a cell line that expresses HDAC1 under the control of the lac promoter, NIH3T3 cells were transfected with pCMVLacI® (LacSwitch® II Inducible Mammalian Expression System; Stratagene) using Polyfect (Quiagen) according to the manufacturer′s instructions. After selection with hygromycin one clone was used for transfection with either mock (flag sequence)- or mHDAC1-flag-pOPRSVI/MCS expression vector (Stratagene). Individual cell clones were analysed for HDAC expression in the presence of 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Unless otherwise stated, our data refer to the mock- and 3T3-clone 1D after 24 h of IPTG induction.

Antibodies

Antibodies were purchased from the following sources. Anti-flag® M2 from Sigma; anti-HDAC1, rabbit polyclonal antibody from Zymed; anti-acetyl-lysine and anti-acetyl-histone H4, rabbit polyclonal antibodies from Upstate; anti-UBF and anti-pRb, rabbit polyclonal antibodies from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against the Pol I subunits PAF53 and RPA116 have been described elsewhere (20).

Immunoprecipitation assays

Samples (100 µl) (equivalent to 200 µg of total protein) were incubated at 4°C overnight with either 5 µg/ml antibody (30 min at 4°C) before binding to 30 µl of protein A–Sepharose or with 30 µl of equilibrated anti-flag® M2 agarose (Sigma). After centrifugation (1000 g for 5 min) the pellets were washed with 40 vol of buffer E [10 mM Tris–HCl, pH 7.5, 100 mM KCl, 1.5 mM MgCl₂, 10% (v/v) glycerol], and proteins were analysed on western blots.

Pull-down experiments

Recombinant flag-UBF from insect cells was immunoprecipitated with anti-flag® M2 agarose at 4°C overnight. [^{14}C]acetylated or non-acetylated flag-UBF beads (30 µl) were incubated with nuclear extracts (100 µl) in the presence of 0.1 µM TSA at 4°C overnight, washed with 40 vol buffer E and analysed on western blots.

Immunofluorescence

Cells grown on cover slips were fixed with 1% (v/v) formaldehyde in phosphate-buffered saline (PBS) at 4°C for 15 min, rinsed with PBS and permeabilized in 80% (v/v) ethanol for 4 h at 4°C and 0.25% (w/v) Triton X-100 in PBS at 4°C for 5 min and incubated with antibodies at room temperature for 2 h. Slips were incubated with FITC- or TRIC-conjugated secondary antibody (diluted 1:40; DAKO) at room temperature for 2 h and visualized in a Zeiss Axioplan fluorescence microscope with a photoimaging appliance.

Preparation of nuclear and nucleolar extracts

Nuclear extracts were prepared using the NE-PER™ Nuclear and Cytoplasmic Extraction kit (Pierce). For chromatographic separation, extracts were prepared in a different way. The cell pellet was washed with PBS and suspended in NI buffer [0.1% (w/v) sodium citrate, 0.1% (w/v) Triton X-100, pH 7.4]. The suspension was incubated for 10 min and disruption of cells was obtained by vortexing; cell disruption was checked by microscopy. Isolated nuclei were washed with PBS, suspended in extraction buffer [10 mM Tris–HCl, pH 7.8, 200 mM NaCl, 1.5 mM MgCl₂, 0.5% (v/v) NP-40, supplemented with protease inhibitor cocktail (Roche)] and extracted under shaking for 90 min. After centrifugation at 12 000 g for 15 min the supernatant was taken as the nuclear extract.

Nucleoli were isolated as described elsewhere (21) with modifications. Isolated nuclei, suspended in 5 ml of 0.25 M sucrose, were centrifuged through 5 ml of 0.88 M sucrose at 1650 g for 10 min at 4°C, washed with 5 ml of 0.34 M sucrose (+0.1 mM phenylmethylsulfonyl fluoride) and sonicated on ice with 15 pulses (cycle 0.5, amplitude 40; UP200; Hilscher GmbH). Disruption of nuclei was checked by microscopy and staining. Nucleoli were purified by centrifugation through a cushion of 0.88 M sucrose at 2200 g for 20 min. Nucleoli were washed with PBS, suspended in extraction buffer [10 mM Tris–HCl, pH 7.8, 300 mM NaCl, 1.5 mM MgCl₂, 0.075% (w/v) NP-40, supplemented with protease inhibitor cocktail (Roche)] and incubated under shaking for 90 min. After centrifugation at 12 000 g for 15 min the supernatant was taken as the nucleolar extract. For some experiments, pure nucleoli were directly suspended in Laemmli Sample Buffer without extraction.

In vitro acetylation assay

For *in vitro* acetylation, recombinant flag-UBF from insect cells was immobilized on M2 agarose and 50 µl flag-UBF beads were equilibrated in buffer E (supplemented with 0.1 µM TSA, 0.1 mM EDTA, 1 mM DTT and protease inhibitor cocktail), suspended in a final volume of 150 µl and mixed with 30 µl of recombinant acetyltransferase p300-His (0.1 µg/ml) and 10 µl of [^{14}C]acetyl-CoA (57 µCi/µmol, 50 µCi/ml; Amersham Biosciences). Reaction mixtures were incubated at 30°C for 2 h. ^{14}C-acetyl-labelled flag-UBF beads were washed with 50 vol of buffer E and used for FDAC-assays or pulldown experiments.

For autoradiography, ^{14}C-acetyl-labelled flag-UBF beads were centrifuged at 1000 *g* for 5 min, mixed with an equal vol of 2× Laemmli sample buffer and heated to 95°C for 5 min. After centrifugation, the supernatant was subjected to SDS–PAGE. Gels were dried, exposed on phosphoimager screens and analysed on a STORM 840 (Molecular Dynamics).

