

CK2-mediated stimulation of Pol I transcription by stabilization of UBF–SL1 interaction

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ABSTRACT

High levels of rRNA synthesis by RNA polymerase I are important for cell growth and proliferation. *In vitro* studies have indicated that the formation of a stable complex between the HMG box factor [Upstream binding factor (UBF)] and SL1 at the rRNA gene promoter is necessary to direct multiple rounds of Pol I transcription initiation. The recruitment of SL1 to the promoter occurs through protein interactions with UBF and is regulated by phosphorylation of UBF. Here we show that the protein kinase CK2 co-immunoprecipitates with the Pol I complex and is associated with the rRNA gene promoter. Inhibition of CK2 kinase activity reduces Pol I transcription in cultured cells and *in vitro*. Significantly, CK2 regulates the interaction between UBF and SL1 by counteracting the inhibitory effect of HMG boxes five and six through the phosphorylation of specific serines located at the C-terminus of UBF. Transcription reactions with immobilized templates indicate that phosphorylation of CK2 phosphoacceptor sites in the C-terminal domain of UBF is important for promoting multiple rounds of Pol I transcription. These data demonstrate that CK2 is recruited to the rRNA gene promoter and directly regulates Pol I transcription re-initiation by stabilizing the association between UBF and SL1.

INTRODUCTION

RNA polymerase I (Pol I) is responsible for the synthesis of the large ribosomal RNA (rRNA) precursor, which is then processed into the three large ribosomal RNAs, 28S, 18S and 5.8S in mammals (1,2). rRNA transcription is critical for cell survival and its activity is exquisitely regulated during cell cycle progression and cell proliferation. Transcription of the rRNA gene is initiated by the assembly of RNA polymerase I and a defined set of transcription factors at the rRNA

gene promoter to form the pre-initiation complex (PIC). The nucleation of these factors at the promoter requires a complex network of protein–protein and protein–DNA interactions. Upstream binding factor (UBF) is an HMG box-containing factor that binds to the rRNA gene promoter and is responsible for the recruitment of the species-specific selectivity factor 1 (SL1). SL1 is a complex composed of TBP and three TBP-associated factors (TAFs), TAF₁₄₈, TAF₁₆₃ and TAF₁₁₀ (3–11). Studies using a cell-free system indicated that SL1 binds directly to UBF and is brought to the promoter by specific protein interaction between two of its subunits, TBP and TAF₄₈, and UBF (12–14). The formation of the UBF–SL1 complex at the rRNA gene promoter promotes the recruitment of the RNA polymerase I enzyme, which occurs via interactions between UBF and Pol I, and between SL1 and the bridging factor Rrn3 (also termed TIF-IA) (15–17). The assembly of the PIC and the transition from a closed to an open complex leads to promoter clearance and transcription elongation. As RNA polymerase I moves away from the promoter, a new Pol I/Rrn3 complex would then be recruited through interactions with the promoter-bound UBF–SL1 complex. Based on this model, a stable UBF–SL1 complex at the rRNA gene promoter would support high rates of rRNA synthesis by promoting multiple rounds of transcription initiation. Although the key role of UBF in nucleating the PIC at the rRNA promoter has been recently challenged (18), recent *in vivo* data have clearly demonstrated that SL1 and Pol I are recruited to chromatin through protein interactions with UBF (19,20).

The activity of UBF is regulated by posttranslational modifications such as acetylation and phosphorylation. CBP-dependent acetylation of UBF stimulates Pol I transcription by counteracting the inhibitory effect of pRb (21–23) and a recent study has shown that acetylation of UBF stimulates its interaction with Pol I, suggesting that the acetylation status of UBF influences the assembly of the PIC at the rRNA promoter (24).

In addition to acetylation, the activity of UBF is regulated by phosphorylation. Metabolic labeling studies of cultured mammalian cells demonstrated that UBF is phosphorylated under normal growth condition and both UBF phosphorylation and RNA polymerase I transcription increase upon

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serum stimulation of quiescent cells (25,26). The critical function of phosphorylation in the regulation of UBF activity has been demonstrated in several studies. SV40 large T antigen, a viral oncogenic protein that promotes cell growth, stimulates Pol I transcription by recruiting to the rRNA gene promoter a cellular kinase that phosphorylates UBF (27,28). Pol I transcriptional activity during the progression of the cell cycle is modulated, at least in part, by phosphorylation of UBF by cyclin-dependent kinases (CDK)-cyclin complexes (29,30). Reversible UBF phosphorylation of two N-terminal HMG boxes by ERK plays an important role in stimulation of rRNA gene transcription (31,32). Collectively, these studies provide compelling evidence for the important role that phosphorylation of UBF plays in the regulation of Pol I transcription. Phosphorylation of UBF has been shown to affect its DNA binding activity (31) and its interaction with other components of the transcriptional apparatus (14-33). By employing *in vitro* protein-protein interaction and DNase I footprinting assays we have shown that the phosphorylation status of UBF plays a key role in modulating the interaction between UBF and SL1 and in the recruitment of SL1 to the promoter elements of the rRNA genes (14). Moreover, mitogen-induced phosphorylation of UBF has been shown to promote its association with TBP, one of the SL1 subunits (34). However, the amino acid residues in UBF whose phosphorylation is necessary for SL1 binding and the cellular kinase responsible for their phosphorylation remain to be identified. The C-terminal region of UBF is particularly rich in phosphorylation sites for the protein kinase CK2, a ubiquitous serine/threonine kinase involved in cell growth, proliferation and survival, and kinase assays with purified factors have shown that UBF is phosphorylated by CK2 *in vitro* (25,35). CK2 is composed of two catalytic α and/or α' subunits, and two regulatory β subunits (36). Genetic studies in yeast and mammalian cells have indicated that CK2 is required for cell viability and for cell to progress through the cell cycle. CK2 is thought to participate in a wide array of cellular processes as a growing number of physiological targets for CK2 have been identified (36). Notably, a number of recent studies have also shown that CK2 directly regulates RNA polymerase II and III transcription (37-40).

In this study we examined the role of CK2 in the regulation of Pol I transcription. Our data indicate that CK2 is physically associated with the RNA polymerase I/Rrn3 complex and is present at the promoter region of the rRNA genes. Studies with a CK2-specific inhibitor and reconstituted transcription assays demonstrate that CK2 activity influences Pol I transcription *in vitro* and in cultured cells. Importantly, our results indicate that CK2-mediated phosphorylation of UBF counteract the negative effect of HMG boxes five and six and stabilizes the interaction of this factor with SL1, thus promoting multiple rounds of Pol I transcription.

MATERIALS AND METHODS

Cell lines

HEK293, HEK293T and normal diploid human fibroblast were cultured in DMEM media containing 10% fetal bovine serum in 5% CO₂ at 37 C. HeLa S3 suspension cells were

cultured in MEM media containing 5% newborn bovine serum at 37 C.

Nuclear and Nucleolar fractionation

Nuclear extracts were prepared from HeLa S3 cells as described by Zhai *et al.* (28). Nucleoli were prepared from 4 liters of exponentially growing HeLa S3 ($3-5 \times 10^5$ cells/ml) as described previously (41). Nucleolar proteins were extracted in 2 ml of TM buffer (50 mM Tris-HCl, pH 7.9, 12.5 mM MgCl₂, 1 mM EDTA, 10% glycerol, 1 mM DTT) containing 0.1 M KCl, 0.1% Nonidet P-40 (NP-40) and protease inhibitors. Nucleolar extract was then applied to a Poros HQ column equilibrated in TM buffer containing 0.1 M KCl and 0.1% NP-40, washed with the same buffer, and eluted with a 0.1-0.8 M KCl gradient in TM buffer containing 0.1% NP-40.

Protein purification

Partially purified Pol I, SL1, and UBF were prepared from HeLa nuclear extract as previously described (41). Briefly, nuclear extracts prepared from HeLa S3 cells were applied on a heparin-agarose column and RNA Pol I, UBF and SL1 were eluted with a 0.1-1.0 M KCl linear gradient in TM buffer (50 mM Tris, pH 7.9, 12.5 mM MgCl₂, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride). Fractions containing Pol I/Rrn3 (eluted at ~250 mM KCl) were pooled and dialyzed against TM buffer containing 0.1 M KCl and loaded onto a Poros Q-Sepharose column equilibrated against TM containing 0.1 M KCl. Proteins were eluted with a salt gradient from 0.1 to 0.7 M KCl. The active fractions were pooled, dialyzed to 0.125 M KCl, aliquoted and stored at -80 C. The RNA polymerase I fraction does not contain any detectable UBF or SL1, as determined by immunoblot analysis. Fractions from the heparin to agarose column containing SL1 (eluted at ~550 mM KCl) were pooled and dialyzed against TM/0.2 M KCl. The SL1 pool was loaded on to a SP-Sepharose column (Pharmacia) pre-equilibrated in TM/0.2 M KCl, and after extensive washes with TM/0.2 M KCl, SL1 was eluted with TM/0.8 M KCl and dialyzed against TM/0.1 M KCl. The fractions from the heparin to agarose column containing UBF (eluted at ~350 mM KCl) were pooled and further purified by fractionation on a Q-Sepharose (Poros) column. Recombinant UBF used in the transcription assays in-solution was purified from baculovirus-infected cells as previously described (14). Recombinant flag-tagged UBF FL, UBF670C, UBF9A/G and UBF9D/E, that were used in the transcription assays with immobilized template (IT) were expressed and purified from Sf9 insect cells infected with recombinant baculoviruses as the following protocol: 24 h post infection, Sf9 cells were collected, washed with PBS, and lysed in RIPA buffer. Cell lysates were incubated with anti-flag M2 agarose (Sigma) for 1 h. Extensive washes were carried out first with RIPA buffer and then sequentially with TM buffer containing 0.1% NP-40 decreasing salt concentrations (0.6, 0.3 and 0.1 M NaCl). The bound proteins were then eluted by incubation with TM buffer containing 0.1% NP-40, 0.1 M NaCl and 0.3 mg/ml flag peptide, and subjected to dialysis in TM buffer containing 0.1 M NaCl and 0.1% NP-40 to remove the flag peptide. All the buffers contain a cocktail of protease inhibitors and 1 mM DTT.

Chromatin immunoprecipitation

HeLa or HEK293 cells were cross-linked by incubation with 1% formaldehyde for 10 min at room temperature. Cells were swelled in hypotonic buffer [3 mM MgCl₂, 10 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 0.1% NP-40], and nuclei were then pelleted by centrifugation. Nuclei were lysed in nuclear lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1)]. The nuclear lysate was sonicated to generate 0.5–1 kbp chromatin fragments. After centrifugation, the supernatant was diluted 1:5 with dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl], and pre-cleared with protein A/G Sepharose mixture that was pre-blocked with BSA and yeast tRNA. For each immunoprecipitation, the pre-cleared lysate (equivalent to $\sim 6 \times 10^6$ cells) was incubated with 5 μ l of the appropriate rabbit antiserum or 3 μ g of antibodies purchased from Santa Cruz, overnight at 4°C, followed by incubation with blocked protein A/G Sepharose mixture for 1 h, or incubated with blocked anti-FLAG M2 agarose beads for 2 h. Beads were sequentially washed in low salt buffer (20 mM Tris-HCl at pH 8.0, 2 mM EDTA, 0.2% SDS, 0.5% Triton X-100, 150 mM NaCl), high salt buffer (20 mM Tris-HCl at pH 8.0, 2 mM EDTA, 0.2% SDS, 0.5% Triton X-100, 500 mM NaCl), LiCl wash buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% SDS, 0.5% NP-40), low salt buffer, and TE (pH 8.0). The bound DNA was then eluted, and reverse cross-linked. After phenol/chloroform-extraction, and ethanol-precipitation, DNA was resuspended in 30 μ l of water. PCR was performed in 50 μ l of reaction mixture containing 2 μ l of DNA, 25 μ l of 2 \times SYBR Green PCR Master Mix (Bio-Rad) and 250 nM primers. Accumulation of fluorescent product was monitored by real-time PCR using iCycler detection system (Bio-Rad). The cycling conditions were 95°C for 2 min, followed by 45 cycles of 95°C for 30 s, 52°C for 30 s and 72°C for 30 s. The sequences of primers used in the PCR are as follows: rRNA promoter region: 5'-GGTATATCTTTTCGCTCCGAG-3' and 5'-AGC-GACAGGTCGCCAGAGGA-3'; 18S coding region: 5'-AG-TCCGGTTGCTTGGGAATGC-3' and CCCTTACGGTACTTGTGTAGCT-3'; termination region: 5'-ACCTGGCGCTA-AACCATTTCGT-3' and 5'-GGACAAACCCTTGTGTCGAGG-3'. A series of dilutions of input DNA were run alongside the chromatin immunoprecipitation (ChIP) samples to establish the standard curve for each pair of primers. Statistical significance of the differences between two groups was determined by the two-tailed Student's *t*-test.

