Mass spectrometric identification of phosphorylation sites of rRNA transcription factor upstream binding factor

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rRNA transcription is a fundamental requirement for all cellular growth processes and is activated by the phosphorylation of the upstream binding factor (UBF) in response to growth stimulation. Even though it is well known that phosphorylation of UBF is required for its activation and is a key step in activation of rRNA transcription, as yet, there has been no direct mapping of the phosphorylation sites of UBF. The results of the present studies employed sophisticated nanoflow HPLC-microelectrospray-ionization tandem mass spectrometry (ESI-MS/MS) coupled with immobilized metal affinity chromatography (IMAC) and computer database searching algorithms to identify 10 phosphorylation sites on UBF at serines 273, 336, 364, 389, 412, 433, 484, 546, 584, and 638. We then carried out functional analysis of two of these sites, serines 389 and 584. Serine-alanine substitution mutations of 389 (S389A) abrogated rRNA transcription in vitro and in vivo, whereas mutation of serine 584 (S584A) reduced transcription in vivo but not in vitro. In contrast, serine-glutamate mutation of 389 (S389E) restored transcriptional activity. Moreover, S389A abolished UBF-SL1 interaction in vitro, while S389E partially restored UBF-SL1 interaction. Taken together, the results of these studies suggest that growth factor stimulation induces an increase in rRNA transcriptional activity via phosphorylation of UBF at serine 389 in part by facilitating a rate-limiting step in the recruitment of RNA polymerase I: i.e., recruitment of SL1. Moreover, studies provide critical new data regarding multiple additional UBF phosphorylation sites that will require further characterization by the field.

immobilized metal affinity chromatography; mass spectrometry; UBF; TBP; RNA polymerase I

ACTIVATION of rRNA transcription is required for sustained growth of all cells. In vitro, eukaryotic RNA transcription can be reconstituted by the addition of three factors: RNA polymerase I (Pol I), selectivity factor I (SL1), consisting of the TATA binding protein (TBP), and three Pol I-specific TBP-associated factors (48, 63, and 110) and the upstream binding factor (UBF). It has been well recognized for nearly a decade that UBF is a necessary component for the activation of efficient rRNA transcription, and that phosphorylation of UBF dramatically enhances rRNA transcription in vitro. Furthermore, UBF phosphorylation is upregulated in states of cellular growth, consistent with a model whereby phosphorylation of UBF is a key mechanism linking cellular growth to activation of rRNA transcription. However, relatively little is known regarding the mechanisms by which UBF phosphorylation regulates transcriptional activity. Previous studies in our laboratory (17) and others (33, 40) demonstrated that growth-induced phosphorylation of UBF resulted in increased binding between SL1 (or TBP) and UBF. UBF and SL1 (known as TIF-I/B in mouse) are the first factors that bind rDNA to initiate formation of a stable transcription complex in vitro (30). However, unlike UBF, SL1 alone does not bind efficiently to the rDNA promoter, which lacks a TATA box. However, the addition of UBF substantially extends the in vitro DNase footprint generated by SL1 alone, suggesting cooperative interactions between these factors (1, 18). Thus UBF phosphorylation may control the kinetics and stability of transcription initiation complex assembly via recruitment of SL1 to the rDNA promoter. Taken together, the results suggest that growth factor-induced increases in rRNA transcription are mediated in part by phosphorylation of UBF and subsequent enhanced recruitment of SL1/TBP to rDNA promoters.

Recently, two kinase families have been implicated in UBF phosphorylation. Drakash et al. (7) demonstrated that PI3 kinase phosphorylates UBF as a result of insulin-like growth factor signaling. This is of particular functional significance, as insulin-like growth factor signaling has been shown to play a role in cellular growth and hypertrophy. Likewise, ERK1/2 plays a well-characterized role in phosphorylation of cell growth and was recently implicated in UBF phosphorylation by Stefanovsky et al. (32). However, the role of this phosphorylation in UBF-mediated rDNA transcription initiation complex has not been completely elucidated.

UBF phosphorylation may also mediate subnuclear localization of UBF. Under conditions of cellular quiescence or serum starvation, UBF is localized diffusely throughout the nucleus. In contrast, agonist or serum stimulation of the cells induced rapid nucleolar localization of UBF (13, 23), suggesting that spatial localization of components of the transcription initiation complex may contribute to regulation of rRNA transcription (3, 8).

