The EMBO Journal Vol.18 No.7 pp.1891–1899, 1999

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

Renate Voit[1], Manuela Hoffmann and Ingrid Grummt

Division of Molecular Biology of the Cell II, German Cancer Research Center, D-69120 Heidelberg, Germany

[1]Corresponding author
e-mail: R.Voit@DKFZ-Heidelberg.de

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 cdks–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 TFIIIB, 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 (Grummt *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 (Matsushime *et al.*, 1994), the level of cyclin D1 was reduced in starved cells (Figure 1B, lane 1) and increased

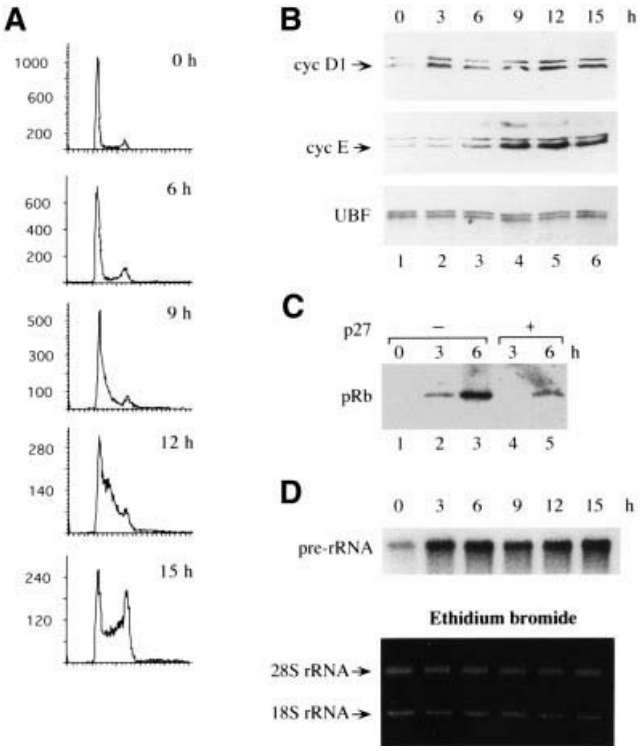

Fig. 1. Pre-rRNA synthesis after mitogenic stimulation of NIH 3T3 fibroblasts. (**A**) Flow cytometric cell cycle analysis (FACS). Cells were arrested in G_0 by serum starvation and stimulated by adding 10% FCS. After serum stimulation, aliquots of cells were analyzed at the times indicated. (**B**) Western blot. Equal numbers of cells were analyzed on immunoblots to monitor levels of cyclin D1, cyclin E and UBF. (**C**) Cdk4-associated kinase assay. NIH 3T3 cells were serum starved for 40 h and stimulated by serum addition for 3 and 6 h, as indicated. Extracts were immunoprecipitated with α-cdk4 antibodies, and immune complexes were assayed for pRb kinase activity in the absence (lanes 1–3) or presence (lanes 4 and 5) of p27. (**D**) Pre-rRNA analysis. Total RNA (2.5 μg) prepared from either quiescent cells (0 h) or at different times after serum stimulation (3–15 h) was subjected to Northern blot analysis to monitor 45S pre-rRNA synthesis (top). An ethidium bromide stain of cellular RNA is shown below, demonstrating that equal amounts of cellular RNA were analyzed.

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-asssociated 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 G_1 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 in G_0-arrested cells rDNA transcription is down-regulated, and up-regulated upon re-entry into the cell cycle.

G_1 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 G_1 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 [^{32}P]orthophosphate, 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 G_1-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 G_2–M and synchronous progression through M and G_1 phase, respectively, were monitored by FACS analysis (data not shown). In Figure 2D and E, the tryptic phosphopeptide maps from asynchronous and G_1 cells are