Antibodies

Rabbit antisera against UBF, TAF₁₁₀, and affinity-purified rabbit antibody against TBP were described previously (41). Polyclonal rabbit antiserum against Pol I β' subunit (194 kDa) was a gift from Dr Rothblum. Goat polyclonal antibodies against CK2 α (sc-6480 and sc-6479) and TAF₁₁₃₂ (sc-1248) were purchased from Santa Cruz.

TBB treatment and RNA analysis

HEK293 cells were seeded at 1.5×10^6 per 9.6 mm plate 1 day before treatment. The drug treatment was performed by adding TBB (CalBiochem) to the medium at final

concentration of 80 μ M. After incubation for the indicated time length, total cellular RNA was isolated using the Trizol reagent. RNA was analyzed by nuclease S1 protection assay or RT-PCR. The nuclease S1 protection assays employed a 5' end-labeled DNA oligonucleotide complementary to the region from -20 to 40 of rRNA gene as previously described (14). Reverse transcription (RT) was performed for 50 min at 60°C using 2 μ g of RNA, 20 pmole pre-rRNA or GAPDH reverse primer, and 15 U thermoscript reverse transcriptase (Invitrogen) in a total volume of 20 μ l of cDNA synthesis buffer containing 5 mM DTT and 1 mM dNTP mix. One microliter of pre-rRNA or GAPDH cDNA was amplified by PCR with 23 or 18 cycles, respectively. Primers used were pre-rRNA forward primer (5'-CCTGCTGTTCTCTCGCGC-GTCCGAG-3'), pre-rRNA reverse primer (5'-AACGCCT-GACACGCACGGCAGGAG-3'), GAPDH forward primer (5'-ACCACAGTCCATGCCATCAC-3'), and GAPDH reverse primer (5'-TCCACCACCCTGTTGCTGTA-3').

In solution transcription reactions

Transcription assays were performed with partially purified Pol I, SL1, and recombinant UBF. Transcription assays and the analysis of *in vitro*-synthesized RNA by nuclease S1 protection assays were performed as previously described (41).

Immobilized DNA template assay

The IT was generated by PCR using prHu3 as the template, and a 5'-biotinylated primer (5'-CGAATTCGTTTTCCGAGATCCCCGTGG) and 3'-primer (5'-CACGGTGGCCCTC-GCCGCCTTC). The template was purified by Qiagen gel extraction kit and then attached with the Dynal M-280 magnetic beads as described in the manufacturer's instructions. Approximately 30 ng DNA template was immobilized on 1 μ l of beads (1 mg/ml). Beads attached to the DNA template were pre-blocked with BSA at final concentration of 5 mg/ml for 30 min. Once prepared, the IT was immediately used for PIC formation as described below. IT (5 μ l at 1 mg/ml bead conc.) was first incubated with ~ 0.1 μ g of recombinant wild type UBF or mutated UBF variants in TM buffer containing 75 mM NaCl and 0.1% NP-40 (30 min, 4°C), and then washed three times with the same buffer at 4°C. IT assembled with wild-type UBF or mutated UBF derivatives was then incubated with 7 μ g HeLa nuclear extract in TM buffer containing 75 mM NaCl and 0.03% NP-40 (30 min, 4°C), followed by extensive washes with 75 mM NaCl and 90 mM NaCl in TM buffer containing 0.03% NP-40 and 0.2 mg/ml BSA at 4°C. *In vitro* transcription reactions were carried out in a final salt concentration of 90 mM NaCl.

In vitro protein-protein interaction assays

Flag-tagged or GST-fusion UBF deletion mutants were expressed in insect (Sf9) cells and affinity-purified on anti-flag M2 agarose (Sigma) or glutathione Sepharose beads by nutating at 4°C for 1 h and then washing extensively. For the analysis of the phosphorylation-dependent SL1 binding, each immobilized UBF variant was then divided into two aliquots. One aliquot was incubated in alkaline phosphatase (AP) reaction buffer containing 1 U shrimp intestine AP and the other in AP reaction buffer only, for 30 min at 30°C. Immobilized proteins were then washed three times

with TM (50 mM Tris [pH 7.9], 12.5 mM MgCl₂, 1 mM EDTA, 10% glycerol) containing 0.4 M KCl and 1% NP-40 and two times in TM buffer containing 0.1 M KCl and 0.1% NP-40. Ten micrograms of partially purified SL1 from HeLa cells was then added and nutated with the immobilized proteins for 4 h at 4°C. The resulting complex was washed four times in TM buffer containing 0.1 M KCl and 0.1% NP-40, eluted with 70 µl of BCO buffer (20 mM Tris [pH 8.0], 0.5 mM EDTA, 20% glycerol, 1 M KCl, 1% DOC) for 30 min at 4°C, and precipitated with a 1/4 volume of 100% trichloroacetic acid (TCA) containing 4 mg/ml sodium deoxycholate (DOC) at 4°C for 20 min. The pellet was washed with 100% acetone, air dried, resuspended in SDS sample buffer, and heated at 95°C for 3 min. Complexes were separated by SDS-8% PAGE and transferred to nitrocellulose membranes for western blot analysis. SL1 was detected with anti-TAF_I110 and anti-TBP polyclonal antibodies. All washes and elution buffers contained a cocktail of protease inhibitors and 1 mM DTT.

Transfection and co-immunoprecipitation assays

HEK293T cells were seeded 1 day before transfection. Cells were transfected by calcium phosphate method with ~12 µg of DNA. At 19 h post-transfection, cells were lysed in TM buffer containing 0.5 M NaCl and 0.1% NP-40. Cell lysate was centrifuged at 44 K r.p.m. for 20 min and the supernatant was then dialyzed in TM buffer containing 0.1 M NaCl and 0.1% NP-40 using a microdialyzer (Gibco). Flag-tagged proteins were captured by incubation with anti-flag M2 agarose (Sigma). The resins were extensively washed with TM buffer containing 0.1 M NaCl and 0.1% NP-40 and the bound proteins were resolved by SDS-8% PAGE and transferred to nitrocellulose membranes. Flag-tagged proteins were visualized by Ponceau S staining and co-immunoprecipitated proteins were detected by western blot analysis.

RESULTS

CK2 associates with the Pol I/Rrn3 complex and is detected on the promoter but not on the coding and termination regions of the rRNA genes

To determine whether CK2 is directly involved in Pol I transcription, we examined if CK2 physically associates with any of the essential components of the Pol I transcriptional machinery. For this purpose, HeLa nuclear extracts were subjected to several steps of column chromatography to separate Pol I/Rrn3 and the two auxiliary factors, SL1 and UBF. The resulting fractions were examined by western blot analysis with antibodies specific for the β' subunit of Pol I, the TAF_I110 subunit of SL1, UBF, Rrn3, and the α subunit of CK2. CK2 was detected in the fraction containing Pol I/Rrn3 but not in the SL1 and UBF fractions (Figure 1A, lanes 1–4). The finding that CK2 cofractionates with the Pol I/Rrn3 complex but not with UBF and SL1 was confirmed in chromatographic fractionation of nucleolar extracts (Figure 1B), which contain a population of proteins primarily involved in the transcription and processing of rRNA. To determine whether the subpopulation of CK2 that copurifies with the Pol I/Rrn3 complex is physically associated with this complex, the fraction isolated from the nuclear extracts

that contains the Pol I/Rrn3 complex (Figure 1A, lane 4) was then subjected to an immunoprecipitation reaction with anti-CK2α antibody. The results of this experiment show that Pol I and Rrn3 co-immunoprecipitate with CK2α (Figure 1A, lane 5) but not with an unrelated nuclear protein (TAF_{II}32) (Figure 1A, lane 6), indicating that at least a fraction of cellular CK2 is bound to the Pol I/Rrn3 complex.

To investigate the relevance of this interaction in Pol I transcription, we then determined whether CK2 is recruited to the rDNA sequences *in vivo*. The association of CK2 with the rDNA was examined in ChIP assays using sets of primers specific for the promoter, the coding (18S RNA) and the termination regions of the rRNA genes. We found that Pol I (β' subunit) and UBF are associated with all three regions of the rRNA genes (Figure 1C). CK2α is also present at the rRNA gene promoter but in contrast to Pol I and UBF, it is not detected at the transcribed and the termination regions, suggesting that this kinase may play a specific role in initiation of Pol I transcription. Moreover, if CK2 is recruited to the promoter via interactions with the Pol I/Rrn3 complex, the absence of detectable CK2 at the coding and termination regions suggest that CK2, like Rrn3 (42), is released from the polymerase upon transcription elongation.

CK2 kinase activity is required for Pol I transcription in cultured cells and *in vitro*

To determine whether the kinase activity of CK2 plays a role in the regulation of Pol I transcription, 293 cells were incubated with 4,5,6,7-tetrabromobenzotriazole (TBB), a specific inhibitor of CK2 kinase activity (43), or DMSO (vehicle) for either 1, 2 or 4 h, and the endogenous level of 45S pre-rRNA was determined by nuclease S1 protection assay or by reverse transcriptase RT-PCR. Since the probe and primers used in these assays detect the extreme 5' end of the external transcribed sequence of the rRNA precursor, which is rapidly processed in the cell, these assays primarily measure the rate of transcription initiation. As shown in Figure 2A, both assays indicate that the addition of TBB causes a sharp decrease in Pol I transcription (upper panel, lanes 2, 3, 5 and 7; lower panel, lanes 2–4). The concentrations of TBB used in this assay inhibit CK2 activity ~80% (43). In contrast, the level of a control mRNA (GAPDH) is not affected by TBB (lower panel, lanes 2–4). A similar level of inhibition of Pol I transcription by TBB was also observed in studies carried out with human primary fibroblasts (data not shown). In a complementary set of experiments, we compared Pol I transcriptional activity in nuclear extract prepared from TBB- and DMSO-treated 293 cells using *in vitro* transcription assays with an rDNA reporter construct. The results of these experiments indicate that nuclear extracts prepared from TBB-treated cells have ~2-fold lower Pol I transcriptional activity than nuclear extracts prepared from DMSO-treated cells (Figure 2B), providing additional evidence that CK2 kinase activity is required for optimal Pol I transcription. In addition, these data suggest that inhibition of CK2 activity likely influences the activity of one or more transcription factor. To investigate further the direct requirement for CK2 activity in Pol I transcription, we performed *in vitro* reconstituted transcription assays with purified Pol I/Rrn3 fraction, which contains