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A major limitation in the field is that there are no reported studies that have directly mapped which of the 70 serine residues in the primary sequence of UBF modulate its transcriptional activity and by what mechanism they do so. Our laboratory (17) and others (23) have shown that UBF is phosphorylated exclusively on multiple serine residues in vivo and is extremely complex. For example, results of two-dimensional phosphoprotic mapping studies in our laboratory provided compelling evidence that growth factor stimulation of cells increased the stoichiometry of phosphorylation on at least 11 different sites compared with quiescent cells in positive transcriptional activity of UBF in vitro represented a major advance for the field. However, there is no direct evidence that these sites are phosphorylated on full-length UBF and many additional sites remain to be identified and functionally characterized. In summary, despite it being widely acknowledged for nearly a decade that regulation of UBF phosphorylation is a key rate-limiting step in control of rRNA transcription and sustained cell growth, very little is known as to how this critical process is regulated.

Here, we report the first direct mapping of phosphorylation sites on full-length UBF1 (see Table 1) obtained using nanoflow HPLC-microelectrospray-ionization tandem mass spectrometry (nHPLC-MS/MS) coupled with immobilized metal affinity chromatography (IMAC) and computer database searching algorithms and demonstrate that at least one of these critical processes is necessary for transcriptional activation via SL1 nucleation to the rDNA transcription initiation site.

**MATERIALS AND METHODS**

**Cell culture, cotransfection assays, and in vitro transcription.** Culture of rat aortic VSMCs has been described previously (13). For transient transfection experiments, VSMCs were plated at a density of 10⁴ cells/cm². After 24 h, cells were transfected in triplicate with FuGENE6, (Roche) following the manufacturer’s protocol. Transfection was with equal (1:1) ratio of pGRIL rDNA-INES luciferase construct to plasmid of interest [pcDNA3 or pcDNA3 carrying wild-type or mutant rUBF1 cDNA (pDUBF, pCDTR2, pCD389A, pCD389E, or pCD584A)]. One day after transfection, the cells had reached confluence were rendered quiescent (11) in a defined serum-free medium for 24 h, after which they were re-fed with serum-containing media for 9 additional hours. They were then lysed with 150 µl × passive lysis buffer (Promega) for 30 min at room temperature. Luciferase reporter activity was assayed on Monolight 2010 luminometer using 20 µl cell lysate and 100 µl luciferase assay substrate (Promega) with 10-s measurements. Values were normalized using protein concentration assayed by Coomassie Plus protein assay reagent (Pierce) according to the manufacturer’s directions.

In vitro transcription assays were performed as previously described (40). For information on plasmid construction, see the online supplemental figures and table.

**Purification and in vitro phosphorylation of UBF.** Full-length FLAG-rUBF1 or mutant FLAG-rUBF1 was prepared by infecting a SF9 culture at 5 × 10⁵ cells/ml with a UBF expression baculovirus, MOI 1.0–10 (constructed using the Baculogold cotransfection kit from Pharmingen exactly as described in product materials). Cells were incubated as a suspension in a spinner flask at 27°C for 44–48 h. At the termination of the incubation, the cells were pelleted by centrifugation, washed once in PBS (Invitrogen), and snap frozen in liquid N₂. Cells were lysed by hypotonic lysis buffer (10 mM Tris-HCl, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂) by sonication (50% duty cycle, 600 We-40% power). After sonication, nuclear debris was pelleted by centrifugation, 12,000 × g for 10 min at 4°C. Nuclear debris was then extracted by Extraction buffer (50 mM Tris-HCl, pH 7.9, 600 mM NaCl, 5 mM MgCl₂, 25% glycerol, 0.5 mM EDTA); extract was then cleared by centrifugation, 12,000 × g for 10 min at 4°C. Lysate and extract were combined, adjusted to pH 7.9 using 1 M Tris base, added to anti-FLAG M2 agarose beads, and rotated at 4°C for 2 h. Beads were extensively washed in >10 volumes of Tris-buffered saline, pH 7.9, and FLAG-rUBF1 (or mutant) eluted in 100 mM glycine, pH 2.5. Fractions were immediately neutralized to 50 mM Tris by the addition of 1 M Tris base and dialyzed against 50 mM Tris, pH 7.9, 150 mM KCl, and 10% glycerol. Purified FLAG-rUBF1 (50 µg) was phosphorylated by 2 µg of ANG II-stimulated VSMC nuclear extract in vitro for 1 h at 37°C in the presence of phosphatase inhibitor cocktail (Sigma). Samples were then snap frozen in liquid N₂ and stored at −80°C until ready for proteolytic digestion and MS/MS analysis. Final concentration of UBF was ~100 µg/ml. All buffers contained Complete protease inhibitor cocktail (Roche), 0.5 mM DTT, and 1 mM MBS unless otherwise stated.