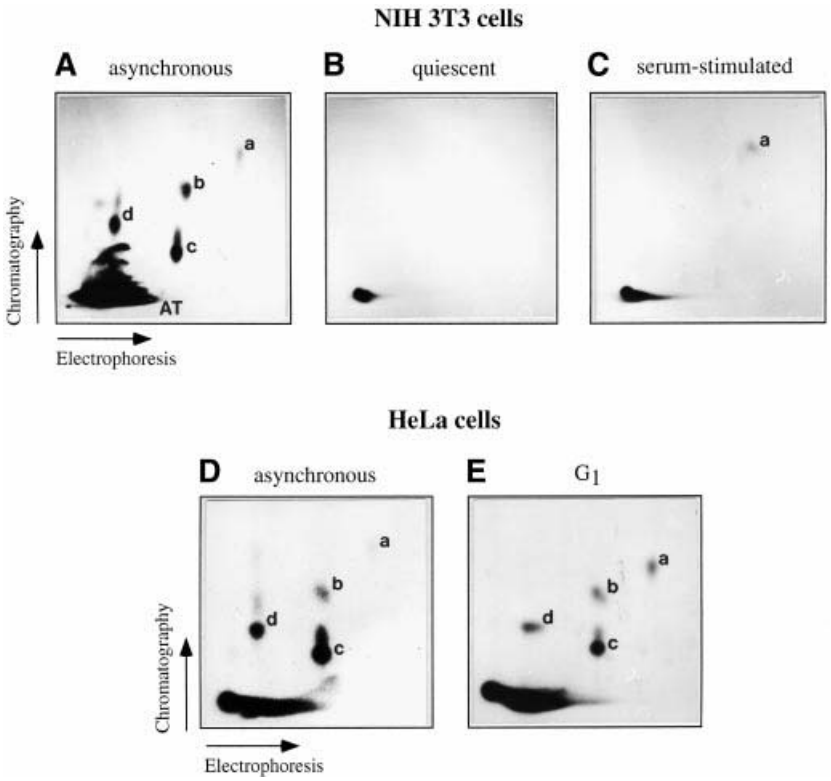

Fig. 2. Tryptic phosphopeptide maps of UBF labeled in NIH 3T3 and HeLa cells. (A–C) Phosphopeptides of UBF labeled in asynchronous (**A**), quiescent (**B**) and serum-stimulated (**C**) NIH 3T3 cells. Cells were metabolically labeled by culturing for 4 h in the presence of [^{32}P]orthophosphate (2.5 mCi/ml), and UBF was immunoprecipitated and subjected to two-dimensional tryptic phosphopeptide mapping. For quiescense, cells were kept in DMEM/0.1% FCS for 40 h prior to labeling, followed by addition of [^{32}P]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 G_1 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 G_0 block and during the G_1 phase of cycling cells suggests that G_1-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 [^{32}P]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 G_1-specific cdks and phosphorylation of peptide **a**, Sf9 cells were co-infected with different combinations of FLAG-UBF1, cdks and cyclins (Figure 3D and E). In the absence of exogenous kinases, the phosphorylation pattern of UBF1 overexpressed in Sf9 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 G_1-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 G_1 progression.

