Phosphorylation by $G_1$-specific cdk–cyclin complexes activates the nucleolar transcription factor UBF

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Transcription of rRNA genes by RNA polymerase I increases following serum stimulation of quiescent NIH 3T3 fibroblasts. To elucidate the mechanism underlying transcriptional activation during progression through the $G_1$ phase of the cell cycle, we have analyzed the activity and phosphorylation pattern of the nucleolar transcription factor upstream binding factor (UBF). Using a combination of tryptic phosphopeptide mapping and site-directed mutagenesis, we have identified Ser484 as a direct target for cyclin-dependent kinase 4 (cdk4)–cyclin D1- and cdk2–cyclin E-directed phosphorylation. Mutation of Ser484 impairs rDNA transcription $in vivo$ and $in vitro$. The data demonstrate that UBF is regulated in a cell cycle–dependent manner and suggest a link between $G_1$ cdk–cyclins, UBF phosphorylation and rDNA transcription activation.

Keywords: cdk–cyclin complexes/cell cycle/ phosphorylation/RNA polymerase I/UBF

Introduction

Progression through the cell cycle is driven by the periodic activation of several cyclin-dependent kinases (cdks) which play a prominent role in the regulation of cellular metabolism. The mechanisms by which these cdks regulate cell cycle progression are not fully understood, mainly because few targets of these kinases have been identified. Periodically transcribed genes encode proteins that directly control cell cycle progression or, alternatively, gene products that are required for periodically occurring metabolic processes. Examples of the latter are the genes transcribed by RNA polymerase I (Pol I) and RNA polymerase III (Pol III), respectively. With regard to class III gene transcription, Pol III activity increases gradually as $G_1$ progresses, and reaches maximal levels at the end of S phase (White et al., 1995). This augmentation of Pol III transcription is accompanied by activation of TFIIB, indicating that this factor is an important target for cell cycle-dependent regulation of Pol III transcription. By analogy, rRNA synthesis by Pol I is repressed at mitosis and increases during $G_1$ progression. Moreover, cells deprived of serum cease to progress through the cell cycle and shut down rRNA synthesis (for recent reviews, see Grummt, 1998, 1999). Thus, by responding to variations in growth rate, changes in Pol I transcription regulate ribosome production and thus determine the potential for cellular proliferation.

Biochemical studies have revealed a plethora of control mechanisms that operate to modulate the activity of components of the Pol I transcription machinery. These studies revealed that targets of regulation are: TIF-IB/SL1, the Pol I-specific TBP–TAF complex (Heix et al., 1998; Kuhn et al., 1998); TIF-IA/TFIC/factor C*, a Pol I-associated factor which is inactivated in quiescent cells (Buttgereit et al., 1985; Tower and Sollner-Webb, 1987; Mahajan et al., 1990; Schnapp et al., 1990, 1993; Brun et al., 1994); and the ‘upstream binding factor’ UBF (O’Mahony et al., 1992; Voit et al., 1992; Glibetic et al., 1995). It has become apparent that growth-dependent control of rRNA synthesis is mediated primarily by phosphorylation of basal transcription factors which either positively or negatively modulate Pol I transcription. A major player in the control of rDNA transcription is UBF. UBF is hypophosphorylated and transcriptionally inactive in quiescent or serum-deprived cells. The DNA-binding activity of UBF was not altered, indicating that the activating function rather than DNA binding was impaired (O’Mahony et al., 1992; Voit et al., 1992). Despite the clear correlation between UBF phosphorylation and rDNA transcription, the cellular kinases involved in this process and the functional consequence of UBF phosphorylation on Pol I transcription activity remain unknown. In this study, we demonstrate that activation of rDNA transcription upon serum stimulation correlates with phosphorylation of one serine residue of UBF, i.e. Ser484, by $G_1$-phase cdk–cyclin complexes. Mutation of this serine impairs rDNA transcription $in vivo$ and $in vitro$. The results not only reveal UBF as a target for cdk4–cyclin D1 and cdk2–cyclin E, but also establish a link between $G_1$ progression, UBF activity and cellular rRNA synthesis.