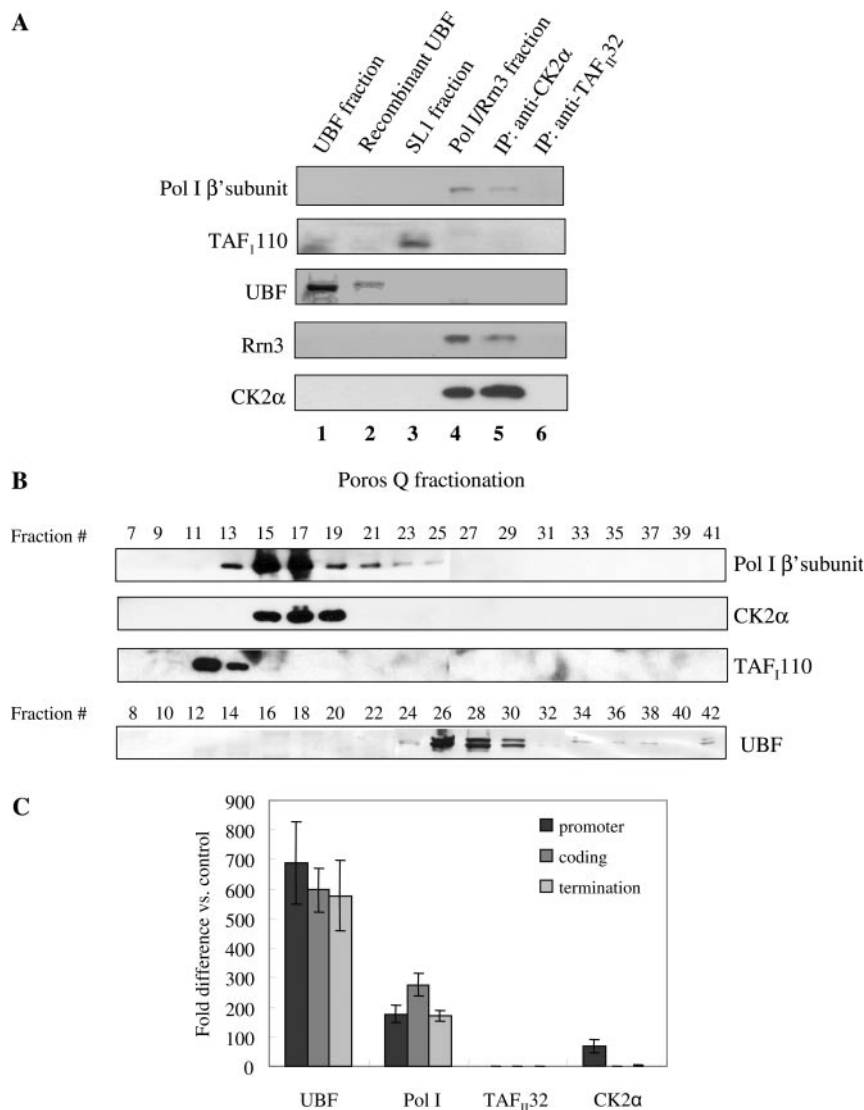


Figure 1. CK2 copurifies with the Pol I/Rrn3 complex and is present on the rRNA gene promoter region. (A) The protein kinase CK2 cofractionates and co-immunoprecipitates with the RNA polymerase I/Rrn3 complex. Nuclear extracts prepared from HeLa S3 cells were fractionated by column chromatography and the peak fractions containing UBF (lane 1), SL1 (lane 3), Pol I/Rrn3 (lane 4) were resolved by SDS-PAGE and analyzed for the presence of the indicated proteins by western blot (lanes 1–4). Lane 2 contains recombinant UBF purified from baculovirus-infected Sf9 insect cells. The peak Pol I/Rrn3 fraction (lane 4) was subjected to immunoprecipitation using goat polyclonal antibodies against CK2 α (lane 5) or TAF₁₁₀ (lane 6), and the resulting products were analyzed by western blot with antibody against the indicated factors. (B) Nucleolar extracts were prepared from four liters of HeLa S3 cells and loaded on a Poros HQ column. The column was then subjected to a continuous salt gradient and aliquots of indicated fractions were tested for the presence of Pol I, SL1, CK2 and UBF by western blot analysis as described in the Materials and Methods. (C) CK2 associates with the rRNA gene promoter but not with the coding and termination regions. Cross-linked chromatin isolated from HeLa cells was immunoprecipitated with rabbit non-immune serum (control), rabbit antisera against UBF and Pol I β' subunit (194 kDa), and goat polyclonal antibodies against TAF₁₁₀ and CK2. Immunoprecipitated chromatin was quantified by real-time PCR using primers specific to the core promoter, 18S coding, and termination regions of the rRNA genes. The relative associations of indicated proteins with the three regions of the rRNA genes are shown as the fold increases over non-immune serum (control). Graph shows means and standard deviations from triplicate real-time PCR reactions and are representative of two independent ChIP assays.

CK2, SL1 and recombinant UBF. A western blot analysis of the purified proteins is shown in Figure 1A (lanes 2–4). The partially purified components were mixed and preincubated with TBB before nucleotides were added to start the transcription reaction. As shown in Figure 2C, upper panel, preincubation of purified factors with increasing amounts of TBB resulted in a dose-dependant decrease of Pol I transcriptional activity, indicating that inhibition of CK2-mediated phosphorylation of one or more components of the Pol I

machinery drastically reduces transcription. This result is further supported by studies showing that addition of a CK2 phosphoacceptor peptide but not an unrelated peptide (flag peptide) to an *in vitro* transcription reaction causes a decrease in Pol I transcription (Figure 2C, lower panel). This CK2 phosphoacceptor peptide contains the consensus CK2 phosphorylatable sequence and has been shown to act as a competitive substrate and inhibit CK2-dependent transcription by Pol II and Pol III (37,40).

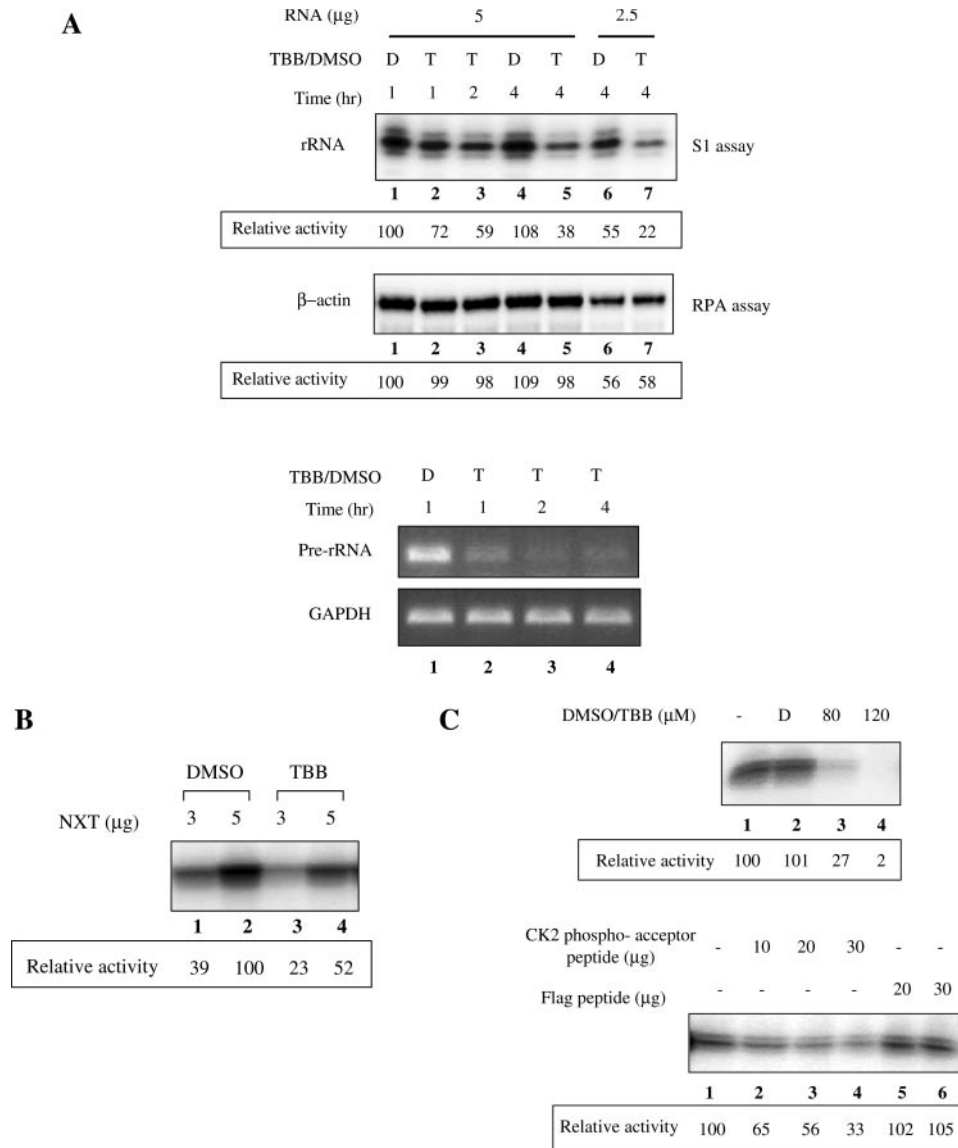


Figure 2. CK2 kinase activity is required for Pol I transcription in cultured cells and *in vitro*. (A) Inhibition of CK2 activity by treatment of 293 cells with TBB down-regulates Pol I transcription. TBB was added to cells in culture at the final concentration of 80 μM. 45S pre-rRNA level was analyzed by nuclease S1 protection assay using 5' end-labeled DNA oligonucleotide complementary to the region from -20 to +40 of the rRNA gene (upper panel) or by RT-PCR (lower panel). Either 5 μg (lanes 1-5) or 2.5 μg (lanes 6 and 7) of total RNA were used in the nuclease S1 protection assay. Products were resolved on an 8% polyacrylamide gel and analyzed by autoradiography. Ribonuclease protection assays (RPA) to detect β-actin transcripts were used as an internal standard to normalize the amounts of RNA used in the nuclease S1 protection assays. Intensities of bands were quantitated by phosphoimager analysis and are shown as relative activities. (B) Nuclear extracts from TBB-treated cells have lower transcriptional activity than extracts from mock treated cells. *In vitro* transcription reaction with a human rRNA template were carried out with nuclear extracts (3 μg, lanes 1 and 3; 5 μg, lanes 2 and 4) prepared from DMSO (mock)-treated (lanes 1 and 2) and TBB-treated (lanes 3 and 4) HeLa cells and *in vitro*-synthesized RNA was analyzed by S1 nuclease protection assays. Relative activities were determined by phosphoimager analysis. (C) Addition of TBB or a CK2 phosphoacceptor peptide to a reconstituted Pol I transcription system inhibits Pol I transcriptional activity. Purified recombinant UBF was mixed with partially purified SL1 and Pol I/Rrn3 complex and incubated with TBB (upper panel) or CK2 phosphoacceptor peptide (lower panel) for 20 min at 30 C before *in vitro* transcription reaction were initiated by the addition of NTPs and a human rDNA template. RNA products were analyzed by nuclease S1 protection assays as described in (A). Relative activities were determined by phosphoimager analysis. The isoelectric point (pI) of the CK2 and flag peptides is 3.7 and 3.5, respectively. Data shown in this figure are representative of at least three independent experiments.