Deacetylase assay

HDAC- or FDAC-activities were determined as described (22) using [^{3}H]acetate prelabelled chicken reticulocyte histones or [^{14}C]acetate prelabelled flag-UBF beads. Samples (50 µl) were mixed with 10 µl prelabelled core histones (40 µg) or 30 µl of ^{14}C-prelabelled flag-UBF (4 µg) and incubated at 37°C for 2 h. The reaction was stopped by addition of 1 M HCl/0.4 M acetate and ethylacetate. After centrifugation aliquots of the upper phase were counted for radioactivity.

Gel filtration chromatography

Nuclear extracts were subjected to gel filtration chromatography, using a Tosoh TSK-G4000 PWXL column (Tosoh Biosep). The column was equilibrated [10 mM Tris–HCl, pH 7.8, 100 mM NaCl, 0.5 mM EDTA, 10% (v/v) glycerol], the flow rate was maintained at 0.4 ml/min, and fractions of 400 µl were collected.

RESULTS

Characterization of a cell line that overexpresses HDAC1 under the control of an inducible promoter

We have established an NIH3T3 cell line that expresses flag-tagged HDAC1 under the control of an IPTG-inducible lac promoter placed upstream of an RSV-promoter. After extensive characterization of different clones, one clone (termed 3T3-clone 1D) and a corresponding mock clone were chosen for the subsequent experiments. Unless otherwise stated, our data were derived from the mock- and the 3T3-clone 1D, respectively, 24 h after induction of HDAC1 expression by IPTG induction.

Overexpression of flag-tagged HDAC1 caused a significant increase of total HDAC activity in 3T3-clone 1D, being ∼50% higher compared to uninduced cells (Figure 1A, upper panel). In contrast, HDAC-activity did not change in mock-transfected cells upon IPTG treatment. The increase in HDAC activity correlated with an increase in HDAC1 mRNA levels measured by semi-quantitative RT–PCR (data not shown). As expected, an anti-flag antibody detected HDAC1-flag in 3T3-clone 1D at 24 and 48 h but not in the mock cell line; a corresponding result was obtained with an anti-HDAC1 antibody (Figure 1A, lower panel). Densitometric evaluation of immunoblots revealed that the amount of overexpressed HDAC1-flag was 22% of the endogenous HDAC1 at 24 h after induction and 37% at 48 h.

To test whether HDAC1 overexpression would reduce the global acetylation level of core histones, we compared the extent of histone H4 acetylation in nuclei and nucleoli. The immunoblot in Figure 1B shows that the acetylation level of histone H4 was slightly reduced in nuclear lysates from cells overexpressing HDAC1 compared with control cells (left panel). This effect was more pronounced in lysates of isolated nucleoli (right panel). Densitometric evaluation revealed that acetylation of histone H4 was decreased by 14% in nuclear lysates and by 26% in nucleolar lysates.

To test whether recombinant HDAC1-flag has a correct physiological localization we performed immunofluorescence microscopy with anti-flag antibodies. Recombinant HDAC1 was localized in the nuclei of HDAC1-overexpressing cells, while mock cells exhibited negligible background staining (Figure 1C). Immunofluorescence analysis of HDAC1-flag and the nucleolar marker UBF at higher magnification revealed that HDAC1-flag not only localized to nuclei but was also present in the nucleoli (Figure 1D).

These results show that upon IPTG induction enzymatically active HDAC1-flag is expressed which is correctly located in the nucleus and deacetylates histones.

UBF is hypoacetylated in cells overexpressing HDAC1

When nuclear extracts from 3T3-clone 1D and mock cells were size-fractionated by gel filtration, HDAC activity eluted in two peaks, a main fraction of ∼800 kDa and a minor one of ∼1.3 MDa (Figure 2A). Flag-tagged HDAC1 was present in both peaks, albeit with higher abundance in the 1.3 MDa fractions (data not shown). Two lines of evidence suggest that overexpressed HDAC1-flag was assembled into physiologically relevant protein complexes. First, NIH3T3 cells displayed an identical distribution of HDAC activity after gel filtration chromatography, and second 3T3-clone 1D showed a 20% higher HDAC activity in the 1.3 MDa peak compared with the mock control.

Western blot analysis of individual fractions from the gel filtration column (Figure 2A) with anti-UBF antibodies showed that the 1.3 MDa complex (fraction 15) contains UBF (Figure 2B, upper panel). Significantly, immunoblotting with anti-acetyl-lysine antibodies indicated that UBF is acetylated in control cells but not in 3T3-clone 1D (Figure 2B, centre panel). Fraction 15 also contained pRb (Figure 2B, centre and lower panel; marked by asterisk) that was acetylated both in mock control cells and 3T3-clone 1D. The finding that UBF, but not pRb is hypoacetylated in 3T3-clone 1D indicates that the HDAC1 complex displays a distinct substrate specificity.

To further prove that UBF is deacetylated in HDAC1-overexpressing cells we incubated fraction 15 of the gel filtration column (Figure 2A) with anti-acetyl-lysine antibodies and monitored precipitation of UBF and pRb on immunoblots (Figure 2C). Clearly, anti-acetyl-lysine antibodies precipitated UBF from mock cells, but not from 3T3-clone