Results

Increase of pre-rRNA synthesis in $G_1$ phase

Previous studies established that pre-rRNA synthesis was decreased in serum-starved cells (Grunmt et al., 1976; O’Mahony et al., 1992; Glibetic et al., 1995). To analyze Pol I transcription after release from serum starvation, quiescent NIH 3T3 fibroblasts were stimulated mitogenically by serum, harvested at different times and analyzed by fluorescence-activated cell sorting (FACS), Western and Northern blotting (Figure 1). The FACS analysis reveals that the cells were arrested in $G_0$ (0 h) and entered S-phase ~9 h after serum stimulation (Figure 1A). In parallel, the expression of cyclin D1, cyclin E and UBF was monitored on immunoblots. Consistent with published data (Matsushima et al., 1994), the level of cyclin D1 was reduced in starved cells (Figure 1B, lane 1) and increased...
rapidly, reaching maximal levels 3 h after serum addition (lane 2). Expression of cyclin E, on the other hand, was maximal 9–12 h after mitogenic stimulation. Both the amount and ratio of the two splice variants of UBF, i.e. UBF1 and UBF2, were the same in quiescent and serum-stimulated cells, indicating that serum deprivation and refeeding did not affect cellular UBF levels quantitatively.

To prove that induction of cyclin D1 correlates with activation of D-type cdk4–cyclin complexes, quiescent or serum-stimulated cells were lysed, cdk4–cyclin complexes were immunoprecipitated with polyclonal antibodies against cdk4, and the immunoprecipitates tested for their ability to phosphorylate bacterially produced retinoblastoma protein (pRb). No cdk4-associated kinase activity could be detected in immunoprecipitates from quiescent cells (Figure 1C, lane 1). However, the immune complexes from serum-stimulated cells contained cdk4-associating kinase activity, as revealed by pRb phosphorylation. Significant levels of pRb phosphorylation were observed 3 h after serum stimulation (lane 2), being maximal after 6 h (lane 3). To demonstrate that phosphorylation of pRb was brought about by cdk4 and not by other kinases that are present in the immunoprecipitates, phosphorylation was performed in the presence of recombinant p27, a potent inhibitor of cdk4 and cdk2 activity (Toyoshima and Hunter, 1994). Indeed, p27 significantly inhibited pRb phosphorylation (lanes 4 and 5), indicating that the majority of kinase activity was due to cdk4–cyclin complexes.

Ribosomal gene transcription during G1 progression was determined on Northern blots using a riboprobe which hybridizes to the extreme 5’ end of 45S pre-rRNA. As the 5’ terminus is removed rapidly by a processing event (Kass et al., 1987), the amount of ~14 kb primary transcripts reflects the cellular Pol I transcriptional activity at a given time. Consistent with previous results, pre-rRNA synthesis was decreased in serum-starved cells (Figure 1D). After serum addition, the rDNA transcription recovered within 3 h and then remained constant. This result demonstrates that G1-arrested cells rDNA transcription is down-regulated, and up-regulated upon re-entry into the cell cycle.

**G1 phase-specific phosphorylation of UBF**

UBF is phosphorylated at multiple sites, and the tryptic phosphopeptide pattern is different in growing and resting cells (Voit et al., 1995). To investigate whether progression through the G1 phase of the cell cycle is accompanied by changes in UBF phosphorylation, the tryptic phosphopeptide pattern of UBF derived from asynchronous, starved and serum-stimulated NIH 3T3 cells was compared. In the experiment shown in Figure 2, cells were metabolically labeled by culturing for 4 h in the presence of 32Porthophosphate, and UBF was immunoprecipitated and subjected to tryptic phosphopeptide analysis. Consistent with previous data demonstrating that the acidic tail of UBF (amino acids 675–765) is phosphorylated efficiently by casein kinase II (CKII) in vivo and in vitro (Voit et al., 1995), most of the label was found in a large tryptic fragment (labeled AT) which encompasses the C-terminal part of UBF. In asynchronous cells, several additional peptides (labeled a–d) were phosphorylated (Figure 2A). The different intensity of the individual spots indicates that the tryptic peptides contain more than one phosphate residue or are phosphorylated differentially during the cell cycle.

Consistent with the latter possibility, a different pattern of phosphopeptides was observed when UBF from serum-starved cells or mitogenically stimulated cells was analyzed. UBF that was labeled within 4 h post-stimulation was phosphorylated at a unique peptide, designated peptide a (Figure 2C). Labeling of peptide a was very weak in interphase cells (Figure 2A) and was not observed in starved cells (Figure 2B). Thus, phosphorylation of peptide a correlates with Pol I transcriptional activation upon transition from the resting to the growing state.