**Indirect immunofluorescence.** After transient transfection (described above), cells were allowed to recover for 48 h at 37°C. Cells were washed 3 times with PBS, fixed in 2% paraformaldehyde for 15 min, and permeabilized with 0.25% Triton X-100 for 15 min. Primary antibodies, anti-FLAG M2 1:500 (Kodak) and anti-fibrillarin 1:100 (Sigma) were applied with 0.1% Triton X-100, 5% goat serum, and 1% horse serum in PBS for 2 h at room temperature. Cells were then washed three times with PBS and stained with 300 nM 4,6-diamidino-2-phenylindole and secondary antibodies 1:2,000 Alexa Fluor 488 anti-mouse IgG, 1:2,000 Alexa Fluor 555 anti-human IgG, 1:2,000 ( Molecular Probes). Before being visualized, the cells were washed three times with PBS. Images were acquired on an Olympus inverted fluorescence microscope and a cooled digital camera (Roper Scientific).

**Mass spectrometric identification of phosphorylation sites.** One hundred microliters (~100 pmol) of purified, phosphorylated UBF1 was reduced, alkylated, and digested with trypsin (14) and aliquots of the digest corresponding to 12.5 pmol of UBF1 were subjected to sample cleanup and IMAC enrichment as previously described (10), with minor modifications. As a single, highly purified protein was being analyzed, no attempt was made to reduce the binding of nonphosphorylated peptides to the IMAC material by utilizing the Fisher esterification. Rather, an aliquot was loaded directly onto a 360 µm outer diameter (OD) × 200 µm inner diameter (ID) fused-silica cleanup column containing a LiChrosorb frit and packed with 8 cm of 5–20 µm C-18 particles. The peptide-loaded clean-up column was rinsed extensively with 0.1% (vol/vol) H₂O₂ and connected to a 360 µm OD × 100 µm ID fused-silica column, packed with 8 cm POROS 20 MC IMAC packing material and activated with 200 µl 100 mM FeCl₃. Peptides were eluted from the cleanup column to the IMAC column with 80% acetonitrile containing 0.1% TFA.
column with a solution of 40% (vol/vol) acetonitrile and 1% (vol/vol) HOAc. The IMAC column was disconnected from the cleanup column and rinsed with a solution containing 100 mM NaCl in 25% (vol/vol) MeCN and 1% (vol/vol) HOAc to remove nonspecifically bound peptides. The IMAC column was reequilibrated with a solution of 0.1% (vol/vol) HOAc, and the phosphopeptides were eluted to an HPLC analytical column/precolumn assembly containing an integrated laser-pulled electrospray ionization emitter tip (21). Samples were analyzed by nanoflow HPLC-microelectrospray ionization on a Finnigan LCQ DECA ion-trap mass spectrometer, operated in data dependent mode. Rapid identification of phosphopeptide candidates from MS/MS spectra was accomplished with an in-house computer program called Neutral Loss Tool (39), which screens for the loss of phosphoric acid from the phosphopeptide precursor (6). Candidate MS/MS spectra were searched against the UBF1 protein sequence using the SEQUEST algorithm with “No Enzyme” specificity (9). Search parameters included a differential modification of 80 Da on serine, threonine, and tyrosine, representing the presence or absence of phosphate, and a static modification of 57 Da on cysteine, representing alkylation with iodoacetamide.

**Peptide synthesis.** Wash resins, 9-fluorenylmethoxycarbonyl-protected amino acids, and phosphorylated amino acids (Calbiochem, San Diego, CA) were used to synthesize peptides on an AMS 422 multiple peptide synthesizer (Gilson Medical Electronics, Middleton, WI). Peptides were characterized by mass spectrometry.