G_1-specific cdks 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

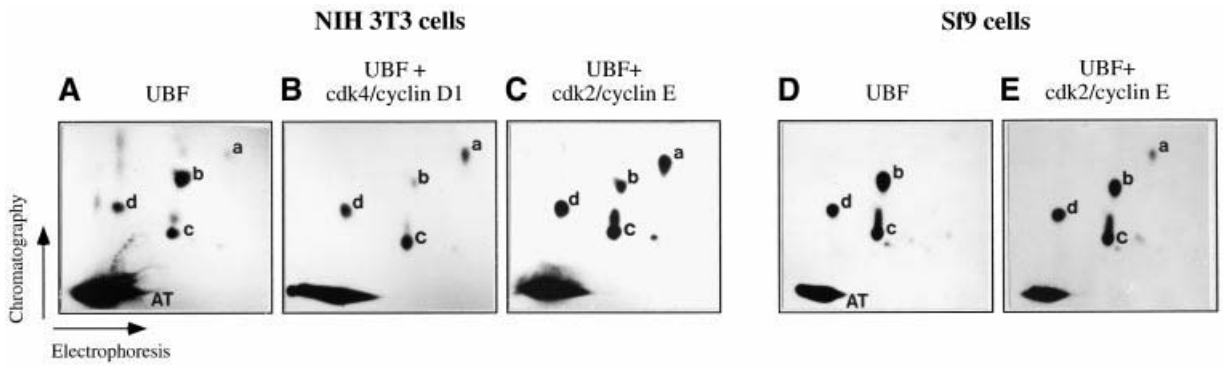

Fig. 3. Tryptic peptide maps of UBF from cells overexpressing cdk–cyclin complexes. (A–C) NIH 3T3 cells (1×10^5) were transfected with 2 μg of pRc/CMV-FLAG-UBF1 alone (**A**) or together with 0.8 μg each of pCMV-cyclin D1 and pCMV-cdk4 (**B**), and pCMV-cyclin E and pCMV-cdk2 (**C**). At 30 h after transfection, the cells were labeled with [^{32}P]orthophosphate for 10 h. UBF was immunoprecipitated and subjected to two-dimensional tryptic peptide mapping. (D and E) Sf9 cells were infected with baculoviruses expressing FLAG-UBF1 (**D**) or FLAG-UBF1, cdk2 and cyclin E (**E**), and labeled for 4 h with [^{32}P]orthophosphate (0.5 mCi/ml). UBF was isolated by immunoprecipitation and subjected to two-dimensional tryptic peptide mapping.

amino acids 481–486 of UBF was found to co-migrate precisely with peptide **a**, suggesting that UBF is phosphorylated at Ser484 by G_1-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 G_1 kinases, indicates that Ser484 is targeted by G_1-specifc cdk–cyclin complexes.

Mutation of Ser484 decreases rDNA transcription

The results presented so far suggest that phosphorylation of UBF by G_1 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 artifical 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

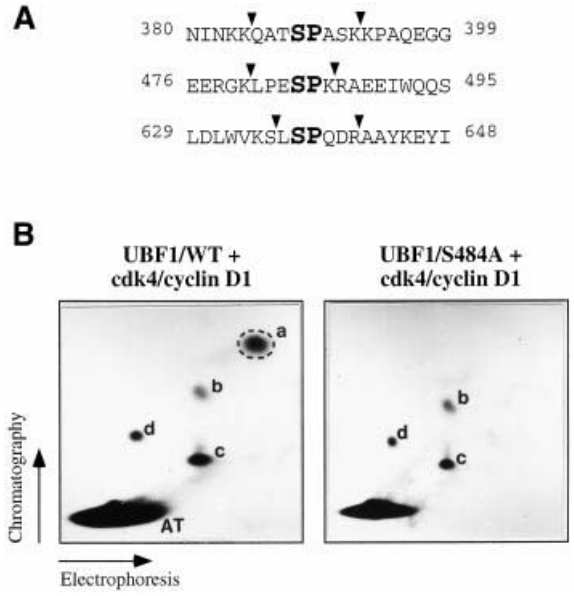

Fig. 4. UBF is phosphorylated at Ser484. (**A**) Alignment of putative cdk target sites in UBF. The cleavage sites for trypsin are marked by arrowheads. (**B**) NIH 3T3 cells (1×10^5) were transfected with 2 μg of pRc/CMV-FLAG-mUBF1 or pRc/CMV-FLAG-mUBF1/S484A together with 0.8 μg each of pCMV-cyclin D1 and pCMV-cdk4. After labeling for 10 h with 0.5 mCi/ml [^{32}P]orthophosphate, UBF was immunoprecipitated and analyzed by tryptic fingerprinting. To identify the sequence of peptide **a**, a synthetic phosphopeptide (L-P-E-phosphoS-P-K) was added to the sample before electrophoresis. The dashed circle indicates the position of the synthetic peptide visualized by fluorescamine staining (Sigma).

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.