As a first step in examining whether phosphorylation of peptide a is brought about by G1-specific cdk(s) rather than by mitogen-activated kinase(s), HeLa cells were arrested in M-phase by nocodazole treatment and then released from the mitotic block by drug withdrawal. The arrest at G1–M and synchronous progression through M and G1 phase, respectively, were monitored by FACS analysis (data not shown). In Figure 2D and E, the tryptic phosphopeptide maps from asynchronous and G1 cells are
Phosphorylation of UBF by G1 cdk–cyclin complexes

Fig. 2. Tryptic phosphopeptide maps of UBF labeled in NIH 3T3 and HeLa cells. (A–C) Phosphopeptides of UBF labeled in asynchronous (A), quiescent (B) and serum-stimulated (C) NIH 3T3 cells. Cells were metabolically labeled by culturing for 4 h in the presence of [32P]orthophosphate (2.5 mCi/ml), and UBF was immunoprecipitated and subjected to two-dimensional tryptic phosphopeptide mapping. For quiescence, cells were kept in DMEM/0.1% FCS for 40 h prior to labeling, followed by addition of [32P]orthophosphate (2.5 mCi/ml) for 4 h in the presence of either 0.1% FCS (B) or 10% FCS (C). The peptide encompassing the hyperphosphorylated acidic tail of UBF is marked (AT). The autoradiographs were exposed for 3 days (A) and 8 days (B and C). (D and E) Phosphopeptides of UBF from cycling HeLa cells. Tryptic fingerprints of UBF are shown from metabolically (4 h) labeled asynchronous cultures (D) or cells that were released from nocodazol-induced M-phase block for 4 h (E).

compared. The two-dimensional phosphopeptide pattern of UBF from HeLa cells closely resembles that of UBF from mouse cells (compare Figure 2A and D). Significantly, 4 h after release from the mitotic block, labeling of peptide a was more pronounced than in interphase cells (Figure 2E). Thus, phosphorylation of peptide a occurs both after mitogenic stimulation of quiescent cells and during G1 phase transition in cycling cells.

Site-specific phosphorylation of UBF by cdk4–cyclin D1 and cdk2–cyclin E

The specific labeling of peptide a both at early times after release from the G0 block and during the G1 phase of cycling cells suggests that G1-specific protein kinase(s) phosphorylate and thus activate UBF. To test this, UBF was transiently overexpressed in NIH 3T3 fibroblasts in the absence or presence of co-transfected expression vectors encoding cdk4–cyclin D1 and cdk2–cyclin E, respectively. The cells were labeled with [32P]orthophosphate and UBF was subjected to tryptic fingerprinting. The phosphopeptide pattern of recombinant UBF was similar to endogenous UBF labeled in untransfected cells. However, the relative intensities of individual phosphopeptides were different. Peptide b was labeled preferentially in cells transfected with UBF alone, whereas phosphorylation of peptides c and d was less pronounced (compare Figures 2A and D, and 3A). It is noteworthy that co-transfection with cdk4–cyclin D1 increased labeling of peptide a (Figure 3B). Peptide a was also enhanced by overexpressing cdk2–cyclin E (Figure 3C), but not cdc2–cyclin A (data not shown), indicating that peptide a is phosphorylated by both cdk4–cyclin D1 and cdk2–cyclin E.

To substantiate the correlation between the activity of G1-specific cdk4 and phosphorylation of peptide a, S9 cells were co-infected with different combinations of FLAG-UBF1, cdk4s and cyclins (Figure 3D and E). In the absence of exogenous kinases, the phosphorylation pattern of UBF1 overexpressed in S9 cells closely resembles that of NIH 3T3 cells (compare Figure 3A and D). Significantly, co-infection with baculoviruses encoding human cdk2 and cyclin E resulted in labeling of phosphopeptide a (Figure 3E). The close correlation between: (i) induction of G1-specific cdk–cyclin complexes; (ii) labeling of peptide a; and (iii) activation of pre-rRNA synthesis suggests that phosphorylation of peptide a may be causally involved in activation of rDNA transcription during G1 progression.

G1-specific cdk4 phosphorylate UBF at Ser484

Consistent with UBF being phosphorylated exclusively at serine residues (Voit et al., 1995), peptide a contains a phosphoserine (data not shown). To map the serine residue in peptide a, synthetic phosphopeptides which correspond to either of the three predicted tryptic peptides of UBF containing the Ser/Pro motif (Figure 4A) were analyzed for co-migration with peptide a on two-dimensional electrophoresis and chromatography. Indeed, one of the synthetic phosphopeptides (LPESPK) encompassing
amino acids 481–486 of UBF was found to co-migrate precisely with peptide a, suggesting that UBF is phosphorylated at Ser484 by G1-specific kinases.