**SL1 interaction assay.** The UBF-SL1 interaction assay was performed largely as previously described (33). Briefly, 500 ng purified wild-type FLAG-rUBF1 or mutant was incubated with anti-FLAG M2 agarose bead (Sigma) for 2 h at 4°C, washed extensively in TM+ + 0.1 (50 mM Tris-HCl, pH 7.9, 100 mM KCl, 12.5 mM MgCl2, 10% glycerol, 0.1% Nonidet P-40), and incubated with 20 μg of partially purified SL1 overnight. Beads were washed extensively in TM+ + 0.1, rUBF1-bound SL1 was eluted in BCO (20 mM Tris-HCl, pH 8.0, 10% glycerol, 1 M KCl, 0.5 mM EDTA, and 1% deoxycholic acid) buffer, TCA precipitated, resolved on 10% SDS-PAGE and analyzed by anti-TAF110 Western.

**RESULTS**

**Effective expression, purification, and phosphorylation of UBF.** The development of a reliable and reproducible method for the efficient expression and purification of UBF was the first major step in our attempts to identify phosphorylation sites within the protein. Indeed, efficient production of full-length recombinant UBF continues to represent a major challenge for the field (L. Comai, L. Rothblum, and C. H. Lin, unpublished observations), and a number of studies continue to truncate forms of the protein for biochemical studies (32). Baculovirus-infected, eukaryotic SF9 cells were chosen due to the fact that 1) the expression of eukaryotic proteins in bacteria often results in incorrectly folded, insoluble molecules (20); 2) there is no known limit for the size of the heterologous gene insertion in baculovirus (16); and 3) the expression of the gene occurs after the virus has been packaged, so that the expressed gene should not affect viral production (11, 16, 31). The incorporation of the FLAG tag was also an important advance, as the tagging did not interfere with the function of UBF (as assessed in in vitro rRNA transcription assays), but it did allow for purification of the protein to apparent homogeneity (see online supplemental Fig. 1a). In addition, this technique was readily applied to the production of phosphorylation site mutants and was also used in the overexpression of full-length UBF in mammalian cells.

Purified FLAG-rUBF was then treated with nuclear extract from ANG II-stimulated VSMC, which we have previously shown leads to rapid phosphorylation of the protein (see online supplemental Fig. 1b) and binding to TBP (17) and activation of its ability to stimulate RNA transcription in vitro (Kihm and Owens, unpublished results). Alternatively, purified recombinant UBF phosphorylated by a Large T antigen associated kinase was used.

**Sequence coverage of FLAG-rUBF using multiple proteolytic enzymes.** Because there are 70 serine residues in UBF, multiple enzymes were used to digest the protein before nHPLC-μESI MS in an effort to visualize all potential phosphorylation sites. Online supplemental Fig. 2 shows that reduction, alkylation, and parallel digestion of FLAG-rUBF with Asp-N, Glu-C, and trypsin resulted in 35.7%, 53.4%, and 64.4% sequence coverage, respectively. Combining the coverages obtained using each individual enzyme provided over 80% sequence coverage, allowing direct visualization of 46 of the 47 serine residues present in the main body of the protein. Importantly, the serine residue not visualized in this analysis is converted to an alanine residue in the human sequence of UBF1. One phosphopeptide was identified in this reversed phase analysis: the serine 584 species FQELLpSNGELNHLPLK.

**IMAC enrichment for the identification of phosphorylation sites.** To selectively enrich the sample for phosphorylated peptides, immobilized metal affinity chromatography (IMAC) using Fe3+ was used (10, 25, 29). This approach separates phosphopeptides from their non-phosphorylated counterparts and permits subsequent online (22, 38) or offline (26) mass spectrometric analysis. In this study, offline IMAC enrichment used in conjunction with nHPLC-μESI MS permitted the identification of nine specific sites of phosphorylation in UBF.