1D (left panel). In contrast, anti-acetyl-lysine antibodies precipitated pRb both in mock and 3T3-clone 1D (centre panel, marked by asterisk; right panel). Thus, overexpressed HDAC1 specifically deacetylates UBF in the 1.3 MDa complex.
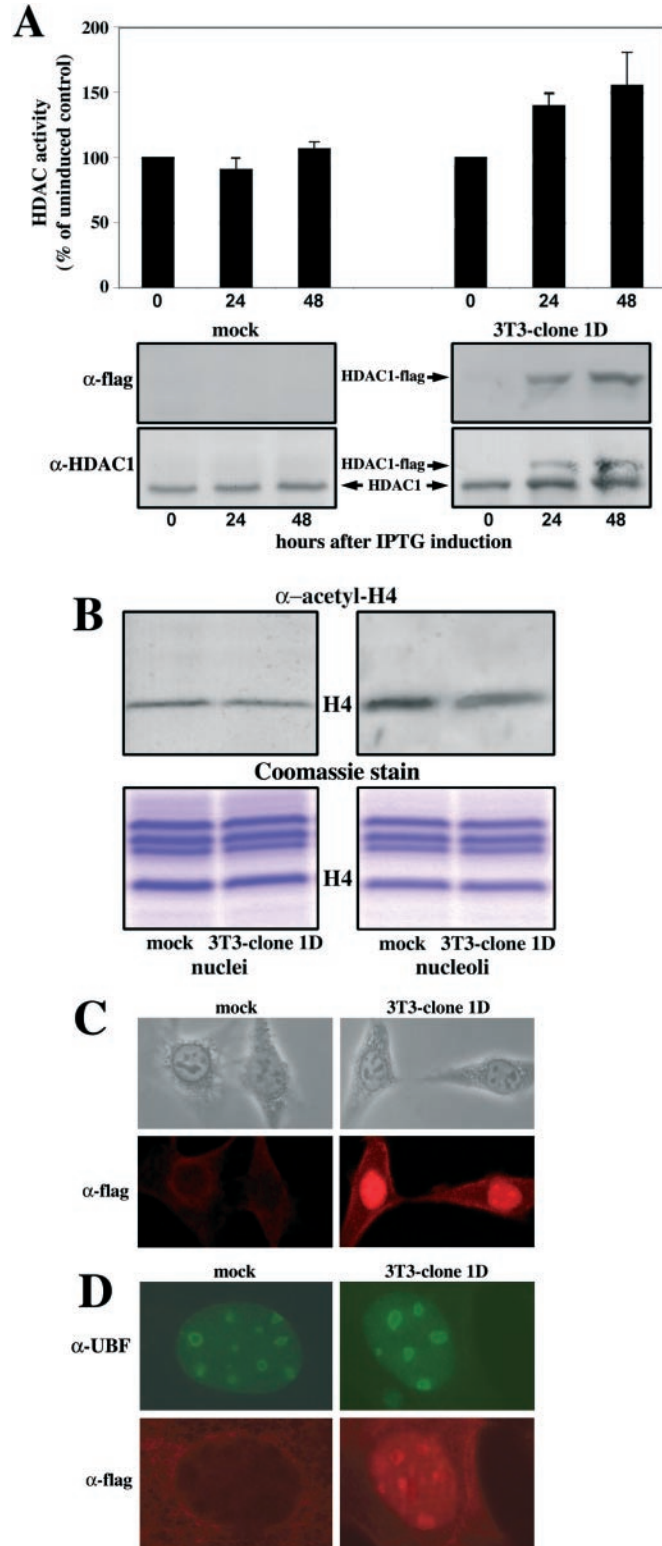
As UBF is an essential Pol I transcription factor, we examined the level of UBF acetylation in extracts from isolated nucleoli. While the amount of UBF was similar in mock cells and 3T3-clone 1D (Figure 2D), anti-acetyl-lysine antibodies precipitated significant amounts of UBF from nucleolar extracts of mock cells, whereas practically no UBF was precipitated from 3T3-clone 1D (Figure 2D). This indicates that UBF is acetylated in nucleoli of mock cells and is deacetylated upon induction of HDAC1.

In vitro acetylation and deacetylation of UBF

To investigate the functional significance of UBF acetylation, we expressed flag-tagged murine UBF in insect cells and monitored acetylation with anti-acetyl-lysine antibodies. As shown in Figure 3A, the acetylation level of UBF expressed in Sf9 cells is very low (Figure 3A, lane 3). However, recombinant UBF was efficiently acetylated *in vitro* after incubation with [^{14}C]acetyl-CoA and recombinant acetyltransferase p300, as revealed by measuring incorporated radioactivity, autoradiography and immunoblotting with anti-acetyl-lysine antibodies (Figure 3B).

Next, we used *in vitro* labelled UBF as a substrate to assay deacetylase activity. Fraction 15 from the TSK gel filtration column (Figure 2A) containing the 1.3 MDa HDAC1-activity peak deacetylated ∼10% of UBF and ∼30% of core histones compared to input levels (Figure 3C). A similar result was obtained with purified recombinant HDAC1, demonstrating that UBF is deacetylated by HDAC1 *in vitro*.

Acetylation modulates the interaction of UBF with Pol I and transcription complex formation

To investigate the functional consequences of UBF deacetylation, we examined the ability of acetylated and deacetylated UBF to interact with the Pol I transcription apparatus. UBF has been shown to interact with PAF53, a 53 kDa protein that is tightly associated with Pol I (23). Western blot analysis showed that the 1.3 MDa fraction of the gel filtration column