To prove this, Ser484 was converted into alanine by site-directed mutagenesis and the phosphopeptide pattern of wild-type and mutant UBF (FLAG-UBF1/S484A) was compared. Peptide a was labeled strongly in NIH 3T3 cells that were co-transfected with wild-type UBF and cdk4–cyclin D1, but not in the S484A mutant (Figure 4B). This result, together with the observation that significant labeling of peptide a requires overexpression of G1 kinases, indicates that Ser484 is targeted by G1-specific cdk–cyclin complexes.

**Mutation of Ser484 decreases rDNA transcription**

The results presented so far suggest that phosphorylation of UBF by G1 kinases may activate UBF which, in turn, would increase rDNA transcription. To demonstrate a correlation between UBF phosphorylation at Ser484 and Pol I transcription activation, wild-type and mutant UBF were transfected into NIH 3T3 cells together with an rDNA reporter plasmid, and Pol I-dependent transcription was monitored. The reporter plasmid used (pMr1930-BH) is an artificial ribosomal minigene in which a murine rDNA fragment harboring the promoter and the enhancer is fused to a 3′-terminal fragment containing two Pol I terminator elements (Figure 5). Pol I transcripts were analyzed by hybridization of RNA from transfected cells to a radiolabeled probe specific for the reporter used (Figure 5A, upper panel). Expression of wild-type and mutant UBF was monitored on quantitative Western blots (Figure 5A, lower panel). Co-transfection of pMr1930-BH with increasing amounts of expression vectors encoding wild-type UBF augmented Pol I-specific transcription ~4-fold (Figure 5A, lanes 1–4). In contrast, transcriptional activation by mutant UBF1/S484A was strongly impaired (lanes 5–7), a finding that underscores the physiological relevance of Ser484 for UBF-directed transcriptional activation.

To demonstrate unambiguously the functional relationship between phosphorylation of Ser484 and cellular rDNA transcriptional activity, NIH 3T3 cell lines were established which stably express epitope-tagged wild-type or mutant UBF, respectively. Total RNA was isolated from two cell clones which express similar levels of wild-type or mutant UBF, and the relative amount of 45S pre-rRNA was determined on Northern blots (Figure 5B, upper panel). Clearly, the amount of pre-rRNA in cells expressing recombinant mutant UBF was ~2-fold lower than in cells expressing wild-type UBF. Moreover, the growth rate of the mutant cell line was significantly reduced (~25%) as compared with cells expressing wild-type UBF (data not shown). Thus, expression of UBF that cannot be phosphorylated at Ser484 impairs cellular pre-rRNA synthesis and retards cell growth.
Phosphorylation of UBF by G1 cdk–cyclin complexes

Fig. 5. Phosphorylation of Ser484 is required for UBF activity in vivo. (A) Mutant UBF does not activate Pol I transcription in vivo. Upper panel: NIH 3T3 cells were co-transfected with 12.5 μg of pMr1930-BH together with 0.5–2.5 μg of pRe/C-MV-FLAG-mUBF1 or pRe/C-MV-FLAG-mUBF1/S484A. At 44 h after transfection, RNA was isolated, and 10 μg of total RNA were separated on agarose gels. Transcripts synthesized from the rDNA minigene were detected on Northern blots using a riboprobe that is complementary to the pUC-derived sequence present in pMr1930-BH. To normalize for variations of RNA loading, the filter was hybridized with a riboprobe detecting β-actin mRNA. Lower panel: to monitor UBF expression, identical amounts of cell extract protein (10 μg) derived from cultures transfected with 0.5 (lanes 2 and 5), 1.0 (lanes 3 and 6) or 2.5 μg (lanes 3 and 6) of the respective UBF expression plasmid were subjected to Western blot analysis using α-UBF antibodies. A scheme representing the rDNA reporter plasmid used for transfections is shown at the top. Bars mark rDNA sequences, open boxes the enhancer repeats, dark lines pUC-derived sequences, and black boxes the two termination signals T1 and T2. Transcripts initiated at the start site and terminated at the first terminator (700 nucleotides) are indicated by a dotted line. (B) Levels of pre-rRNA in NIH 3T3 cells expressing wild-type or mutant UBF1. Upper panel: total RNA was isolated from stable cell lines overexpressing either FLAG-UBF1/WT (lanes 1–3) or FLAG-UBF1/S484A (lanes 4–6). RNA derived from 1×10^5 (lanes 1 and 4), 2.5×10^5 (lanes 2 and 5) and 5×10^5 (lanes 3 and 6) cells was analyzed on Northern blots using a 32P-labeled riboprobe which is complementary to nucleotides 1–155 of mouse pre-rRNA. The blot subsequently was reprobed for cytochrome c oxidase (cox) mRNA. Lower panel: Western blot demonstrating that identical levels of FLAG-UBF1/WT and FLAG-UBF1/S484A are expressed in the stable cell lines. Each lane contains protein from 1×10^6 cells.