Ten different UBF serine phosphorylation sites were identified by LC-MS/MS on an ion trap mass spectrometer. Ten different phosphorylation sites in rUBF1 were identified in toto, including serines 273, 336, 364, 389, 412, 433, 484, 546, 584, and 638 (Table 1, Fig. 1, and see online supplemental Fig. 3). All assigned sequences were confirmed by comparing the fragmentation pattern of the experimental spectrum to that of a synthetic phosphopeptide (example shown in online supplemental Fig. 1b). Importantly, all phosphorylations occurred on serine residues, in agreement with previously conducted phosphoamino acid analysis (13). Mutation of serine 389 decreased UBF induced rRNA transcriptional activation in vitro and in vivo whereas mutation of serine 584 alanine had no effect. Given the large technical burden of producing purified, recombinant full-length UBF, we chose to focus on two phosphorylation sites for further functional characterization. Since our interest has been in determining the phosphorylation events that lead to UBF-SL1 interaction, we selected sites that may contribute to controlling this interaction. Recent structural studies have revealed conserved amino acid residues in negative cofactor 2 (NC2) that interacts directly with TBP (15) bound to the TATA sequence, and this motif is found on UBF at several sites (see online supplemental Fig. 4), including one adjacent to serine 389. This suggested
Fig. 1. Microelectrospray ionization tandem mass spectrometry (MS/MS) spectrum of a tryptic phosphorylated peptide derived from mutant upstream binding factor 1 (rUBF1). A: identification of phosphorylation at serine 389. B: MS/MS spectrum of the synthetic phosphopeptide VLGEEKMLNKKQTPSPASK. C: identification of phosphorylation at serine 584. Predicted masses for the ions of type b and y are shown above and below the sequence, respectively. The \( b^{(y)} \) and \( y^{(b)} \) masses are average values while the singly charged b and y ion masses are monoisotopic. Ions observed in the spectrum are underlined and those that lose phosphoric acid are presented in bold type. The label \( \Delta \) denotes a loss of phosphoric acid from the corresponding ion of type b or y.
that UBF-TBP interaction may be regulated by phosphorylation at serine 389.

Next, we chose to focus on serine 584 as it was identified without requiring IMAC enrichment, and was therefore one of the first sites identified.

To test the function of the serine 389 and 584 phosphorylation sites on rRNA transcription, serine to alanine substitution mutations of rUBF1 were tested in an in vitro reconstitution rRNA transcription assay. In addition, to determine whether the phosphorylation site mutations altered rRNA transcription in intact cells, a series of co-transfection studies were performed in cultured VSMCs with wild-type or mutant UBF expression plasmids and a unique rDNA promoter luciferase reporter plasmid, pGRIL (see Supplemental Methods).

Results showed that wild-type rUBF1 activated rRNA transcription by nearly twofold in an in vitro reconstitution assay (Fig. 2A) and nearly threefold in vivo transient cotransfection assays (Fig. 2B). In contrast, mutation of serine 389 to alanine virtually abolished UBF-activated transcription in both systems. To simulate the negative charge of a phosphate group at residue 389, this site was also mutated from a serine to a glutamate residue, and mutation of serine 389 to glutamate fully restored activity in vitro (data not shown) and restored ~80% activity in vivo (Fig. 2B). In contrast to effects of mutation of serine 389, mutation of serine 584 to alanine residue did not seem to affect transcription in vitro but decreased transcription in vivo by 75%. Interestingly, transient transfection of pcDNA3 encoding for a truncation mutation (TR), in which the COOH terminus was removed downstream of amino acid 674 repressed transcription from the rDNA-ires-luciferase construct in vivo below the activity of samples containing the empty vector (pcDNA3) control.

Mutation of serine 389 to alanine abrogated UBF-SL1 interaction in vitro, whereas serine 389 to glutamate mutation partially restored interaction. UBF phosphorylation has been shown to modulate UBF-SL1 interactions (17, 33). To determine whether phosphorylation of serine 389 is required for UBF-SL1 interaction, we performed in vitro interaction assays; results showed that wild-type rUBF1 bound to SL1 with an efficacy equivalent to 10–20% of input SL1 (Fig. 3, lanes 4, 1, and 2, respectively). Mutation of serine 389 to alanine abolished this UBF-SL1 interaction (Fig. 3, lane 5) as did removal of the acidic tail (TR) (Fig. 3, lane 7), while mutation of serine 389 to glutamate partially restored UBF-SL1 interaction (Fig. 3, lane 6).

Phosphorylation of serine 389 and 584 sites are not necessary for nucleolar localization of UBF. We and others (13, 23) previously demonstrated that UBF is distributed throughout the nucleus in quiescent cells and is localized specifically to the nucleolus within 15 min of growth factor stimulation. Because of the rapid kinetics of this event, we hypothesized that localization of UBF may be due to phosphorylation of serine 389 or 584. Under our transfection culture conditions (cells were asynchronous and in logarithmic growth phase), we found that wild-type FLAG-UBF (green) in most cells was localized to the nucleoli (red). Consistent with previous reports, removal of the acidic tail (UBF 674C, green) abrogated nucleolar localization of UBF (19) (Fig. 4B) such that the protein was distributed diffusely throughout the nucleus (blue). In contrast, mutation of serines 389 and 584 to alanine had no affect on nucleolar localization of UBF. Furthermore, mutation of serine 389 to glutamate also did not significantly affect subnuclear localization of UBF.