Figure 1. Characterization of a stably transfected NIH3T3 cell line overexpressing HDAC1 under the control of an inducible promoter. (**A**) HDAC activity (upper panel) and expression of HDAC1-flag protein (lower panels) in mock cells and 3T3-clone 1D after IPTG induction. HDAC activity was measured by the standard assay with chicken reticulocyte core histones as substrate. Enzyme activity is expressed as percentage of the uninduced cell line. Error bars indicate the SD from the mean of three independent experiments. Production of recombinant flag-tagged HDAC1 protein was determined by immunoblotting with anti-flag antibodies. Total HDAC1 (endogenous HDAC1 and recombinant flag-tagged HDAC1) was detected by immunoblotting with anti-HDAC1 antibodies; 30 μg of total protein were applied to each lane. (**B**) Acetylation of histone H4 in mock cells and 3T3-clone 1D. Bulk histone H4 acetylation was determined in lysates of nuclei (left panels; 25 μg total protein per lane) and nucleoli (right panels; 20 μg total protein per lane) of IPTG induced mock- and HDAC1-overexpressing cells by immunoblotting with an anti-acetyl-H4 antibody. Alkaline phosphatase conjugated secondary antibodies were used for visualization of immunosignals (upper panels). The corresponding Coomassie stained gels are provided as loading control (lower panels). (**C**) Subcellular localization of overexpressed flag-tagged HDAC1. Exponentially growing mock- and HDAC1-overexpressing cells were analysed by phase contrast (upper panels) and immunofluorescence microscopy (lower panels) with anti-flag antibodies. Magnification, 400×. (**D**) Subnuclear localization of overexpressed flag-tagged HDAC1 and UBF. Exponentially growing mock- and HDAC1-overexpressing cells were analysed by immunofluoresence microscopy with anti-UBF antibodies (green fluorescence) and anti-flag antibodies (red fluorescence). Magnification, 1000×.

(fraction 15) also contained Pol I (Figure 4A; input). If anti-PAF53 antibodies were used to precipitate Pol I from this fraction, UBF was co-precipitated from mock-transfected cells but not from 3T3-clone 1D (Figure 4A). Similar amounts of RPA116, the second largest subunit of Pol I, were co-precipitated in both mock cells and 3T3-clone 1D, suggesting that deacetylation of UBF by HDAC1 impairs the interaction of UBF with Pol I.

To test whether acetylation of UBF augments the interaction with Pol I, pull-down assays were performed using bead-bound *in vitro* acetylated flag-UBF and nuclear extracts in the presence of Trichostatin A (Figure 4B; [^{14}C] panel). Trichostatin was added to prevent deacetylation during the

incubation time. Analysis of captured proteins by immunoblotting revealed that acetylated UBF retained PAF53, while non-acetylated UBF did not interact with PAF53 (Figure 4B). Likewise, RPA116 was only retained by acetylated UBF, suggesting that acetylation is a major determinant for the association of UBF with Pol I. In addition, the pull-down experiments show that endogenous HDAC1 is more efficiently retained by acetylated UBF, consistent with acetylation facilitating the interaction of UBF and Pol I (Figure 4B).

To test whether UBF acetylation modulates the nucleolar retention of UBF or Pol I, we compared the amount of UBF and Pol I in nucleoli isolated from 3T3-clone 1D and mock cells. For this purpose, cells were synchronized in G$_1$- and S-phase, as initial experiments have shown that the effect of HDAC1 on rRNA synthesis was most pronounced in S-phase. In G$_1$-phase, i.e. 10 h after release from serum starvation, no significant differences in the level of UBF and Pol I were observed in nucleoli of 3T3-clone 1D and mock cells (Figure 4C). However, in S-phase (16 h after refeeding), the level of RPA116 and PAF53 that was retained in nucleoli was considerably lower in 3T3-clone 1D than in the mock control. The amount of UBF, on the other hand, remained unchanged (Figure 4C). The amount of total cellular PAF53 and RPA116 was equal (data not shown). Thus, overexpression of HDAC1 either impairs the recruitment of Pol I to rDNA or weakens the association of Pol I with nucleolar structures. Given that the amount of Pol I that is associated with rDNA reflects cellular pre-rRNA synthetic activity, the level of 45S pre-rRNA should be lower in S-phase cells overexpressing HDAC1 than in G$_1$-phase cells or mock control. Indeed, the decrease of PAF53 and RPA116 levels in 3T3-clone 1D in S-phase was accompanied by a pronounced reduction of rDNA transcription (Figure 4C, pre-rRNA panel), supporting the view that HDAC1-mediated deacetylation of UBF abrogates Pol I transcription.