Phosphorylation of Ser484 is required for rDNA transcription activation

If phosphorylation of Ser484 is important for UBF function, then mutant UBF1/S484A should exhibit a lower transcriptional activity. To test this, UBF was isolated by immunopurification from cells expressing epitope-tagged wild-type or mutant UBF, and identical amounts were assayed in a UBF-responsive reconstituted transcription system (Figure 6A). In this system, no transcription of the murine template occurs in the absence of UBF (lane 1). Addition of increasing amounts of immunopurified wild-type UBF stimulated transcription ~10-fold (lanes 2–5). Remarkably, transactivation by UBF1/S484A was strongly impaired (lanes 6–9), demonstrating the importance of Ser484 for UBF activity.

The decrease in the activity of mutant UBF was even more pronounced when using UBF that was purified from baculovirus-infected Sf9 cells. As shown in Figure 6B, similar amounts of FLAG-tagged UBF1 and UBF1/S484A exhibited marked differences in transcriptional activity. Whereas as little as 0.5 ng of wild-type UBF stimulated transcription in the reconstituted system, 20 times higher levels of UBF1/S484A were required to yield the same amount of transcripts (compare lanes 2 and 9). Thus, substitution of Ser484 by alanine reduces UBF activity by more than one order of magnitude.

Discussion

In this study, we have investigated the role of phosphorylation of UBF by G1 phase-specific cdk–cyclin complexes in rDNA transcriptional regulation. Previous studies suggested that UBF is phosphorylated at multiple sites, and the changing patterns of phosphorylation are targets of regulatory mechanisms which coordinate cell proliferation and rDNA transcription. In quiescent cells, UBF is hypophosphorylated and transcriptionally inactive (O’Mahony et al., 1992; Voit et al., 1992, 1995). After refeeding, the pattern of UBF phosphorylation is altered and transcriptional activity is restored. We present several lines of evidence to indicate that specific phosphorylation of UBF is causally involved in activation of pre-rRNA synthesis upon mitogenic stimulation of quiescent cells. First, Pol I transcription during G1 phase progression correlates with de novo expression of cyclin D and activation of cdk4–cyclin D. Secondly, G1-specific cdks, i.e. cdk4–cyclin D
and cdk2–cyclin E (but not cdc2–cyclin A), phosphorylate UBF at a unique serine residue at position 484. Phosphorylation of this serine, which is conserved in UBF from different species, augments UBF activity. Mutation of Ser484 to alanine impaired UBF-directed transcriptional activation both in vitro and in vivo. Consistent with Ser484 being a target for G1-specific protein kinases, phosphorylation at Ser484 occurred early after serum stimulation. At time points later than 9 h, little de novo phosphorylation at this site was observed. The available data indicate complex interdependent phosphorylation and dephosphorylation reactions, and suggest a hierarchy of phosphorylation events that may modulate UBF activity.

The G0 to S-phase transition in mammalian cells is known to be regulated through the sequential and concerted actions of cdks and their regulatory partners, the cyclins (Sherr, 1994). Our finding that activation of cellular pre-

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### Coomassie stain

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is supported by the recent finding that recombinant, unphosphorylated pRb interacts with UBF and abrogates Pol I transcription in vitro (Cavanaugh et al., 1995; Voit et al., 1997). By analogy to E2F, it is intriguing to speculate that in vivo repression of Pol I transcription in G0 involves association of UBF with pRb, and this interaction depends on the phosphorylation state of either UBF or both UBF and pRb. Like UBF, the phosphorylation state of pRb fluctuates through different phases of the cell cycle. Hypophosphorylated pRb predominates in early G1, and phosphorylation by G1-specific cdks relieves transcriptional inhibition of E2F-dependent genes. It will be important to establish whether complexes between pRb and UBF exist in G0 and/or early G1, which dissociate after phosphorylation by G1 kinases. In initial experiments, we have compared the ability of wild-type UBF and mutant UBF1/S484 to interact with recombinant GST–pRb. These experiments did not reveal any difference in UBF–pRb interaction (R.Voit, unpublished results) and, therefore, phosphorylation of Ser484 is unlikely to be causally involved in the dissociation of complexes between UBF and pRb. Our data are consistent with Ser484 being unphosphorylated in quiescent cells, and this hypophosphorylation accounts for the low transcriptional activity. Mitogenic stimulation results in phosphorylation of this site and transcriptional activation. Given the potential for redundancy in the mitogenic signaling pathways, one could argue that Ser484 is phosphorylated by members of the MAP kinase family rather than by G1-specific cdks. Like cdks, MAP kinases phosphorylate a number of transcription factors after mitogenic induction, and target S/T-P sites. Nevertheless, we consider this possibility to be unlikely because UBF was not phosphorylated by p42mapk or SAPK from different sources (data not shown). Furthermore, efficient phosphorylation of peptide a in vivo was only observed in Sf9 or NIH 3T3 cells overexpressing either cdk4–cyclin D or cdk2–cyclin E.