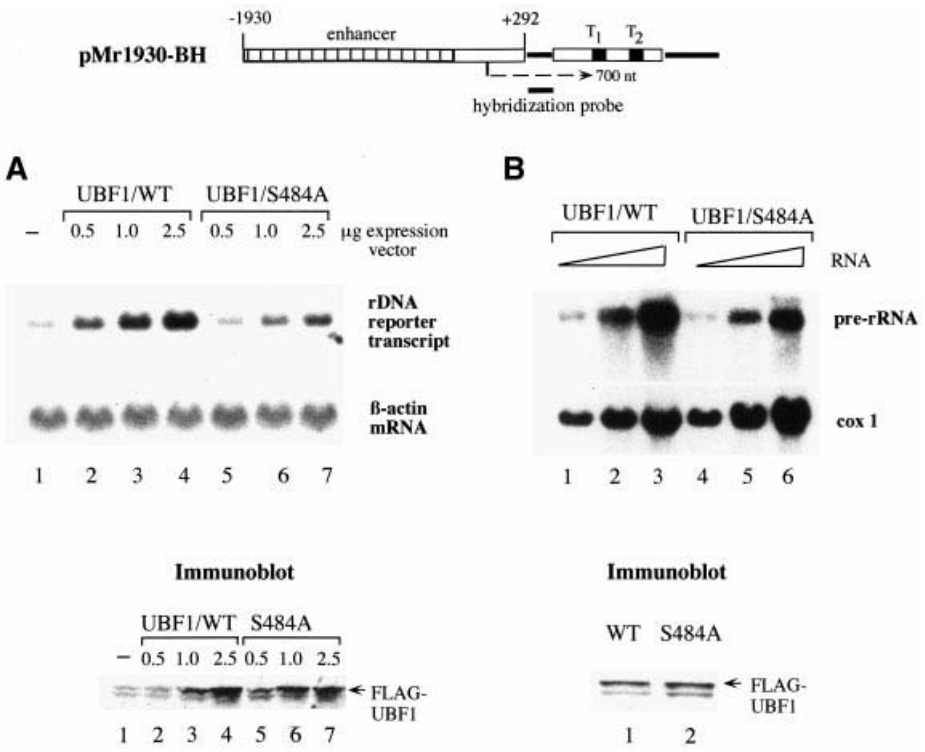

Fig. 5. Phosphorylation of Ser484 is required for UBF activity *in vivo*. (**A**) Mutant UBF does not activate Pol I transcription *in vivo*. Upper panel: NIH 3T3 cells were co-transfected with 12.5 µg of pMr1930-BH together with 0.5–2.5 µg of pRc/CMV-FLAG-mUBF1 or pRc/CMV-FLAG-mUBF1/S484A. At 44 h after 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 T_1 and T_2. 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 ^{32}P-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 1×10^4 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 G_1 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 G_1 phase progression correlates with *de novo* expression of cyclin D and activation of cdk4–cyclin D. Secondly, G_1-specific cdks, i.e. cdk4–cyclin D

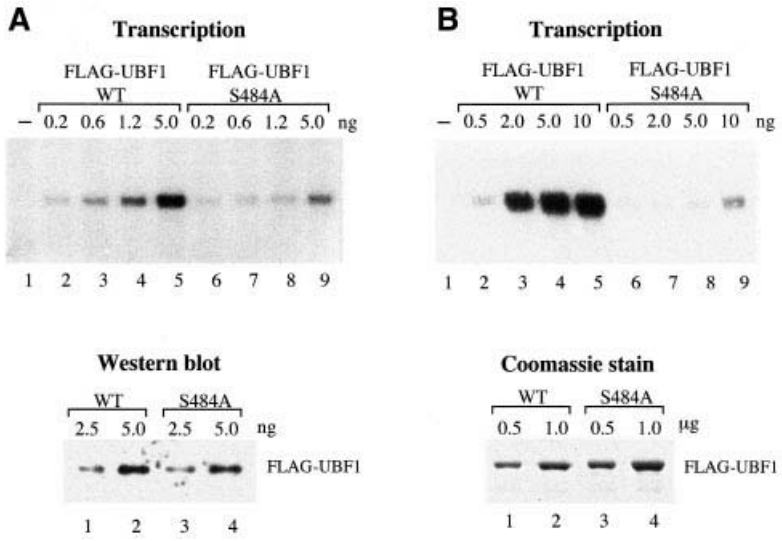

Fig. 6. UBF1/S484A is transcriptionally inactive. (**A**) FLAG-UBF1/WT and FLAG-UBF1/S484A were immunopurified from NIH 3T3 cells that stably express the respective cDNAs, and equal amounts were analyzed on immunoblots using α-UBF antibodies. Increasing amounts of wild-type (lanes 2–5) or mutant UBF1 (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.