CK2 activity influences the interaction between SL1 and UBF in cultured cells

To investigate the molecular mechanism by which CK2 regulates Pol I transcription, we determined whether CK2 activity affects the network of protein-protein interactions involved in the assembly of the Pol I PIC. For this analysis, we focused on the interactions between UBF and SL1 and

between Pol I and Rrn3, since these interactions are known to be modulated by phosphorylation (14). Moreover, UBF and Rrn3 appear to be cellular targets of CK2 since phospho-peptide mapping of *in vivo*-labeled UBF and Rrn3 revealed that peptides bearing CK2 phosphorylatable sites on both proteins are indeed phosphorylated (44,45). 293T cells were transfected with vectors expressing flag-UBF (pCMV-flag-UBF)

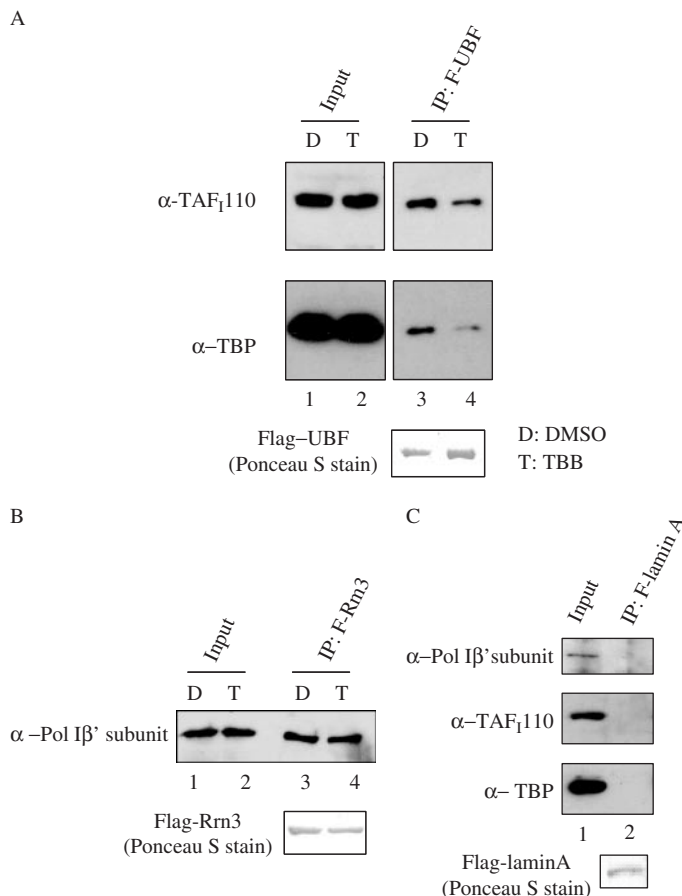


Figure 3. Inhibition of CK2 activity impairs the interaction between UBF and SL1 in cultured cells. 293T cells were transfected with pCMV-flag-UBF, pCMV-flag-Rrn3 and pCMV-flag-lamin A (negative control) using the calcium phosphate method. At 19 h post-transfection, cells were treated with TBB for 2 h and then lysed in TM buffer containing 0.5 M NaCl and 0.1% NP-40. The lysates were dialyzed and incubated with anti-flag M2 agarose resin. The products of the immunoprecipitation reactions from the cells transfected with the constructs expressing flag-UBF (panel A, lanes 3 and 4), flag-Rrn3 (panel B, lanes 3 and 4) or flag-lamin A (panel C, lane 2) were separated by SDS-8% PAGE, transferred to nitrocellulose membranes and analyzed by western blot with antibodies against two subunits of SL1 (panels A and C; TAF₁₁₀ and TBP) and antibody against the largest subunit of RNA polymerase I (panels B and C; pol I β'). To assure that approximately equal amounts of flag-tagged proteins were immunoprecipitated from the TBB- and DMSO-treated cell extracts the membranes were stained by Ponceau S (lower panels).

or flag-Rrn3 (pCMV-flag-Rrn3) and treated with either the CK2 kinase inhibitor TBB or DMSO (vehicle). After 2 h of continuous treatment, cells were lysed and subjected to immunoprecipitation with anti-flag agarose resin. Western blot analysis of the immunoprecipitated products shows that a smaller amount of SL1 is coimmunoprecipitated with UBF in the extract from cell treated with TBB compared to the extract from control cells (Figure 3A, lanes 3 and 4), suggesting that the interaction between UBF and SL1 is reduced in the cells treated with TBB. By contrast, the interaction of Rrn3 with the polymerase is not affected by the treatment with the CK2 kinase inhibitor, as similar amounts of Pol I β' subunit were coimmunoprecipitated with Rrn3 from TBB- and DMSO-treated cells (Figure 3B, lanes 3 and 4). SL1 and Pol I did not co-immunoprecipitate with an unrelated

flag-tagged protein (flag-lamin A, Figure 3C), confirming that the observed protein interactions were not due to non-specific binding to the anti-flag resin. Taken together, these results provide evidence that the kinase activity of CK2 specifically influences the interaction between UBF and SL1.

SL1 makes direct contact with a 40 amino acid domain in the C-terminus of UBF but the phosphorylation-dependent regulation of the UBF-SL1 interaction requires an extended region comprising HMG boxes 5 and 6 and the C-terminus

We have previously shown that the C-terminal region of UBF is required for SL1 binding and further demonstrated that phosphorylation of UBF is necessary for establishing a stable UBF-SL1 complex at the rRNA gene promoter (14). However, the phosphoamino acid residues and the cellular kinase involved in the phosphorylation-dependent binding of SL1 have not been identified. Notably, the C-terminal region of UBF from amino acids 670 to 764 contains a series of serine residues that are embedded within CK2 consensus sites (S/TXXE/D). Moreover, this region is highly phosphorylated *in vivo* and mutation of nine conserved serine sites within this region of mouse UBF abolishes CK2 phosphorylation within the acidic tail (44). To investigate the potential role that phosphorylation within this region plays in the regulation of SL1 binding, we first mapped the minimal SL1 binding region of UBF and then examined its dependence on phosphorylation. For this purpose, a series of UBF mutants containing progressive truncations of the C-terminal region were expressed and purified from baculovirus-infected insect cells and incubated with partially purified SL1. After extensive washes, the presence of bound SL1 was determined by western blot analysis with TBP and TAF₁₁₀ antibodies. As shown in Figure 4A, deletion of the last 18 amino acids of UBF (UBF746C) does not affect the binding to SL1 (lane 4), while further deletion of an additional 18 amino acid residues (UBF728C) results in a significant loss of binding (lane 3). Further deletions of the C-terminus (UBF706C and UBF670C) completely eliminate SL1 binding (lanes 1 and 2), indicating that the region of UBF between amino acids 706 and 746 is required for this protein interaction. To determine whether this 40 amino acid region can bind to SL1 by itself, we then carried out protein interaction assays using GST-fusion UBF mutants spanning the C-terminal region of UBF (Figure 4B). The results of this experiment show that GST-UBF(706-746) (lane 2) binds to SL1 as well as GST-UBF(670-746) (lane 3) and GST-UBF746C (lane 1), confirming that the region from amino acids 706 to 746 within the C-terminal domain of UBF makes direct contact with SL1.

The treatment of recombinant full length UBF purified from insect cells with AP causes a significant reduction in SL1 binding (14,27) (see also Figure 6C, lanes 1 and 2), suggesting that phosphorylation of UBF regulates the interaction between UBF and SL1. To determine whether the region of UBF between amino acids 706 and 746 is also necessary and sufficient for the phosphorylation-dependent regulation of this interaction, a series of UBF mutants were expressed and purified from insect cells and treated with AP or buffer before incubation with SL1. After extensive washes, the

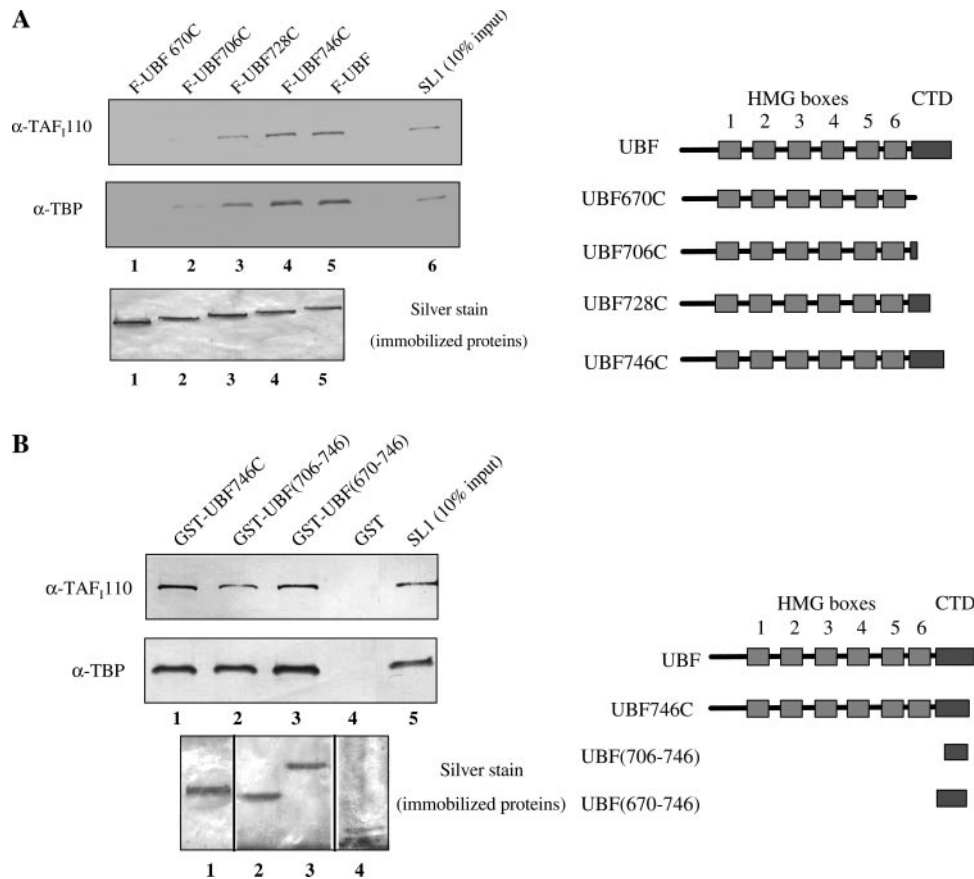


Figure 4. Mapping the SL1 binding domain of UBF. (A) Flag-tagged UBF (lane 5) and a set of UBF mutants containing progressive deletion of the C-terminal region (UBF670C, lane 1; UBF706C, lane 2; UBF728C, lane 3; UBF746C, lane 4) were purified from insect cells infected with the respective recombinant baculoviruses, immobilized on anti-flag resins, and incubated with partially purified SL1. After extensive washes, the bound proteins were eluted and analyzed by western blotting with antibodies against two subunits of SL1 (TAF₁₁₀, upper panel; TBP, lower panel). Lane 6 contains 10% of the SL1 fraction used in the interaction assays. Silver stained gel containing 20% of the beads-immobilized UBF mutants used in the protein interaction assays is shown in the bottom panel. (B) GST-UBF746C (lane 1), GST-UBF(706–746) (lane 2), GST-UBF(670–746) (lane 3) and GST (lane 4) were purified from insect cells infected with the respective recombinant baculoviruses, immobilized on glutathione resin and used in protein interaction assays with SL1 as described in (A). Lane 5 contains 10% of the SL1 fraction used in the interaction assays. Silver stained gel containing 20% of the beads-immobilized UBF mutants used in the protein interaction assays is shown in the lower panel. A schematic representation of UBF with its major functional domains [HMG boxes 1–6 and the C-terminal domain (CTD)] and the mutants tested in the respective interaction assay is shown below each western blot. Each experiment was repeated three times with identical results.

bound proteins were resolved on an SDS–polyacrylamide gel and the presence of SL1 was detected by western blot with TBP antibody. The results of this experiment, shown in Figure 5, indicate that the binding of SL1 to UBF (706–746) was not significantly affected by treatment with AP (lanes 9 and 10), suggesting that this domain of UBF binds to SL1 independently of its phosphorylation state. This result was confirmed in a protein interaction assay with bacterially expressed UBF (706–746) (data not shown). A similar result was also obtained with a slightly longer UBF mutant [UBF (670–746), lanes 7 and 8]. In contrast, the phosphorylation-dependency of the interaction was restored in the reaction containing UBF (491–746), a UBF mutant that contains HMG boxes five and six in addition to the C-terminal domain of UBF, since the AP-treated protein shows a dramatic reduction in SL1 binding compared to the mock-treated counterpart (lanes 5 and 6). These results suggest that although SL1 binds to the region of UBF between amino acids 706 and 746, the phosphorylation-dependent regulation of this molecular interaction requires the presence of

an additional region from amino acids 491 to 670 (HMG boxes five and six). This interpretation is strongly supported by the result showing that UBFdx, a UBF mutant missing HMG boxes five and six, binds to SL1 equally well with and without treatment with AP (lanes 3 and 4).