To verify that acetylation regulates the interaction of UBF and Pol I, we analysed the interaction of these proteins in cells that do not overexpress HDAC1. Nucleolar extracts
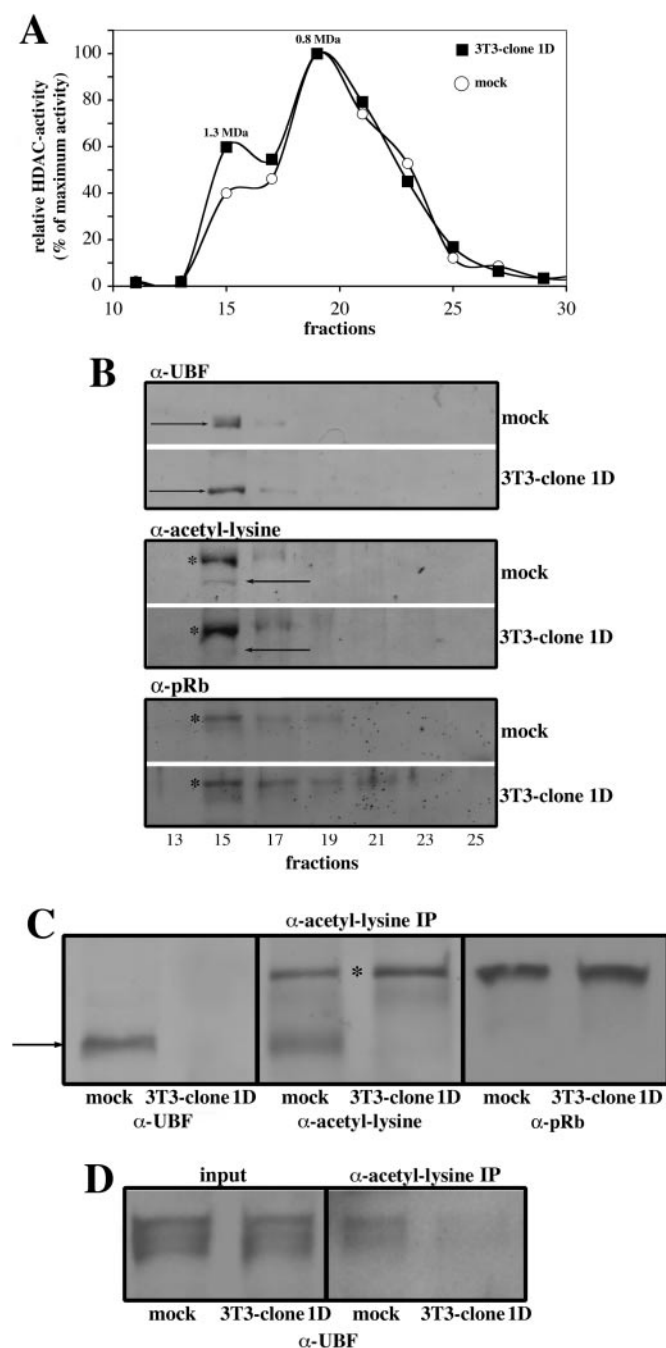
Figure 2. Hypoacetylation of UBF in HDAC1-overexpressing NIH3T3 cells during S-phase. Nuclear extracts of mock and 3T3-clone 1D in S-phase were subjected to TSK gel filtration chromatography. Chromatographic fractions (ranging from 0.2 to 1.6 MDa) were analysed by HDAC activity assay (**A**), immunoblotting (**B**) and immunoprecipitation (**C**). (A) HDAC activity of nuclear extracts after TSK gel filtration chromatography. Fractions were assayed for histone deacetylase activity which was expressed as percentage of the maximum activity (open circles, mock-transfected cells; closed squares, HDAC1-overexpressing cells). (B) Immunoblot analysis. Fractions 13–25 of the TSK chromatography (mock control and 3T3-clone 1D) were subjected to SDS–PAGE with subsequent immunoblotting using anti-UBF antibodies (upper panel), anti-acetyl-lysine antibodies (centre panel), and anti-pRb antibodies (lower panel). Arrows mark the position of UBF, asterisks mark the position of the retinoblastoma protein (pRb). (C) Immunoprecipitation of acetylated UBF. TSK fractions 15 of the mock control and 3T3-clone 1D, respectively, were subjected to immunoprecipitation with anti-acetyl-lysine antibodies. Immunoprecipitated proteins were subjected to SDS–PAGE and western blotting. Proteins were immunodetected with antibodies against UBF (left panel), acetyl-lysine (centre panel) and pRb (right panel). An arrow indicates the position of UBF, an asterisk marks the position of the retinoblastoma protein. (**D**) Immunoprecipitation of acetylated UBF in nucleolar extracts. Nucleolar extracts of mock control and 3T3-clone 1D in S-phase were subjected to immunoprecipitation with anti-acetyl-lysine antibodies. Input extracts (left panel, input) and immunoprecipitates (right panel; IP) were subjected to immunoblotting using anti-UBF antibodies for detection.
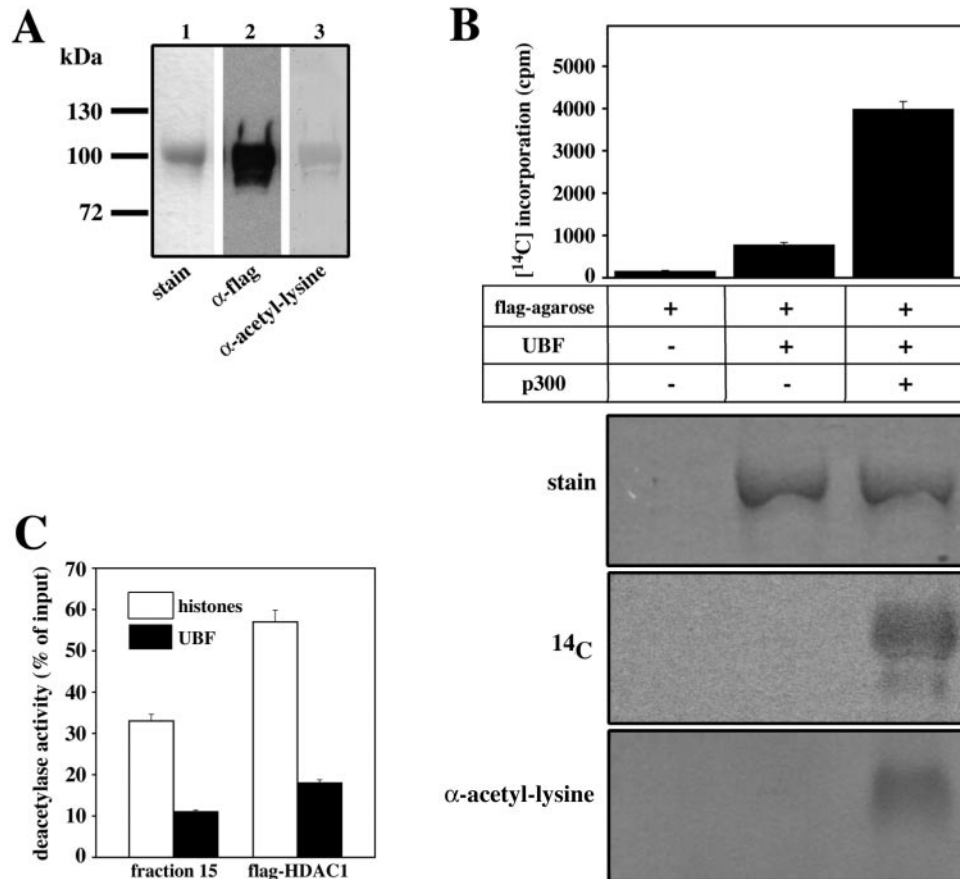
Figure 3. Acetylation and deacetylation of UBF. Recombinant flag-tagged UBF was expressed in insect cells, acetylated *in vitro* by recombinant acetyltransferase p300 and subsequently deacetylated by HDAC1. (**A**) Expression of recombinant UBF in insect cells. Flag-UBF was affinity purified by anti-flag-agarose elution. Lane 1, SDS–polyacrylamide gel (Comassie stained) of affinity-purified flag-UBF; lane 2, immunoblot with anti-flag antibodies; lane 3, immunoblot with anti-acetyl-lysine antibodies. (**B**) Acetylation of UBF by p300. Affinity-purified flag-UBF was acetylated *in vitro* by recombinant p300 and [^{14}C]acetyl-CoA. Acetyltransferase activity is expressed as radioactivity incorporation in cpm (upper part). Aliquots of the acetyltransferase assay (different composition shown in diagram table) were subjected to SDS–PAGE (Coomassie stain; upper panel) with subsequent autoradiography (^{14}C; centre panel) or immunoblotting with anti-acetyl-lysine antibodies (lower panel). (**C**) Deacetylation of UBF by HDAC1. [^{14}C]acetyl labelled flag-UBF was used as a substrate for deacetylase assays using fraction 15 (50 µl) of TSK gel filtration chromatography (Figure 2A) from 3T3-clone 1D or affinity purified flag-tagged HDAC1 (0.2 µg) from 3T3-clone 1D as enzyme source. Deacetylase activity with acetylated UBF (4 µg) as substrate (closed columns). As a reference, enzyme preparations were incubated with prelabelled chicken reticulocyte core histones (40 µg; open columns). Deacetylase activities are expressed as percent of the corresponding input radioactivity.