DISCUSSION

The importance of UBF phosphorylation in activation of rRNA transcription was first demonstrated nearly a decade ago by Voit and co-workers (35, 36). Since then, several effects of growth stimulation on UBF have been defined. First, UBF is hyperphosphorylated in response to growth stimulation (13, 23, 36). Second, UBF is localized specifically to the nucleolus within 15 min of stimulation (13). Third, UBF affinity for SL1/TBP is increased in response to growth stimulation (17, 23, 36). Fourth, UBF activation of rRNA transcription is increased by growth stimulation (34–37). As such, observations in the present studies showing direct evidence of phosphorylation of 10 unique serine sites within UBF, and that mutation of serine 389 resulted in significant loss in the transcriptional activity of UBF in vitro and in vivo represent a major advance for the field. These findings will allow further functional analysis of UBF phosphorylation in the control of rRNA transcription in intact cells.
Fig. 3. Effect of phosphorylation site mutation on UBF-SL1 interaction in vitro. Purified recombinant FLAG-tagged WT UBF1 or mutant was immobilized on anti-FLAG M2 agarose beads and then incubated with partially purified SL1 (lanes 4–7). Bound SL1 was eluted in BCO buffer (20 mM Tris-HCl, pH 8.0, 10% glycerol, 0.5 mM EDTA, and 1% deoxycholic acid) and analyzed by anti-TAF110 Western (top). After BCO elution, beads were boiled in Laemmli sample buffer and resolved by 10% SDS-PAGE and stained with Coomassie brilliant blue to verify UBF loading (bottom). Lanes 1 and 2 represent 10% and 20% of SL1 incubated with UBF in each sample, respectively. Lane 3, negative control, in which no UBF was immobilized to bead before incubating with SL1.

To that end, we are presently designing anti-phospho UBF antibodies to directly probe the functional significance of these sites in growth-stimulated cells as present methods precluded the identification of sites differentially phosphorylated in UBF from growth-stimulated vs. unstimulated cells. Of note, we have previously demonstrated that in cells rendered quiescent and maintained in neutral protein balance, the pattern of UBF phosphorylation is similar to that in stimulated cells, and differs only in stoichiometry of phosphorylation at each site (17). Indeed, while MS/MS is an exceedingly powerful tool, the technique cannot rule out phosphorylation at a given site if there is an absence of signal. Therefore, it would not have been possible to unequivocally use the technique to delineate differential phosphorylation between stimulated and unstimulated cells.

Likewise, we cannot completely exclude the possibility of phosphorylation on threonine or tyrosine residues, although we (present manuscript) (13, 17) and others (23, 34, 37) have reported phosphorylation of only serine residues on UBF. Stefanovsky and co-workers have recently demonstrated that UBF peptides mimicking HMG boxes 1 and 2 are phosphorylated in vitro on threonine 117 and threonine 201, respectively (32). Investigators were able to demonstrate in a co-transfection assay that both threonine residues were important in activating rRNA transcription. However, it remains to be determined whether or not the full-length protein is phosphorylated in vivo on these sites as well. Indeed, in the present studies, we have exhaustively attempted to identify phosphorylation on threonine and tyrosine residues on full-length UBF, but were not successful. As previously noted, the technical limitations of tandem mass spectrometry precluded our ability to completely rule out phosphorylation on nonserine residues.

Of the serine phosphorylation sites identified in present studies, the two tested in cotransfection studies both caused disruption of rRNA transcription from our chimeric reporter construct. While not a novel model of rRNA transcription (12), results from the in vivo transfection assay may have uncovered unique roles for different functional regions of UBF. Specifically, mutation of serine 584 to alanine decreased transcription from the reporter construct, but had little effect in vitro. Several explanations may account for this effect, at least three of which may be related to the role of phosphorylation of serine 584.