Phospho-ablation/mimicking mutants of UBF indicate that phosphorylation of the C-terminus of UBF by CK2 regulates SL1 binding

One possible interpretation of the results shown in Figure 5 is that phosphorylation of serine residues within the region between amino acids 491 and 670 regulates SL1 binding. However, these results do not rule out that in the context of the full length protein phosphorylation within the C-terminus or in combination with the phosphorylation in HMG boxes five and six are important for the regulation of this interaction. To address this question and to attempt to identify the phosphorylation sites critical for modulating this protein–protein

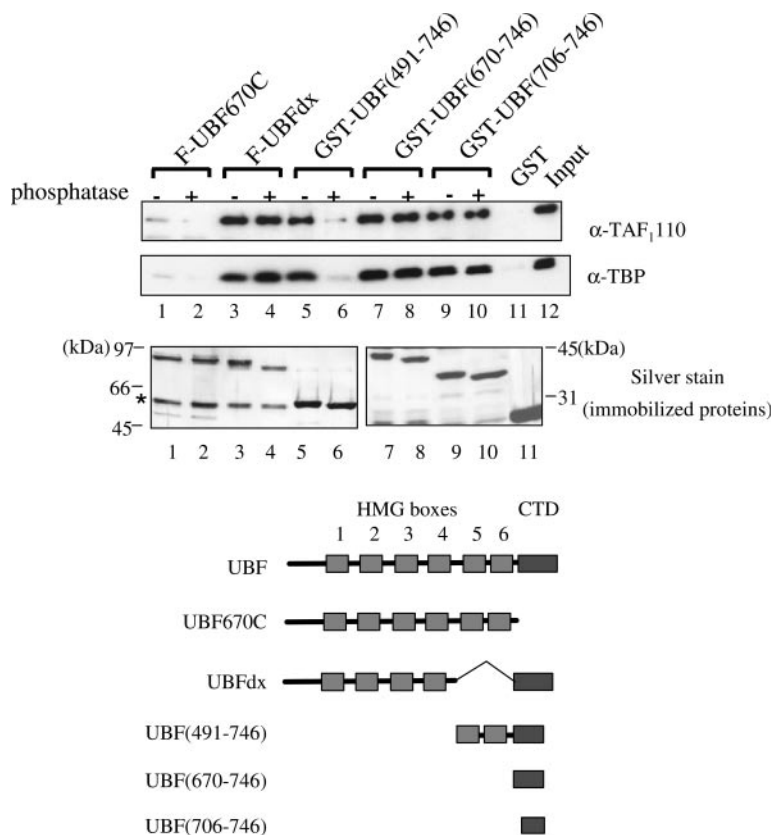


Figure 5. Mapping the region of UBF that is required for the phosphorylation-dependent regulation of this interaction. Flag-tagged or GST-fusion UBF deletion mutants were expressed and purified from baculovirus-infected cells. Proteins were immobilized on the appropriate resins and equally divided into two aliquots. One aliquot was subjected to alkaline phosphatase treatment while the other was incubated with buffer only. After the treatment, the immobilized proteins were washed extensively, and tested in protein interaction assays with SL1 as described in Figure 4A. A schematic representation of UBF and UBF mutants used in this analysis is shown below the western blot. Equal aliquots of flag-tagged and GST-fusion UBF mutants used in the protein interaction assays were resolved by SDS-PAGE and silver stained (lower panel). Asterisk denotes antibody heavy chain band in lanes 1–4. The protein binding assays were repeated several times with identical results.

interaction, we examined whether CK2-acceptor sites between amino acids 491 and 764 play any role in this regulatory process. This region of UBF contains 12 CK2 phosphorylatable serines and 9 of these sites are located within the C-terminal domain, between amino acid 670 and 764 (Figure 6A). We therefore generated a series of phospho-ablation and phospho-mimicking mutations by replacing CK2 phosphorylatable serine residues with alanine/glycine or glutamic/aspartic acid within the region from HMG box five to the C-terminus of UBF (Figure 6B). Constructs encoding flag-tagged wild-type UBF, UBF670C and the set of aforementioned mutants were transfected into 293T cells, and the interaction of these proteins with endogenous SL1 was assessed by co-immunoprecipitation using anti-flag resin (Figure 6B). As expected, full length UBF binds well to SL1 (lane 1) whereas UBF670C, which lacks the SL1 binding domain, fails to co-immunoprecipitate SL1 (lane 2). The UBF mutant in which three serine residues on HMG box six are mutated to alanines (UBF3A) binds to SL1 as well as the wild-type protein (lane 3). On the other hand, the UBF mutant with nine serines within the C-terminus substituted with alanine/glycine residues (UBF9A/G) binds poorly to SL1 (lane 4). Mutations of these nine residues affect SL1 binding independently of mutations at other CK2-acceptor sites, since UBF with combined Ser to Ala/Gly substitutions in HMG box

six and the C-terminus (UBF3A + 9A/G) displays a SL1 binding capacity similar to UBF9A/G (lane 5). In contrast to the alanine/glycine mutations, the substitution of CK2-acceptor sites in HMG box six or in the C-terminus of UBF with negatively charge residues, which mimic phosphorylated amino acids, results in a similar or slightly better binding than wild-type UBF (lanes 6 and 7, respectively). Analogous results were obtained in protein binding assays with truncated UBF mutants [UBF (479–764) 9A/G and UBF (479–764) 9D/E; data not shown]. These results suggest that in the presence of HMG boxes 5 and 6, phosphorylation of CK2 phosphoacceptor serine residues located within the C-terminal region between amino acids 670 and 764 regulates the interaction with SL1. In contrast, CK2 phosphoacceptor serines outside this region do not seem to influence this interaction. This interpretation is further supported by the observation that in contrast to wild-type UBF, treatment of UBF9D/E with AP does not influence SL1 binding (Figure 6C).

To examine the impact of UBF phosphorylation by CK2 on the recruitment of SL1 to the rRNA gene promoter, we performed ChIP assays. Since endogenous UBF is potentially a heterogeneous population with varying degrees of phosphorylation, we specifically compared and contrasted the recruitment of SL1 by the phospho-ablation and phospho-mimicking UBF mutants UBF9A/G and UBF9D/E.

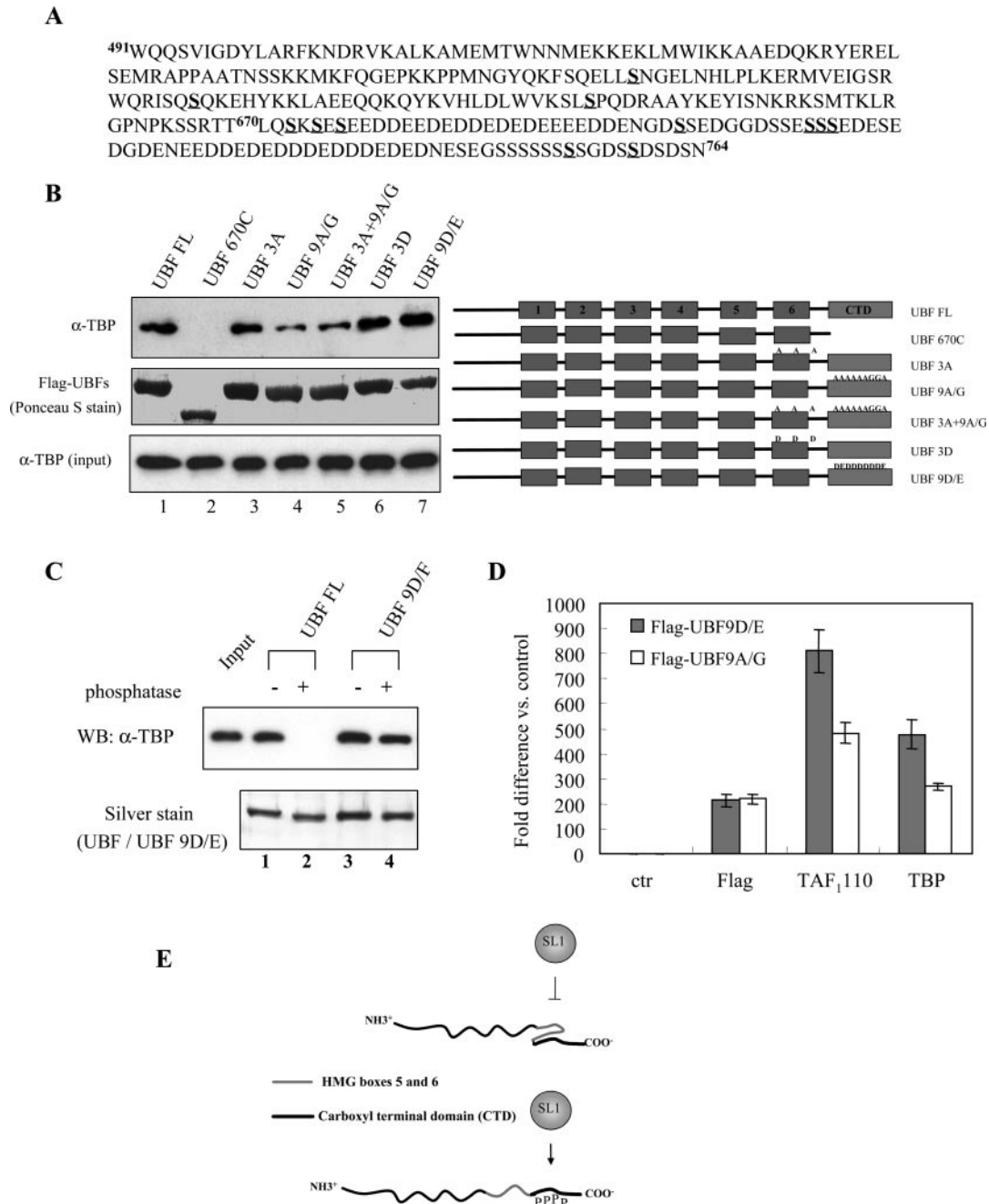


Figure 6. Protein interaction and ChIP assays with phospho-ablation and phospho-mimicking mutants of UBF. (A) Sequence of the region of UBF from amino acids 491 to 764. This region includes HMG box 5 (from amino acids 490 to 546), HMG box six (from amino acids 568 to 634) and the C-terminal domain (CTD; from amino acid 670 to 764). CK2 phosphoacceptor serine residues are shown in bold. (B) 293T cells transfected with constructs expressing wild-type and mutant forms of UBF were lysed and incubated with anti-flag M2 agarose. After extensive washes, the bound proteins were separated by SDS–8% PAGE and transferred to nitrocellulose membranes. The presence of SL1 in the immunoprecipitation products was determined by western blot analysis with antibody against TBP (upper panel). The amounts of wild-type and mutant forms of UBF (middle panel) and TBP (lower panel) in the immunoprecipitation reactions was determined by Ponceau S staining and western blot analysis, respectively. A schematic representation of the mutants generated in this study is shown. Phospho-ablation and phospho-mimicking mutants of UBF were generated by replacing CK2 phosphorylatable serine residues with alanine/glycine or aspartate/glutamate by site-directed mutagenesis. (C) Flag-tagged wild-type UBF and UBF 9D/E were expressed and purified from baculovirus-infected cells. Proteins were immobilized on resins and equally divided into two aliquots. One aliquot was subjected to alkaline phosphatase treatment while the other was incubated with buffer only. After the treatment, the immobilized proteins were washed extensively with dissociation buffer, and examined in protein interaction assays with SL1 as described in Figure 4A. (D) 293 cells in 150 mm dishes were transfected with flag-UBF9A/G (20 µg) or flag-UBF9D/E (20 µg) using Lipofactamine 2000 (Invitrogen). After 30 h transfection, cross-linked chromatin was immunoprecipitated with rabbit non-immune serum (ctr), rabbit antisera against TAF₁₁₀ and TBP, and anti-FLAG agarose beads. Immunoprecipitated chromatin was quantified by real-time PCR using primers specific to the core promoter of the rRNA genes. The relative associations of indicated proteins are shown as the fold increases over non-immune serum (control). Graph shows means and standard deviations from triplicate real-time PCR reactions and is representative of two independent ChIP assays. The differences in rRNA gene promoter recovered from chromatin immunoprecipitations with TAF₁₁₀ and TBP antibodies between the two groups are statistically significant ($p < 0.005$). (E) Hypothetical model showing the phosphorylation-induced conformational change in UBF that exposes the SL1 binding site within the C-terminal domain.