Fig. 6. UBF1/S484A is transcriptionally inactive. (A) FLAG-UBF1/WT and FLAG-UBF1/S484A were immunopurified from NIH 3T3 cells that stably express the respective cDNAs, and equal amounts were analyzed on immunoblots using α-UBF antibodies. Increasing amounts of wild-type (lanes 2–5) or mutant UBF1 (lanes 6–9) were assayed for transcriptional activity in a reconstituted transcription system. In lane 1, no UBF was added. Specific run-off transcripts (371 nucleotides) were analyzed by PAGE and autoradiography. (B) FLAG-UBF1/WT (lane 1 and 2) and FLAG-UBF1/S484A (lanes 3 and 4) were immunopurified from Sf9 cells infected with the respective baculoviruses. The amount and purity of recombinant UBF were analyzed on Coomassie Blue-stained SDS–polyacrylamide gels. Aliquots of 0.5–10 ng of wild-type (lanes 2–5) or mutant UBF1 (lanes 6–9) were assayed for transcriptional activity in a reconstituted transcription system as described above.
Admittedly, we are still ignorant of which function is impaired in mutant UBF1/S484A. Ser484 is located in the C-terminal part of HMG box 4, a region that has been implicated in the synergistic interaction with the TBP-TAF complex SL1/TIF-IB (Jantzen et al., 1992). UBF binds to the rDNA promoter and stimulates transcription through cooperative interactions with SL1. The interaction between UBF and SL1 yields an extended footprint pattern at the human rDNA promoter. Deletion of either box 3 (amino acids 284–371) or box 4 (amino acids 371–491) impaired interactions with SL1 and abrogated transcription, indicating that the C-terminal part of UBF, including HMG boxes 3 and 4 and the acidic tail, plays an important role in mediating protein–protein interactions at the rDNA promoter. To assess the functional consequences of Ser484 phosphorylation, we have compared the ability of wild-type and mutant UBF to interact with TIF-IB, Pol I and the rDNA promoter. However, none of the different assays used, i.e. DNase footprinting, electrophoretic mobility shift assays, ‘pull-down’ experiments or co-immunoprecipitations, revealed significant differences between wild-type and mutant UBF. The phosphorylation pattern can have pronounced effects on UBF activity. In transient transfection experiments, 5×10^5 NIH 3T3 cells were plated on 10 cm dishes and transfected with a total of 20 µg of DNA using the calcium phosphate precipitation technique. RNA was prepared from cells harvested 44 h after transfection by lysis in 4 M guanidinium isothiocyanate, separated on 1% MOPS-formaldehyde gels, and transferred to nylon filters. After UV cross-linking, the blots were hybridized with ^32P-labeled riboprobes. Cellular pre-rRNA was visualized by hybridization to antisense RNA encompassing 5′-terminal rDNA sequences from −170 to +155. Transcripts from the reporter plasmid pMr1930-BH were monitored by hybridization to U1 promoter sequences from nucleotides +235 to +396 (EcoRI–NarI fragment). To monitor RNA recovery, the blots subsequently were hybridized to riboprobes against mRNA of cytochrome c oxidase or β-actin. Hybridization was performed in 50% formamide, 5× SSC, 50 mM sodium phosphate pH 6.5, 8× Denhardt’s, 0.5 mg/ml yeast RNA, 0.1% SDS at 65°C for 16 h. The filters were washed in 0.2× SSC, 0.1% SDS at 65°C. All experiments were performed in duplicate and repeated at least three times. For stable expression of FLAG-tagged wild-type and mutant UBF1, NIH 3T3 cells were transfected with pRe-CMV-FLAG-mUBF1 and pRe-CMV-FLAG-mUBF1/S484A, respectively, followed by selection with 600 µg/ml of G418.