from synchronized NIH3T3 cells in G_0-phase (0 h), late G_1-phase (10 h), S-phase (16 h) and G_2-phase (20 h) were incubated with antibodies against PAF53 and co-immunoprecipitated UBF was analysed on western blots. As shown in Figure 5A, PAF53 did not interact with UBF in G_0- and G_1-phase cells, whereas significant amounts of UBF co-precipitated with Pol I from S- and G_2-phase extracts. The coprecipitation at 16 and 20 h can not be attributed to the moderate increase in UBF input from 0 to 16 h, since absolutely no coprecipitation of UBF takes place at 0 and 10 h. In contrast, anti-PAF53 antibodies co-precipitated RPA116 irrespective of the cell cycle stage (Figure 5A), indicating that PAF53 is rather associated with Pol I during the whole cell cycle. Noteworthy, co-precipitated UBF from S-phase cells (16 and 20 h) was acetylated, consistent with UBF acetylation enhancing the interaction with Pol I. In support of this, anti-acetyl-lysine antibodies immunoprecipitated significant amounts of UBF from S-phase nucleolar extracts, but not from G_0-phase extracts (Figure 5B). Densitometric analysis reveals that the overall amount of nucleolar UBF

increased 1.5-fold during the transition from G_1- to S-phase (0–16 h, input panel), whereas the amount of UBF that precipitated with anti-acetyl-lysine antibodies (IP panel) increased ~5-fold (densitometry not shown in figure). These results suggest that cell cycle-dependent changes of UBF acetylation are involved in the regulation of Pol I transcription.

DISCUSSION

In eukaryotes, transcription of ribosomal RNA represents >50% of total RNA synthetic activity and is tightly regulated according to the cell's biosynthetic demand for ribosomes. Numerous studies have established that almost every component of the Pol I transcription apparatus is targeted by the complex regulatory network that controls pre-rRNA synthesis (5). One of the targets that regulate Pol I transcription is UBF. UBF was shown to bind across the entire rDNA repeat, thereby leading to enrichment of the Pol I transcription machinery

(24). Binding of UBF to the upstream control element is a crucial step in the formation of the Pol I pre-initiation complex. UBF interacts with both Pol I (25) and the TBP-containing promoter selectivity factor SL-1/TIF-1B (26), thereby targeting the Pol I transcription machinery to rDNA and facilitating the assembly of transcription initiation complexes. UBF function is impaired through interacting proteins, such as pRb (15,19), p130 (16) and p53 (18,27). Moreover, UBF activity is modulated by phosphorylation at multiple sites by cyclinD1/CDK4 (28) and cyclinE/CDK2 (10), casein kinase II (9), ERK1/2 MAP-kinase (29), and the p70S6-kinase (30). In addition, UBF has been shown

to be acetylated (14), but the functional significance of this modification remained elusive.

During the last decade it became evident that HATs and HDACs not only modify histones but also non-histone proteins, such as pRb, c-myc and p53 (31–33). In practically all cases of non-histone protein acetylation the modification causes changes of the interaction properties of the protein or stabilization of protein complexes. For example, acetylation of p53 by CBP and p300, but not by PCAF, stabilizes p53 complexes, whereas deacetylation is required for its efficient degradation (34). Another example is TFIIB which has to be acetylated to interact with TFIIF, thereby activating RNA polymerase II transcription (35).

Since active ribosomal genes are characterized by a lack of intact nucleosomes (3), the inhibitory effect of HDAC1 on transcription of ribosomal genes cannot be exclusively due to a decrease of histone acetylation but also has to be attributed to deacetylation of components of the Pol I transcription machinery. The same is true for the effects of HDAC inhibitors on Pol I transcription (13). Furthermore, some of the proteins involved in the regulation of ribosomal gene transcription (e.g. pRb, c-myc and p53) (31–33) are reversibly acetylated.