For this purpose, cells were transfected with flag-UBF9A/G or flag-UBF9D/E and chromatin was immunoprecipitated with antibodies against flag, TAF₁₁₀ and TBP, and a non-immune serum control. Immunoprecipitation with flag antibody show that UBF9A/G and UBF9D/E bind to the rRNA gene promoter sequences with equal affinities (Figure 6D). No detectable amount of rRNA promoter DNA immunoprecipitated with flag antibody in cells transfected with flag-lamin A (data not shown). On the other hand, the recovery of rRNA gene promoter sequences in ChIP reactions with antibodies against TAF₁₁₀ and TBP in cells expressing UBF9A/G was significantly lower than in cells expressing UBF9D/E. This result is likely an underestimate of the difference in SL1 recruitment between the two UBF variants since it assumes that the flag protein occupies all the rRNA promoter sites while it is likely that the transfected proteins are associated with only a fraction of the rRNA promoter sites.

Taken together, these results indicate that phosphorylation of serines located within the carboxy terminal region of UBF play an important role in SL1 binding. However, since the C-terminal domain of UBF binds to SL1 regardless of its phosphorylation status (Figure 5, lanes 7–10), our data suggest that phosphorylation of this region of UBF is not directly involved in SL1 binding but rather influences the accessibility of this region to SL1, possibly through a phosphorylation-induced conformational change (Figure 6E).

Phosphorylation of the C-terminus of UBF promotes multiple rounds of transcription

To investigate the molecular mechanism by which phosphoserine residues located within the C-terminal domain of UBF affects transcription, we examined the effect of the nine aforementioned phospho-ablation and phospho-mimicking mutations on single and multiple rounds of transcription on ITs (Figure 7A). *In vitro* transcription assays with ITs have been useful to study the assembly of the PIC on the promoter and their activities in single and multiple rounds of transcription (46–48). Hence, a biotinylated human rRNA template was immobilized on streptavidin-based magnetic bead and incubated with purified wild-type UBF, UBF670C, UBF9A/G or UBF9D/E. After the unbound proteins were removed by extensive washes, the amounts of wild-type UBF and mutated UBF variants bound to the ITs were determined by western blot analysis. As shown in Figure 7B, wild-type and mutant UBF proteins bind to the ITs with equal affinity. This result is in agreement with previous studies showing that DNA binding of mammalian UBF is mediated by the first four HMG boxes (5,35). The ITs bound to wild-type UBF and UBF mutants were then incubated with nuclear extract prepared from HeLa cells to allow the assembly of the PIC. The unbound proteins were then removed by extensive washes and each PIC-assembled template was split equally into eight aliquots and used in single and multiple rounds of transcription assays. Calf thymus DNA (ctDNA), which prevents RNA polymerase and other factors from reassembling on the DNA template (48,49), was added to the appropriate set of reactions to limit transcription to a single round. Nucleotides were then added to

the PICs-assembled templates to start transcription and the amount of transcripts generated in each reaction was determined at various time points by nuclease S1 protection assays. In the single round of transcription assays the level of transcripts generated by PICs with UBF9A/G or UBF9D/E is not significantly different from those generated by PIC with wild-type UBF (Figure 7C, upper panel, lanes 1, 3–5, 7–9, 11–13, 15 and 16). By contrast, the PIC with UBF670C shows an overall reduction in transcriptional activity at every time point examined (Figure 7C, upper, lanes 2, 6, 10 and 14). This result is in agreement with earlier data showing that the C-terminal region of UBF plays an important role in activation of Pol I transcription (5). Strikingly, while PICs with wild-type UBF, UBF9A/G and UBF9D/E yield similar levels of transcripts in single round reactions, the amount of transcripts generated in the multiple rounds of transcription by PIC with UBF9A/G (Figure 7C, lower panel, lanes 7, 11 and 15) are considerably lower than those generated by PICs with wild-type UBF (lanes 1, 5, 9 and 13) or UBF9D/E (lanes 4, 8, 12 and 16). The graph in Figure 7D shows the profile of multiple rounds of transcription reactions from three independent experiments. These results indicate that the amount of transcripts produced by the UBF9A/G-assembled PIC does not increase significantly after 5 min of reaction, while the level of transcripts generated by PICs with wild-type UBF and UBF9D/E show a steady increase over time. Interestingly, PIC with UBF9D/E, which resembles a constitutively phosphorylated UBF, is slightly more efficient than PIC with wild-type UBF in multiple rounds of transcription. These findings suggest that the phosphorylation of nine CK2-acceptor serines on the C-terminus of UBF is not essential for the initial assembly of a productive initiation complex but is important for transcription re-initiation, possibly through the stabilization of the interaction between UBF and SL1 at the rRNA gene promoter.

DISCUSSION

Here we provide experimental evidence in support for a direct role of CK2 in Pol I transcription. These results in combination with recently published data indicate that this protein kinase regulates gene transcription by all three classes of nuclear RNA polymerase (Pol I, II and III) in human cells. While a relationship between Pol I transcription and CK2 has been implied by studies showing that mammalian UBF is a substrate of CK2 (25,35,44) and CK2 cofractionates with Pol I (50–52), the functional significance of these findings was unknown. Our studies now show that Pol I transcription activity in cultured cells is significantly reduced upon inhibition of endogenous CK2 with a specific chemical inhibitor (TBB). This drug is highly specific for CK2 and the concentrations used in our assays inhibit ~80% of CK2 kinase activity (43,53). TBB-treated nuclear extracts also display lower Pol I transcriptional activity than mock-treated nuclear extracts and *in vitro* transcription assays with partially purified proteins demonstrate that CK2 activity directly regulates Pol I transcription. The fold effect on Pol I transcription by CK2 observed in our transcription assays is comparable to that of other regulatory factors that influence the function of UBF, SL1 or Rrn3 (22,33,54–56) and that

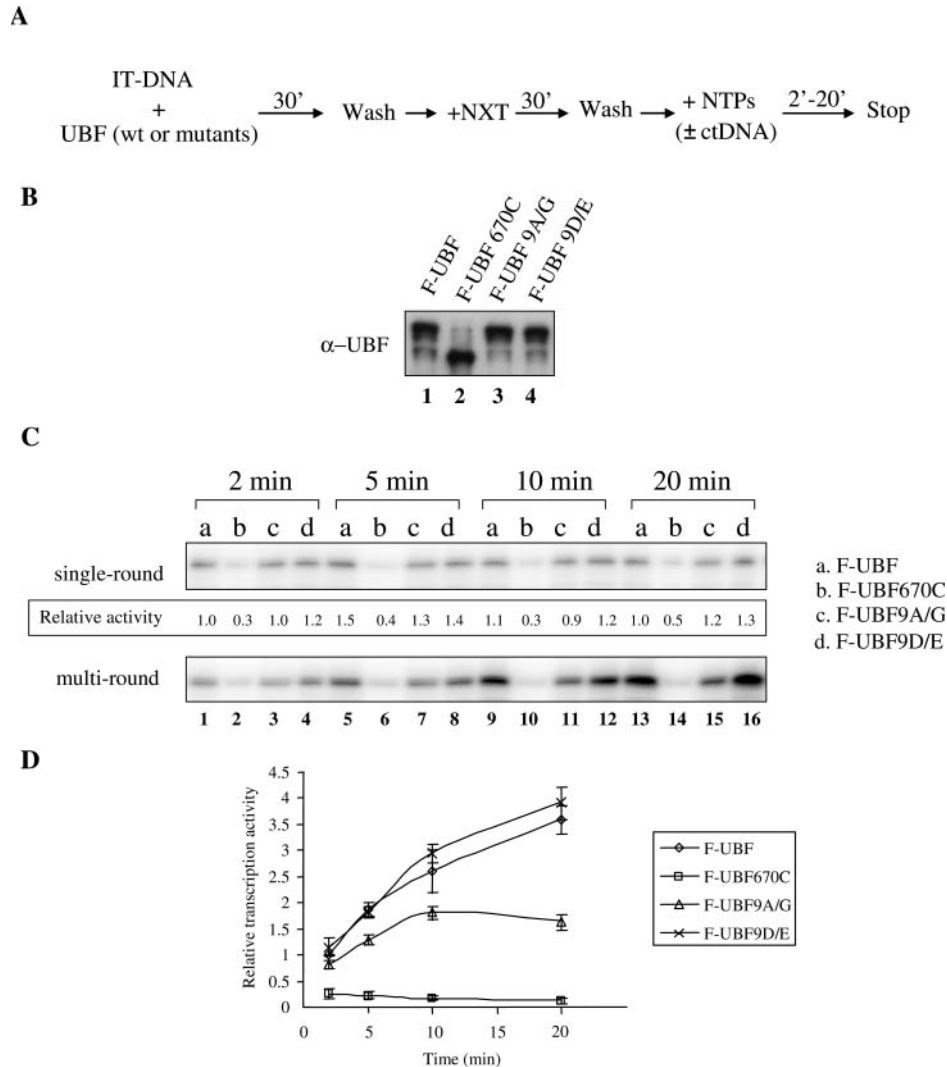


Figure 7. Mutations of the CK2 phosphoacceptor sites in the C-terminus of UBF influence multiple rounds of transcription. (A) Scheme showing the experimental approach used for the analysis of single and multiple rounds of transcription from PICs-assembled on immobilized templates. (B) Recombinant wild type and UBF mutant proteins bind to immobilized rRNA template with similar efficiencies. Amounts of recombinant flag-tagged proteins bound to the immobilized templates were determined by western blot analysis using antibodies against UBF. (C) The activities of wild-type UBF, UBF670C, UBF9A/G, and UBF9D/E were examined in single (in the presence of calf thymus DNA) and multiple (in the absence of calf thymus DNA) rounds of transcription reactions as described in the text. Single or multiple rounds of transcription reactions were initiated by the addition of nucleotides (NTPs) and stopped after either 2, 5, 10 or 20 min. The transcripts generated in each reaction were analyzed by nuclease S1 protection assays and autoradiography, and quantitated by phosphorimager analysis. The results shown are representative of two single round and three multiple rounds of transcription, respectively. (D) Mean and standard deviations of multiple rounds of transcription reactions from three independent experiments are represented graphically.

reported on CK2-mediated regulation of Pol II and III transcription (37,40).