**Materials and methods**

**Plasmids**

pMrWT, the template used for in vitro transcription, contains a 324 bp 5′-terminal murine rDNA fragment including sequences from −170 to +155 with respect to the transcription start site. The template was truncated with NdeI to yield 371 nucleotide run-off transcripts. The artificial ribosomal minigene construct pMrF1930-BH used in transfection experiments represents a fusion between a murine rDNA promoter fragment (from −1930 to +292 relative to the transcription start site) and a 3′-terminal BamHI–HindIII fragment which contains two terminator elements, i.e. T1 and T2 (Grunmt et al., 1985).

pReCMV-mUBF1, the expression vector for murine UBF1, has been described (Kuhn et al., 1994). Mutant UBF1/S484A was constructed by overlap extension PCR using oligonucleotides that replaced Ser484 by alanine. cDNAs encoding wild-type and mutant UBF1 were tagged at the 5′ end with sequences encoding the FLAG epitope peptide DYKDDDDK and cloned into pReCMV for transfection into NIH 3T3 cells. pReCMV-FLAG-mUBF1, pReCMV-FLAG-mUBF1/S484A and generation of recombinant baculoviruses, the respective cDNA was inserted into pVL1392 (pVL1392-FLAG-mUBF1/S484A).

**Cell culture, transfections and RNA analysis**

NIH 3T3 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). For synchronization, cells were cultured in low serum (0.1% FCS) for 40 h and then stimulated by addition of fresh medium containing 10% FCS. For FACS analysis, 10^6 cells were fixed with propidium iodide and analyzed by flow cytometry. S9 insect cells were cultured at 27°C in TC-100 medium supplemented with 10% FCS.

For transient transfection experiments, 5×10^5 NIH 3T3 cells were plated on 10 cm dishes and transfected with a total of 20 µg of DNA using the calcium phosphate precipitation technique. RNA was prepared from cells harvested 44 h after transfection by lysis in 4 M guanidinium isothiocyanate, separated on 1% MOPS-formaldehyde gels, and transferred to nylon filters. After UV cross-linking, the blots were hybridized with ^32P-labeled riboprobes. Cellular pre-rRNA was visualized by hybridization to antisense RNA encompassing 5′-terminal rDNA sequences from −170 to +155. Transcripts from the reporter plasmid pMr1930-BH were monitored by hybridization to U1 promoter sequences from nucleotides +235 to +396 (EcoRI–NarI fragment). To monitor RNA recovery, the blots subsequently were hybridized to riboprobes against mRNA of cytochrome c oxidase or β-actin. Hybridization was performed in 50% formamide, 5× SSC, 50 mM sodium phosphate pH 6.5, 8× Denhardt’s, 0.5 mg/ml yeast RNA, 0.1% SDS at 65°C for 16 h. The filters were washed in 0.2× SSC, 0.1% SDS at 65°C. All experiments were performed in duplicate and repeated at least three times. For stable expression of FLAG-tagged wild-type and mutant UBF1, NIH 3T3 cells were transfected with pRe-CMV-FLAG-mUBF1 and pRe-CMV-FLAG-mUBF1/S484A, respectively, followed by selection with 600 µg/ml of G418.

**In vitro kinase assay**

To assay cdk4-associated kinase activity, 2×10^6 cells were lysed in IP buffer [50 mM HEPES pH 7.6, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 4 mM dithiothreitol], sonicated, centrifuged, and the supernatants precipitated for 4 h at 4°C with α-cdk4 antibodies (Santa Cruz, C-22) coupled to Dynabeads containing α-rabbit IgGs (Dynal) essentially as described by Matsushime et al. (1994). After extensive washing, immunoprecipitates were resuspended in kinase assay buffer (50 mM HEPES pH 7.6, 10 mM MgCl2, 1 mM DTT, 25 µM ATP, 5 µCi of [γ-^32P]ATP) and used to phosphorylate 0.2 µg of recombinant GST–pRb (379–928) in the absence or presence of 10% GST–p27 have been described before (Toyoshima and Hunter, 1994; Voit et al., 1997). The concentration and purity of the isolated proteins were estimated by Coomassie Blue staining of the proteins separated on SDS–polyacrylamide gels.