Regulation of Pol I transcription through competitive recruitment of CBP and HDAC to rDNA, as well as the effect of acetylation upon rDNA transcription has been described recently (13,14,36). These studies have demonstrated that acetylation activates rDNA transcription both by acetylation of histone tails and by recruitment of the Pol I transcription apparatus to the rDNA promoter. UBF is efficiently acetylated by CBP and Tip60, both of which have been shown to stimulate rDNA transcription in a nucleosome-independent manner. Most likely, Tip60 is the responsible acetyltransferase *in vivo* (36), whereas the responsible HDAC had not been identified.

Fractionation of nuclear extracts by gel filtration chromatography has shown that acetylated UBF is part of a 1.3 MDa protein complex which contains other components of the Pol I transcription machinery, and very likely corresponds to a Pol I 'holoenzyme'. The 1.3 MDa complex contains
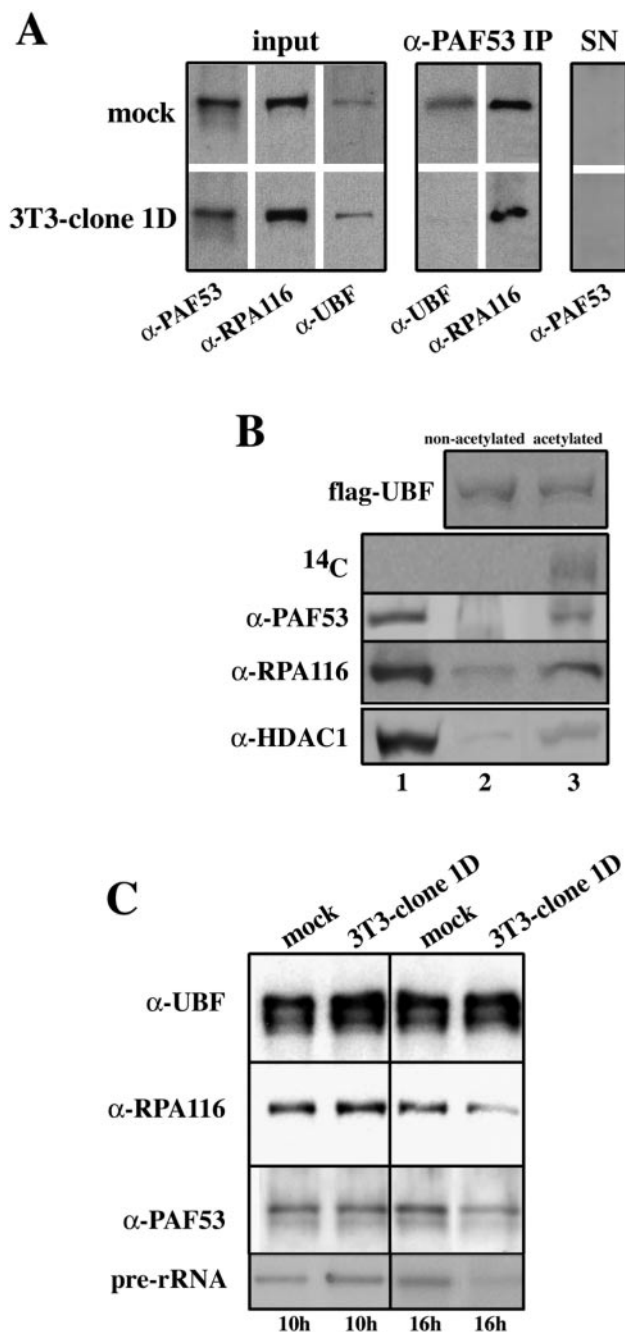