Chromatographic fractionation and immunoprecipitation experiments with extracts from human cells show that CK2 is associated with RNA polymerase I but not with UBF and SL1, as observed in other organisms (50–52). Interestingly, ChIP assays indicate that CK2 is found at the promoter of the rRNA genes. However CK2, unlike Pol I, is not detected at the 18S coding or termination regions of the rRNA genes, suggesting that this protein kinase is recruited to the rRNA gene promoter by Pol I but is then released from the polymerase upon the transition to transcription elongation. In this scenario, CK2 would have access and phosphorylate transcription factors that are only found at the promoter. This

would provide a fine control mechanism that allows the phosphorylation of the subpopulation of UBF found at the promoter and involved in SL1 binding, but prevents that of UBF molecules that are bound to other regions of the rRNA gene. Since CK2 does not bind to UBF or SL1, it is likely released from the rRNA gene promoter upon the transition to transcription elongation. However, we cannot rule out that CK2 remains at the promoter through interactions with UBF or SL1 that may have been disrupted by the experimental conditions used in the extract fractionation studies.

Biochemical analyses have indicated that binding of UBF to the rRNA gene promoter is critical for the recruitment of SL1 and the assembly of a productive preinitiation complex

at the rDNA promoter (3–5,57,58). These *in vitro* data are supported by cell-based studies, which showed that association of UBF with the rRNA gene clusters is sufficient to recruit the Pol I transcriptional machinery to these loci (19,20). A recent report has challenged this model and suggested that SL1 stably binds to the rRNA gene promoter in the absence of UBF (18). We have never observed a detectable binding of human SL1 to DNA by either DNA footprinting or EMSA and our data support a key role for UBF in nucleating the pre-initiation complex at the rRNA gene promoter.

We have previously reported that phosphorylation of UBF regulates the interaction between this factor and SL1 (14,27). The C-terminal region of UBF is highly phosphorylated in active growing cells (25,26,35), and its phosphorylation state is modulated by signaling pathways activated by growth factors (34,55). The C-terminal region of UBF is exceptionally rich in serine, glutamic acid and aspartic acid residues (4). This unusual amino acid composition has made the identification of the phosphorylated amino acid residues by conventional approaches particularly difficult, and attempts to map the phosphorylated sites within this region by mass spectrometry have been unsuccessful. Yet, inspection of UBF amino acid sequence indicates that the C-terminal region contains several CK2 phosphoacceptor sites and phosphorylation within this region by CK2 has been reported by others (25,35,44). However, the function of UBF that is regulated by CK2 phosphorylation was not determined. Here we show that inhibition of CK2 activity in cultured cells specifically affects the interaction between UBF and SL1, suggesting a direct involvement of this protein kinase in a regulatory process that influence transcription initiation. In agreement with this finding, we demonstrated through the analysis of a set of phospho-mimicking/ablation mutants that nine CK2 phosphorylation sites on the carboxy-terminus of UBF are important for binding to SL1. The relevance of CK2-mediated phosphorylation within the C-terminal domain of UBF in the transcription process is underscored by the transcription assays with ITs, which show that the phospho-ablation mutant UBF9A/G are much less efficient than wild-type UBF or phospho-mimicking mutant UBF9D/E in promoting multiple rounds of transcription.

We have previously reported that a kinase activity associated with large T antigen phosphorylates UBF and regulate the interaction of UBF with SL1 (27). Although we have experimental evidence indicating that CK2 associates with large T antigen, we have recently identified additional cellular kinases that bind to large T antigen (S. Navarro and L. Comai, unpublished data) and studies are in progress to determine the relative contribution of these kinases to UBF phosphorylation and SL1 binding.

CK2 is a constitutively active protein kinase. This raises the question of how it plays its regulatory role in Pol I transcription. Since CK2 has been detected in the nucleolus of active growing but not confluent cells (59), it is likely that the subcellular localization of CK2 is regulated by growth signals. Consistent with this hypothesis, it has been shown that CK2 can be transported from the cytoplasm to the nucleus and nucleolus by direct interaction with fibroblast growth factor-2 (FGF-2) (60), suggesting that the recruitment

of CK2 to the rRNA gene promoter can be mediated by interaction with factors other than Pol I. Significantly, a recent study has indicated that FGF-2 can stimulate Pol I transcription by binding to UBF (61).

UBF belongs to a subfamily of the HMG proteins (HMG1 proteins) that have one or more HMG1 box domains similar to the High Mobility Group proteins 1 and 2. This family of proteins is also characterized by the presence of an acidic region at the C-terminus which commonly contains canonical CK2 phosphorylation sites (62). Studies on HMG1 proteins have revealed that CK2-mediated phosphorylation of the acidic C-terminal domain induces a conformational change in the HMG box domain, which affect its DNA binding specificity (62,63). UBF contains six HMG1-like boxes and the first four (HMG boxes 1–4) are involved in DNA binding (5,35). However HMG boxes 5 and 6 are not required for DNA binding and their function was never well understood. The data presented in this study suggest that the region between HMG boxes 5 and 6 is required for conferring the phosphorylation-dependency of the UBF–SL1 interaction (Figure 5). Since this region does not bind directly to SL1, we propose that phosphorylation of the C-terminal domain of UBF induces a structural change in these HMG boxes which makes the C-terminal region of UBF, from amino acids 706 to 746, available for SL1 binding (Figure 6F). In contrast, HMG boxes 1–4 do not appear to be structurally affected by CK2 phosphorylation of the C-terminus since the phospho-mimicking/ablation mutants of UBF bind to the ITs equally well (Figure 7B). Clearly, the phosphorylation-induced conformational change hypothesis can only be rigorously tested through detailed structural studies.

The critical role that CK2 plays in general transcription has been recently emphasized by studies showing that this protein kinase is present on the promoter of RNA Pol II- and III-transcribed genes. Analyses carried out in the Hernandez lab indicated that CK2 associates with the U6 promoter and by phosphorylating components of the transcription complex, it plays both positive and negative regulatory roles in transcription by RNA polymerase III (38,39). Likewise, experiments done in Reinberg lab have shown that CK2 is associated with the downstream promoter element (DPE) of a number of RNA polymerase II-transcribed genes and exerts a positive effect on the transcription of these genes (40). Our study, which provides evidence that CK2 also plays a critical role in RNA polymerase I transcription, reinforces the concept that this protein kinase is an important component of all nuclear transcriptional machineries. In regard to Pol I transcription, the experiments described in this study suggest a model by which CK2 is recruited to the promoter via the RNA polymerase I/Rrn3 complex and stimulates multiple rounds of Pol I transcription by stabilizing the UBF–SL1 complex at the rRNA gene promoter through phosphorylation of the C-terminal domain of UBF. In contrast, lack of phosphorylation at the reported sites would lead to the formation of an unstable complex that rapidly disassembles from the rRNA gene promoter. While this work underscores the functional link between CK2 and UBF, we cannot rule out that phosphorylation of other components of the PIC by CK2 may also influence Pol I transcription and future studies will examine this possibility.

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Conflict of interest statement. None declared.

REFERENCES

- Comai, L. (2004) Mechanism of RNA polymerase I transcription. *Adv. Protein Chem.*, **67**, 123–155.
- Grummt, I. (2003) Life on a planet of its own: regulation of RNA polymerase I transcription in the nucleolus. *Genes Dev.*, **17**, 1691–1702.
- Bell, S.P., Learned, R.M., Jantzen, H.M. and Tjian, R. (1988) Functional cooperativity between transcription factors UBF1 and SL1 mediates human ribosomal RNA synthesis. *Science*, **241**, 1192–1197.
- Jantzen, H.M., Admon, A., Bell, S.P. and Tjian, R. (1990) Nucleolar transcription factor hUBF contains a DNA-binding motif with homology to HMG proteins. *Nature*, **344**, 830–836.
- Jantzen, H.M., Chow, A.M., King, D.S. and Tjian, R. (1992) Multiple domains of the RNA polymerase I activator hUBF interact with the TATA-binding protein complex hSL1 to mediate transcription. *Genes Dev.*, **6**, 1950–1963.
- O'Mahony, D.J. and Rothblum, L.I. (1991) Identification of two forms of the RNA polymerase I transcription factor UBF. *Proc. Natl Acad. Sci. USA*, **88**, 3180–3184.
- Yang-Yen, H.F. and Rothblum, L.I. (1988) Purification and characterization of a high-mobility-group-like DNA-binding protein that stimulates rRNA synthesis *in vitro*. *Mol. Cell. Biol.*, **8**, 3406–3414.
- Comai, L., Tanese, N. and Tjian, R. (1992) The TATA-binding protein and associated factors are integral components of the RNA polymerase I transcription factor, SL1. *Cell*, **68**, 965–976.
- Comai, L., Zomerdijk, J.C., Beckmann, H., Zhou, S., Admon, A. and Tjian, R. (1994) Reconstitution of transcription factor SL1: exclusive binding of TBP by SL1 or TFIID subunits. *Science*, **266**, 1966–1972.
- Eberhard, D., Tora, L., Egly, J.M. and Grummt, I. (1993) A TBP-containing multiprotein complex (TIF-IB) mediates transcription specificity of murine RNA polymerase I. *Nucleic Acids Res.*, **21**, 4180–4186.
- Heix, J., Zomerdijk, J.C., Ravanpay, A., Tjian, R. and Grummt, I. (1997) Cloning of murine RNA polymerase I-specific TAF factors: conserved interactions between the subunits of the species-specific transcription initiation factor TIF-IB/SL1. *Proc. Natl Acad. Sci. USA*, **94**, 1733–1738.
- Beckmann, H., Chen, J.L., O'Brien, T. and Tjian, R. (1995) Coactivator and promoter-selective properties of RNA polymerase I TAFs. *Science*, **270**, 1506–1509.
- Hempel, W.M., Cavanaugh, A.H., Hannan, R.D., Taylor, L. and Rothblum, L.I. (1996) The species-specific RNA polymerase I transcription factor SL-1 binds to upstream binding factor. *Mol. Cell. Biol.*, **16**, 557–563.
- Tuan, J.C., Zhai, W. and Comai, L. (1999) Recruitment of TATA-binding protein-TAFI complex SL1 to the human ribosomal DNA promoter is mediated by the carboxy-terminal activation domain of upstream binding factor (UBF) and is regulated by UBF phosphorylation. *Mol. Cell. Biol.*, **19**, 2872–2879.
- Bodem, J., Dobrev, G., Hoffmann-Rohrer, U., Iben, S., Zentgraf, H., Delius, H., Vingron, M. and Grummt, I. (2000) TIF-1A, the factor mediating growth-dependent control of ribosomal RNA synthesis, is the mammalian homolog of yeast Rrn3p. *EMBO Rep.*, **1**, 171–175.
- Miller, G., Panov, K.I., Friedrich, J.K., Trinkle-Mulcahy, L., Lamond, A.I. and Zomerdijk, J.C. (2001) hRRN3 is essential in the SL1-mediated recruitment of RNA Polymerase I to rRNA gene promoters. *EMBO J.*, **20**, 1373–1382.
- Moorefield, B., Greene, E.A. and Reeder, R.H. (2000) RNA polymerase I transcription factor Rrn3 is functionally conserved between yeast and human. *Proc. Natl Acad. Sci. USA*, **97**, 4724–4729.
- Friedrich, J.K., Panov, K.I., Cabart, P., Russell, J. and Zomerdijk, J.C. (2005) TBP-TAF complex SL1 directs RNA polymerase I pre-initiation complex formation and stabilizes upstream binding factor at the rDNA promoter. *J. Biol. Chem.*, **280**, 29551–29558.
- Mais, C., Wright, J.E., Prieto, J.L., Raggett, S.L. and McStay, B. (2005) UBF-binding site arrays form pseudo-NORs and sequester the RNA polymerase I transcription machinery. *Genes Dev.*, **19**, 50–64.
- Chen, D., Belmont, A.S. and Huang, S. (2004) Upstream binding factor association induces large-scale chromatin decondensation. *Proc. Natl Acad. Sci. USA*, **101**, 15106–15111.
- Hirschler-Laszkiewicz, I., Cavanaugh, A., Hu, Q., Catania, J., Avantaggiati, M.L. and Rothblum, L.I. (2001) The role of acetylation in rDNA transcription. *Nucleic Acids Res.*, **29**, 4114–4124.
- Pelletier, G., Stefanovsky, V.Y., Faubladier, M., Hirschler-Laszkiewicz, I.I., Savard, J., Rothblum, L.I., Cote, J. and Moss, T. (2000) Competitive recruitment of CBP and Rb-HDAC regulates UBF acetylation and ribosomal transcription. *Mol. Cell*, **6**, 1059–1066.
- Cavanaugh, A.H., Hempel, W.M., Taylor, L.J., Rogalsky, V., Todorov, G. and Rothblum, L.I. (1995) Activity of RNA polymerase I transcription factor UBF blocked by Rb gene product. *Nature*, **374**, 177–180.
- Meraner, J., Lechner, M., Loidl, A., Goralik-Schramel, M., Voit, R., Grummt, I. and Loidl, P. (2006) Acetylation of UBF changes during the cell cycle and regulates the interaction of UBF with RNA polymerase I. *Nucleic Acids Res.*, **34**, 1798–1806.
- Voit, R., Schnapp, A., Kuhn, A., Rosenbauer, H., Hirschmann, P., Stunnenberg, H.G. and Grummt, I. (1992) The nucleolar transcription factor mUBF is phosphorylated by casein kinase II in the C-terminal hyperacidic tail which is essential for transactivation. *EMBO J.*, **11**, 2211–2218.
- O'Mahony, D.J., Xie, W.Q., Smith, S.D., Singer, H.A. and Rothblum, L.I. (1992) Differential phosphorylation and localization of the transcription factor UBF *in vivo* in response to serum deprivation. *In vitro* dephosphorylation of UBF reduces its transactivation properties. *J. Biol. Chem.*, **267**, 35–38.
- Zhai, W. and Comai, L. (1999) A kinase activity associated with simian virus 40 large T antigen phosphorylates upstream binding factor (UBF) and promotes formation of a stable initiation complex between UBF and SL1. *Mol. Cell. Biol.*, **19**, 2791–2802.
- Zhai, W., Tuan, J.A. and Comai, L. (1997) SV40 large T antigen binds to the TBP-TAF(I) complex SL1 and coactivates ribosomal RNA transcription. *Genes Dev.*, **11**, 1605–1617.
- Klein, J. and Grummt, I. (1999) Cell cycle-dependent regulation of RNA polymerase I transcription: the nucleolar transcription factor UBF is inactive in mitosis and early G1. *Proc. Natl Acad. Sci. USA*, **96**, 6096–6101.
- Voit, R., Hoffmann, M. and Grummt, I. (1999) Phosphorylation by G1-specific cdk-cyclin complexes activates the nucleolar transcription factor UBF. *EMBO J.*, **18**, 1891–1899.
- Stefanovsky, V.Y., Pelletier, G., Hannan, R., Gagnon-Kugler, T., Rothblum, L.I. and Moss, T. (2001) An immediate response of ribosomal transcription to growth factor stimulation in mammals is mediated by ERK phosphorylation of UBF. *Mol. Cell*, **8**, 1063–1073.
- Stefanovsky, V., Langlois, F., Gagnon-Kugler, T., Rothblum, L.I. and Moss, T. (2006) Growth factor signaling regulates elongation of RNA polymerase I transcription in mammals via UBF phosphorylation and r-chromatin remodeling. *Mol. Cell*, **21**, 629–639.
- Voit, R. and Grummt, I. (2001) Phosphorylation of UBF at serine 388 is required for interaction with RNA polymerase I and activation of rDNA transcription. *Proc. Natl Acad. Sci. USA*, **98**, 13631–13636.
- Kihm, A.J., Hershey, J.C., Haystead, T.A., Madsen, C.S. and Owens, G.K. (1998) Phosphorylation of the rRNA transcription factor upstream binding factor promotes its association with TATA binding protein. *Proc. Natl Acad. Sci. USA*, **95**, 14816–14820.
- O'Mahony, D.J., Smith, S.D., Xie, W. and Rothblum, L.I. (1992) Analysis of the phosphorylation, DNA-binding and dimerization properties of the RNA polymerase I transcription factors UBF1 and UBF2. *Nucleic Acids Res.*, **20**, 1301–1308.
- Litchfield, D.W. (2003) Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem. J.*, **369**, 1–15.
- Johnston, J.M., Allison, S.J., Morton, J.P., Schramm, L., Scott, P.H. and White, R.J. (2002) CK2 forms a stable complex with TFIIB and activates RNA polymerase III transcription in human cells. *Mol. Cell. Biol.*, **22**, 3757–3768.