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 G_1-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 G_0- 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-rRNA synthesis in G_1 correlates with phosphorylation of UBF by G_1-specific cdk–cyclin complexes not only underscores the role of cyclin-dependent kinases in control of cell division, but also identifies UBF as a novel target for G_1 cdks. Cyclin D-dependent kinases are essential for the passage through the restriction point in late G_1, at which cells become committed to proceed in the cell cycle without a further requirement for mitogenic stimuli, whereas cdk2–cyclin E activity is required for the initiation of S-phase (Ohtsubo *et al.*, 1995; Resnitzky and Reed, 1995). Known targets for the G_1-specific cdks are the members of the pocket-protein family, pRb, p107 and p130 (Hinds *et al.*, 1992; Dowdy *et al*., 1993; Ewen *et al.*, 1993; Beijersbergen *et al.*, 1995), which serve as negative regulators of the heterodimeric E2F–DP transcription factor complexes. By reversibly interacting with members of the E2F family, pRb controls the expression of genes whose products are required for cell cycle progression.

The importance of pRb phosphorylation by G_1-specific cdks in allowing cells to go through the cell cycle 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 G_0 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 G_1, and phosphorylation by G_1-specific cdks relieves transcriptional inhibition of E2F-dependent genes. It will be important to establish whether complexes between pRb and UBF exist in G_0 and/or early G_1, which dissociate after phosphorylation by G_1 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 G_1-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 $p42^{mapk}$ 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.

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 (data not shown) and, therefore, the contribution of Ser484 phosphorylation to UBF-dependent transcription is not yet known.

Similarly, the contribution of other protein kinases to UBF function has yet to be investigated. Early studies revealed that multiple serine residues within the C-terminal 'acidic tail' of UBF were phosphorylated by CKII *in vivo* and *in vitro* (O'Mahony *et al.*, 1992; Voit *et al.*, 1992, 1995; Hershey *et al.*, 1995). Since the 'acidic tail' is required for UBF-directed transcriptional activation, phosphorylation by CKII was thought to be causally involved in regulation of UBF activity. However, mutation of the C-terminal serine residues reduced transcriptional activity only 3-fold (Voit *et al.*, 1995), indicating that phosphorylations within the body of UBF play a more important role in growth-dependent regulation of UBF activity. Apparently, subtle changes in the phosphorylation pattern can have pronounced effects on UBF activity. In support of this, phosphorylation of a unique serine in Oct-1 has been shown to initiate an allosteric conformational change of the protein, thereby creating or revealing phosphoacceptor sites for the action of additional protein kinases (Tanaka and Herr, 1990). We hypothesize that formation of a productive transcriptional transactivation surface of UBF may require a phosphorylation-dependent allosteric conformational mechanism which recruits components of the transcription machinery to the rDNA promoter.

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 *Nde*I to yield 371 nucleotide run-off transcripts. The artificial ribosomal minigene construct pMr1930-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 *Bam*HI–*Hin*fI fragment which contains two terminator elements, i.e. T_1 and T_2 (Grummt *et al.*, 1985).

pRc/CMV-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 pRc/CMV for transfection into NIH 3T3 cells (pRc/CMV-FLAG-mUBF1, pRc/CMV-FLAG-mUBF1/S484A). For 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. Sf9 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 ^{32}P-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 pUC9 sequences from nucleotides +235 to +396 (*Eco*RI–*Nar*I 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 pRc/CMV-FLAG-mUBF1 and pRc/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, 1 mM dithiothreitol (DTT), 0.1% Tween-20, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml each of leupeptin, aprotinin, pepstatin,10 mM glycerophosphate, 1 mM KF, 0.1 mM sodium orthovanadate], 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 $MgCl_2$, 1 mM DTT, 25 μM ATP, 5 μCi of [γ-^{32}P]ATP) and used to phosphorylate 0.2 μg of recombinant GST–pRb(379–928) in the absence or presence of 1 μg of recombinant GST–p27. After incubation for 30 min at 30°C, proteins were separated on 10% SDS–polyacrylamide gels and visualized by autoradiography. Expression and purification of recombinant GST–pRb and 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 Sf9 cells infected with recombinant baculoviruses as described (Voit *et al.*, 1997). At 44 h post-infection, cells were lysed by sonification in buffer AM-600 (600 mM KCl, 20 mM Tris–HCl pH 7.9, 5 mM $MgCl_2$, 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 lysates were cleared by centrifugation. The supernatant was 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 KCl, 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-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 (1×10^5) transfected with expression vectors encoding wild-type or mutant UBF (pRc/CMV-FLAG-mUBF1 and pRc/CMV-FLAG-mUBF1/S484A) were labeled for 10 h in phosphate-free DMEM containing 10% dialyzed FCS and 1 mCi/ml [^{32}P]orthophosphate. To label endogenous UBF during G_1 phase progression, 4×10^5 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 [^{32}P]orthophosphate either at low serum (0.1%) or after refeeding 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 EGTA, 20 mM KF, 1 mM sodium orthovanadate, 10 mM K_2HPO_4, 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 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 [^{32}P]orthophosphate of exponentially growing cells was done essentially as described before (Heix *et al.*, 1998). For cell labeling during G_1, exponentially growing HeLa cells were first arrested in S-phase by treatment with 2.5 mM thymidine for 25 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 [^{32}P]orthophosphate, and UBF was immunoprecipitated and processed for tryptic phosphopeptide mapping as described above.