Figure 4. Deacetylation of UBF by HDAC1 inhibits the association with Pol I. (**A**) Co-immunoprecipitation of UBF with Pol I. Extracts from mock- and HDAC1-overexpressing cells were fractionated by gel filtration as shown in Figure 2. The amount of PAF53, RPA116 and UBF present in 20 μg of protein in chromatographic fractions 15 was monitored on immunoblots (input, left panel). Samples were incubated with anti-PAF53 antibodies and the immuno-precipitates were analyzed with antibodies against UBF and RPA116 (centre panel; α-PAF53 IP). To check for the efficiency of the precipitation reaction the supernatant was immunoblotted with anti-PAF53 antibodies (SN, right panel). (**B**) PAF53 does not interact with non-acetylated UBF. Nuclear extracts of NIH3T3 cells in S-phase were incubated with 5 μg immobilized, non-acetylated or [^{14}C]acetylated flag-UBF in the presence of 0.1 μM trichostatin A. Bound proteins were analysed on immunoblots with antibodies against PAF53, RPA116 and HDAC1. Lane 1, pull-down input; lane 2, non-acetylated flag-UBF pull down; lane 3, [^{14}C]acetylated flag-UBF pull down. [^{14}C] panel shows the autoradiograms. Flag-UBF panel shows the Coomassie blue stained lanes of non-acetylated and acetylated immobilized flag-UBF (dilution 1:5). (**C**) Amount of UBF, RPA116 and PAF53 in nucleoli 10 and 16 h after refeeding of serum-starved cells. Nucleoli were isolated from mock cells and 3T3-clone 1D cells at 10 h (G$_1$-phase, left panels) and 16 h (S-phase, right panels) after refeeding. Nucleolar lysates were analysed by SDS–PAGE with subsequent immunoblotting with antibodies against UBF, RPA116 and PAF53. In parallel, cellular RNA was isolated and pre-rRNA levels in the same number of cells (10 μl) was determined on northern blots using a digitonin-labelled probe against the 5′-terminal 150 bases of the primary transcript.
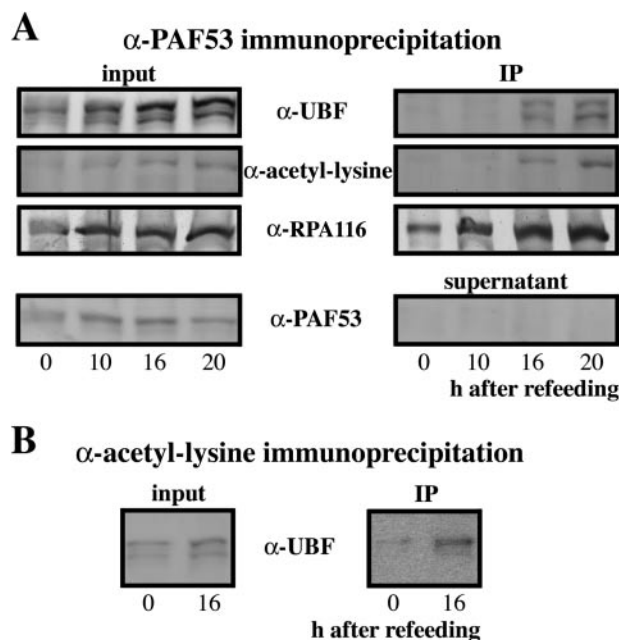
Figure 5. PAF53 interacts with acetylated UBF in S- and G_2-phase in synchronized NIH3T3 cells. (**A**) Immunoprecipitation with anti-PAF53 antibodies of nucleolar extracts of synchronized NIH3T3 cells in serum-starved cells (G_0, 0 h), G_1 (10 h after refeeding), S (16 h after refeeding), and G_2 (20 h after refeeding). Nucleolar extracts were subjected to SDS-PAGE and immunoblotted with antibodies against UBF, acetyl-lysine, PAF53 and RPA116 (input, left panels). Extracts were then immunoprecipitated with anti-PAF53 antibodies and immunoprecipitated proteins were analysed with antibodies against UBF, acetyl-lysine and RPA116 (IP, right panels). The efficiency of the immunoprecipitation reaction was checked by immunoblotting the supernatant with anti-PAF53 antibodies (right panel). (**B**) Antibodies against acetyl-lysine precipitate UBF from nucleolar extracts of S-phase cells. Nucleolar proteins from synchronized NIH3T3 cells in G_0-phase (0 h) and S-phase (16 h after refeeding) were immunoprecipitated with anti-acetyl-lysine antibodies and UBF was detected on western blots.

HDAC1 and pull-down experiments have shown that acetylated UBF retains HDAC1 whereas non-acetylated UBF does not. We analyzed the increased association of HDAC1 to acetylated flag-UBF in the presence and absence of the deacetylase inhibitor trichostatin A (data not shown). Regardless of whether trichostatin was present or not, we observed a significantly increased binding of HDAC1 to acetylated flag-UBF at 4°C; this rather suggests that the association of HDAC1 with acetylated UBF is not due to the direct catalytic interaction of enzyme and substrate, but is due to specific protein-protein interactions, maybe mediated by an interacting protein (e.g. pRb).

In HDAC1-overexpressing cells UBF is deacetylated and pre-rRNA synthesis is down-regulated. The effect of UBF acetylation was most pronounced in S-phase, indicating that reversible acetylation of UBF may be a means to modulate pre-rRNA synthesis during cell cycle progression.

Importantly, our results elucidate the mechanism underlying activation of Pol I transcription by UBF acetylation. Acetylation stimulates the interaction with PAF53, a subunit of the initiation-competent subpopulation of Pol I. The need for such a regulated recruitment becomes more evident, if one considers that yeast contains a 17-fold excess of Pol I compared with the rDNA binding factors UAF and CF (37).

The following observations suggest that reversible UBF acetylation regulates the interaction with PAF53 and thus the recruitment of Pol I to rDNA. First, acetylation of UBF occurs within HMG boxes 1 and 2 (14), the part of UBF that is required for interaction with Pol I (25). Second, overexpression of HDAC1 did not affect the nucleolar localization of UBF, whereas the nucleolar retention of Pol I was strongly decreased. Like the inhibition of Pol I transcription in HDAC1-overexpressing cells, the decrease of Pol I within the nucleolus was most evident in S-phase. This suggests that the interaction between acetylated UBF and PAF53 is important to recruit Pol I, together with associated factors, to the rDNA promoter and facilitates transcription initiation.

With regard to the mechanism by which acetylation regulates UBF function, it is conceivable that acetylation induces a structural change which may be required for the interaction of UBF with Pol I. In support of this, we found that different antibodies against UBF precipitated UBF in its deacetylated form, but failed to precipitate acetylated UBF. On immunoblots, however, all antibodies recognized both acetylated and deacetylated UBF with equal efficiency. It is also likely that UBF acetylation and phosphorylation are interrelated, both fluctuating during cell cycle progression (10,28). Acetylation assays with mutant UBF revealed that replacement of serine 388 by alanine resulted in significantly enhanced p300-mediated *in vitro* acetylation, whereas mutation of serine 484 resulted in a complete inhibition of acetylation by p300 *in vitro* (J. Meraner and P. Loidl, unpublished data). These data strongly argue for a specific and physiologically relevant interdependence of acetylation and phosphorylation events, similar to those described for the retinoblastoma protein (31). Finally, acetylation of TFIIB is necessary for a strong interaction with TFIIF, thereby activating RNA polymerase II transcription (35). Thus, acetylation-based tuning of protein–protein interactions may serve a general role in transcriptional regulation as part of the complex epigenetic regulatory network (38).

ACKNOWLEDGEMENTS

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