38. Hu,P., Wu,S. and Hernandez,N. (2003) A minimal RNA polymerase III transcription system from human cells reveals positive and negative regulatory roles for CK2. *Mol. Cell*, **12**, 699–709.
39. Hu,P., Samudre,K., Wu,S., Sun,Y. and Hernandez,N. (2004) CK2 phosphorylation of Bdp1 executes cell cycle-specific RNA polymerase III transcription repression. *Mol. Cell*, **16**, 81–92.
40. Lewis,B.A., Sims,R.J., 3rd, Lane,W.S. and Reinberg,D. (2005) Functional characterization of core promoter elements: DPE-specific transcription requires the protein kinase CK2 and the PC4 coactivator. *Mol. Cell*, **18**, 471–481.
41. Lin,C.Y., Tuan,J., Scalia,P., Bui,T. and Comai,L. (2002) The cell cycle regulatory factor TAF1 stimulates ribosomal DNA transcription by binding to the activator UBF. *Curr. Biol.*, **12**, 2142–2146.
42. Hirschler-Laszkiwicz,I., Cavanaugh,A.H., Mirza,A., Lun,M., Hu,Q., Smink,T. and Rothblum,L.I. (2003) Rrn3 becomes inactivated in the process of ribosomal DNA transcription. *J. Biol. Chem.*, **278**, 18953–18959.
43. Sarno,S., Reddy,H., Meggio,F., Ruzzene,M., Davies,S.P., Donella-Deana,A., Shugar,D. and Pinna,L.A. (2001) Selectivity of 4,5,6,7-tetrabromobenzotriazole, an ATP site-directed inhibitor of protein kinase CK2 ('casein kinase-2'). *FEBS Lett.*, **496**, 44–48.
44. Voit,R., Kuhn,A., Sander,E.E. and Grummt,I. (1995) Activation of mammalian ribosomal gene transcription requires phosphorylation of the nucleolar transcription factor UBF. *Nucleic Acids Res.*, **23**, 2593–2599.
45. Mayer,C., Bierhoff,H. and Grummt,I. (2005) The nucleolus as a stress sensor: JNK2 inactivates the transcription factor TIF-IA and down-regulates rRNA synthesis. *Genes Dev.*, **19**, 933–941.
46. Yudkovsky,N., Ranish,J.A. and Hahn,S. (2000) A transcription reinitiation intermediate that is stabilized by activator. *Nature*, **408**, 225–229.
47. Aprikian,P., Moorefield,B. and Reeder,R.H. (2001) New model for the yeast RNA polymerase I transcription cycle. *Mol. Cell Biol.*, **21**, 4847–4855.
48. Panov,K.I., Friedrich,J.K. and Zomerdijk,J.C. (2001) A step subsequent to preinitiation complex assembly at the ribosomal RNA gene promoter is rate limiting for human RNA polymerase I-dependent transcription. *Mol. Cell Biol.*, **21**, 2641–2649.
49. Kugel,J.F. and Goodrich,J.A. (1998) Promoter escape limits the rate of RNA polymerase II transcription and is enhanced by TFIIE, TFIIH, and ATP on negatively supercoiled DNA. *Proc. Natl Acad. Sci. USA*, **95**, 9232–9237.
50. Hannan,R.D., Hempel,W.M., Cavanaugh,A., Arino,T., Dimitrov,S.I., Moss,T. and Rothblum,L. (1998) Affinity purification of mammalian RNA polymerase I. Identification of an associated kinase. *J. Biol. Chem.*, **273**, 1257–1267.
51. Albert,A.C., Denton,M., Kermekchiev,M. and Pikaard,C.S. (1999) Histone acetyltransferase and protein kinase activities copurify with a putative *Xenopus* RNA polymerase I holoenzyme self-sufficient for promoter-dependent transcription. *Mol. Cell Biol.*, **19**, 796–806.
52. Saez-Vasquez,J., Meissner,M. and Pikaard,C.S. (2001) RNA polymerase I holoenzyme-promoter complexes include an associated CK2-like protein kinase. *Plant Mol. Biol.*, **47**, 449–459.
53. Battistutta,R., De Moliner,E., Sarno,S., Zanutti,G. and Pinna,L.A. (2001) Structural features underlying selective inhibition of protein kinase CK2 by ATP site-directed tetrabromo-2-benzotriazole. *Protein Sci.*, **10**, 2200–2206.
54. Mayer,C., Zhao,J., Yuan,X. and Grummt,I. (2004) mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. *Genes Dev.*, **18**, 423–434.
55. Hannan,K.M., Brandenburger,Y., Jenkins,A., Sharkey,K., Cavanaugh,A., Rothblum,L., Moss,T., Poortinga,G., McArthur,G.A., Pearson,R.B. *et al.* (2003) mTOR-dependent regulation of ribosomal gene transcription requires S6K1 and is mediated by phosphorylation of the carboxy-terminal activation domain of the nucleolar transcription factor UBF. *Mol. Cell Biol.*, **23**, 8862–8877.
56. Muth,V., Nadaud,S., Grummt,I. and Voit,R. (2001) Acetylation of TAF(I)68, a subunit of TIF-IB/SL1, activates RNA polymerase I transcription. *EMBO J.*, **20**, 1353–1362.
57. Bell,P., Mais,C., McStay,B. and Scheer,U. (1997) Association of the nucleolar transcription factor UBF with the transcriptionally inactive rRNA genes of pronuclei and early *Xenopus* embryos. *J. Cell. Sci.*, **110**, 2053–2063.
58. Bell,S.P., Jantzen,H.M. and Tjian,R. (1990) Assembly of alternative multiprotein complexes directs rRNA promoter selectivity. *Genes Dev.*, **4**, 943–954.
59. Belenguer,P., Baldin,V., Mathieu,C., Prats,H., Bensaïd,M., Bouche,G. and Amalric,F. (1989) Protein kinase NII and the regulation of rDNA transcription in mammalian cells. *Nucleic Acids Res.*, **17**, 6625–6636.
60. Filhol,O., Nueda,A., Martel,V., Gerber-Scokaert,D., Benitez,M.J., Souchier,C., Saoudi,Y. and Cochet,C. (2003) Live-cell fluorescence imaging reveals the dynamics of protein kinase CK2 individual subunits. *Mol. Cell Biol.*, **23**, 975–987.
61. Sheng,Z., Liang,Y., Lin,C.Y., Comai,L. and Chirico,W.J. (2005) Direct regulation of rRNA transcription by fibroblast growth factor 2. *Mol. Cell Biol.*, **25**, 9419–9426.
62. Wisniewski,J.R., Szewczuk,Z., Petry,I., Schwanbeck,R. and Renner,U. (1999) Constitutive phosphorylation of the acidic tails of the high mobility group 1 proteins by casein kinase II alters their conformation, stability, and DNA binding specificity. *J. Biol. Chem.*, **274**, 20116–20122.
63. Krohn,N.M., Stemmer,C., Fojan,P., Grimm,R. and Grasser,K.D. (2003) Protein kinase CK2 phosphorylates the high mobility group domain protein SSRP1, inducing the recognition of UV-damaged DNA. *J. Biol. Chem.*, **278**, 12710–12715.