First, whereas the in vitro reconstitution assay does not include chromatin or tertiary DNA-protein structures, the reporter gene likely forms tertiary and chromatin structures in a manner that may be physiologically relevant (28). Indeed, we have found that UBF preferentially associates with histone H3 methylated-lysine 9 (C. H. Lin, W. L. Cheung, C. P. Allis, and G. Owens, unpublished observations), suggesting a direct interaction between UBF and specific rDNA chromatin structures. Second, whereas activity in the reconstitution assay is primarily dependent upon the total concentration of each factor in solution, the transfection assay is also dependent on intranuclear spatial localization of factors. Third, since the reconstitution assay consists of only factors necessary for transcription, results of the transfection assay may suggest the effect of co-activators or repressors [e.g., Rb(2) or p130(5)]. It may be that phosphorylation of serine 584 plays an important role in one or more of these phenomena; further study of this phosphorylation site may provide insight into as of yet undiscovered roles for UBF phosphorylation.

Importantly, one of the most well-characterized roles of UBF phosphorylation, modulation of the interaction with SL-1,
was perturbed by mutation of serine 389. The fact that mutation of serine 389 virtually abolished UBF-SL1 interaction was somewhat surprising as previous work by Tuan et al. (33) demonstrated that a fragment containing only the amino acid residues of UBF between 491 and 764 was sufficient for UBF-SL1 interaction in an in vitro assay. Furthermore, removal of the COOH-terminal residues between 670 and 764 of UBF completely abrogated UBF-SL1 interaction. While these data suggest that the acidic COOH terminus of UBF is most likely the portion of the protein that directly interacts with the SL1 complex, recent structural studies have revealed a great deal regarding the nature of TBP and cofactor interactions. The NC2 has recently been co-crystallized with TBP bound to a TATA DNA fragment. On the basis of the crystal structure, the investigators (15) identified several conserved amino acid residues in the NC2β chain (helix 5) that interact directly with TBP via its COOH-terminal domain. Interestingly, the motif formed by these residues can be found in several regions in UBF (see online supplemental Fig. 4). More importantly, several UBF phosphorylation sites are located flanking these motifs, including serine 389, consistent with phosphorylation of these putative TBP interaction motifs. Consistent with this hypothesis, the 40 amino acid region surrounding serine 389 contains one theoretical α-helix based on both the Chou-Fasman (4) and Garnier-Robson (27) methods (see online supplemental Fig. 4) that terminates at serine 389. However, Glutamate substitution of serine 389 extends the α-helix through residue 389 to 391 or 393 (Chou-Fasman or Garnier-Robson methods, respectively). Additional studies will be required to further delineate the kinetic and spatial relationship between UBF phosphorylation and SL1 recruitment in the context of transcription initiation.

Indeed, the kinetics of UBF nucleolar localization suggest involvement of a rapid posttranslational modification event such as phosphorylation (13). In fact, recent fluorescence recovery after photobleaching and fluorescence loss in photobleaching experiments in vivo have demonstrated that UBF moves rapidly in and out of the nucleolus (3, 8), such that sustained nucleoplasmic or nucleolar localization is likely due to a shift in equilibrium by some posttranslational modification. However, alanine substitution mutations for serine 389 and 584 did not alter nucleolar localization. Since we have mapped 10 phosphorylation sites so far, and we present data for only 2 sites here, we certainly cannot exclude the possibility that phosphorylation of a single site controls localization of UBF to the nucleolus. Alternatively, it may be that UBF must be phosphorylated on a certain number of sites to reach a threshold negative charge as opposed to phosphorylation at a specific site to be localized to the nucleolus. For example, nuclear import of NFAT requires complete dephosphorylation of 13 sites to expose a nuclear localization signal and mask a nuclear export signal (24). However, to adequately test this hypothesis in UBF would require an exhaustive panel of combinatorial mutations of the ten sites we have mapped.

While present studies are the first direct identification of UBF phosphorylation sites, two key studies by Voit and co-workers (34, 37) significantly advanced the field by presenting the first evidence suggesting specific differential UBF phosphorylation at serine 484 and serine 388 (identical to the site we have called serine 389). These studies employed a combination of two-dimensional phosphotryptic mapping and site-directed mutagenesis to indirectly identify phosphorylation at these sites. In contrast, the present studies report the first unequivocal direct identification of UBF phosphorylation sites by tandem mass spectrometry, and therefore represent a required validation of phosphorylation of UBF on sites also identified by Voit and co-workers (serines 388/389 and 484) as well as eight additional novel phosphorylation sites. These findings represent a major advance for the field and will provide the basis for extensive further studies to elucidate the mechanisms that control growth induced activation of UBF and increased rRNA transcription.

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