**Expression and purification of UBF**

FLAG-tagged UBF1 was purified from S9 cells infected with recombinant baculoviruses as described (Voit et al., 1997). At 44 h post-infection, cells were lysed by sonication in buffer AM-600 (600 mM KCI, 20 mM Tris–HCl pH 7.9, 5 mM MgCl2, 0.1 mM EDTA, 10% glycerol, 0.5 mM DTT) supplemented with protease inhibitors (0.5 mM PMSF; 2 µg/ml each of pepstatin, leupeptin, aprotinin) and phosphatase inhibitors (80 mM β-glycerophosphate, 20 mM potassium fluoride, 1 mM sodium orthovanadate). NP-40 was added to a final concentration of 0.5%, and the supernatants were clarified by centrifugation. The supernatants were incubated at 4°C with α-FLAG antibodies (M2; Kodak) covalently bound to agarose beads. After washing with buffer AM (see above) containing 0.5% NP-40 and 1 M KCI, bead-bound UBF was eluted with buffer AM-300 containing 0.1% NP-40 and 400 µg/ml FLAG-peptide. Alternatively, UBF was isolated from NIH 3T3 cells overexpressing FLAG-mUBF1 or FLAG-mUBF1/S484A, using α-FLAG antibodies as described above.
In vitro transcription assays
The fractionation scheme for purification of murine Pol I and Pol I-specific transcription factors has been described (Schnapp and Grummt, 1996). Standard reactions contained 8 ng of pMrWT/NdeI, 4 μl of partially purified Pol I (H-400 fraction), 1 μl of TIF-IA/TIF-IC (poly-l-lysine–agarose fraction), 3 μl of TIF-IB (CM-400 fraction) and 0.2–10 ng of UBF. After incubation for 1 h at 30°C, run-off transcripts were purified and analyzed by gel electrophoresis and autoradiography.

Western blots
Cells were lysed in sample buffer and sonicated for 10 s. Proteins were separated by SDS–PAGE and transferred to nitrocellulose. The membrane was blocked in PBS containing 5% milk powder and 0.2% Tween-20 for 1 h, probed with specific antibodies, and proteins were visualized by ECL (Amersham). Antibodies against UBF (Voit et al., 1992, 1997), cyclin D1 (Santa Cruz, HD11), cyclin E (Santa Cruz, M-20; UBI, #06-459), cdk2 (Santa Cruz, M2), cdk4 (Santa Cruz, C-22) and the FLAG epitope (M2, Kodak) were used.

In vivo phosphorylation and tryptic phosphopeptide mapping
NIH 3T3 cells (1×10^6) transfected with expression vectors encoding wild-type or mutant UBF (pCR/CMV-FLAG-mUBF1 and pCR/CMV-FLAG-mUBF1/S484A) were labeled for 10 h in phosphate-free DMEM containing 10% dialyzed FCS and 1 mCi/ml [32P]orthophosphate. To label endogenous UBF during G1 phase progression, 4×10^6 NIH 3T3 cells were starved for 40 h by serum deprivation (0.1% FCS), and incubated for 4 h in the presence of 4 mCi of [32P]orthophosphate either at low serum (0.1%) or after refedding with 10% FCS. Extract preparation and immunoprecipitation with α-UBF or α-FLAG antibodies were carried out as described (Voit et al., 1995). Briefly, cells were lysed in RIPA buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl, 0.5% sodium deoxycholate, 0.5% NP-40, 0.5% SDS, 10 mM EDTA, 20 mM KF, 1 mM sodium orthovanadate, 10 mM K2HPO4, 2 μg/ml each of leupeptin, aprotinin and pepstatin) and incubated for at least 6 h with α-UBF antibodies coupled to protein G-agarose or α-FLAG antibodies coupled to protein A-agarose or protein G–agarose or α-FLAG M2–agarose. Immunoprecipitated proteins were separated by 6% SDS–PAGE, transferred to nitrocellulose and visualized by autoradiography. The region containing UBF was cut out and processed for tryptic phosphopeptide mapping as described (Voit et al., 1995).

HeLa cells were grown in DMEM, supplemented with 10% FCS. Metabolic labeling with [32P]orthophosphate of exponentially growing cells was done essentially as described before (Heix et al., 1998). For cell labeling during G1, exponentially growing HeLa cells were first arrested in S-phase by treatment with 2.5 mM thymidine for 23 h. Then the cells were washed and incubated for 6 h in regular medium before the addition of 40 ng/ml of nocodazole. After 5 h, nocodazole was removed, cells were incubated for another 4 h in the presence of phosphate-free DMEM/dialyzed 10% FCS and 5 mCi of [32P]orthophosphate, and UBF was immunoprecipitated and processed for tryptic phosphopeptide mapping as described above.

For in vivo phosphorylation in insect cells, 3×10^8 S9 cells were infected with baculoviruses expressing FLAG-USF1 either alone or together with baculoviruses encoding human His-tagged cdk2 and cyclin E. Metabolic labeling of cellular proteins was performed as described (Heix et al., 1998).

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References


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