For *in vivo* phosphorylation in insect cells, 3×10^6 Sf9 cells were infected with baculoviruses encoding FLAG-UBF1 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).

Acknowledgements

We thank B.Dorr for preparation and fractionation of mouse extracts, R.Laskey and J.Pines for baculoviruses encoding cdk2 and cyclin E, T.Hunter for the pGEX-p27 expression plasmid, S.Mittnacht for plasmid pGEX-Rb(379–928), P.Jansen-Durr for providing various expression plasmids encoding human cdks and cyclins, H.Beckmann for baculovirus stocks expressing FLAG-tagged human UBF, and H.-R.Rackwitz for synthesizing the peptides. This work was supported by the Deutsche Forschungsgemeinschaft (Vo728/1-1 and Priority Program 'Cell Cycle') and the Fond der Chemischen Industrie.

References

Beijersbergen,R.L., Carlee,L., Kerkhoven,R.M. and Bernards,R. (1995) Regulation of the retinoblastoma protein-related p107 by G_1 cyclin complexes. *Genes Dev.*, **9**, 1340–1353.

Brun,R.P., Ryan,K. and Sollner-Webb,B. (1994) Factor C*, the specific initiation component of the mouse RNA polymerase I holoenzyme, is inactivated early in the transcription complex. *Mol. Cell. Biol.*, **14**, 5010–5021.

Buttgereit,D., Pflugfelder,G. and Grummt,I. (1985) Growth-dependent regulation of rRNA synthesis is mediated by a transcription initiation factor (TIF-IA). *Nucleic Acids Res.*, **13**, 8165–8170.

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.

Dowdy,S.F., Hinds,P.W., Louie,K., Reed,S.I., Arnold,A. and Weinberg,R.A. (1993) Physical interaction of the retinoblastoma protein with human D cyclins. *Cell*, **73**, 499–511.

Ewen,M.E., Sluss,H.K., Sherr,C.J., Matsushime,H., Kato,J. and Livingstone,D.M. (1993) Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell*, **73**, 487–497.

Glibetic,M., Taylor,L., Larson,D., Hannan,R., Sells,B. and Rothblum,L. (1995) The RNA polymerase I transcription factor UBF is the product of a primary response gene. *J. Biol. Chem.*, **270**, 4209–4212.

Grummt,I. (1998) Initiation of murine rDNA transcription. In Paule,M.R. (ed.), *Transcription of Ribosomal RNA Genes by Eukaryotic RNA Polymerase I*. Landes Bioscience, Austin, TX, pp. 135–154.

Grummt,I. (1999) Regulation of mammalian ribosomal gene transcription by RNA polymerase I. *Prog. Nucleic Acid Res. Mol. Biol.*, **62**, 109–154.

Grummt,I., Smith,V.A. and Grummt,F. (1976) Amino acid starvation affects the initiation frequency of nucleolar RNA polymerase. *Cell*, **7**, 439–445.

Grummt,I., Maier,U., Ohrlein,A., Hassouna,N. and Bachellerie,J.-P. (1985) Transcription of mouse rDNA terminates downstream of the 3′ end of 28S RNA and involves interaction of factors with repeated sequences in the 3′ spacer. *Cell*, **43**, 801–810.

Heix,J., Vente,A., Voit,R., Budde,A., Michaelidis,T.M. and Grummt,I. (1998) Mitotic silencing of rRNA synthesis: inactivation of the promoter selectivity factor SL1 by cdc2/cyclin B-mediated phosphorylation. *EMBO J.*, **17**, 7373–7381.

Hershey,J.C., Hautmann,M., Thompson,M.M., Rothblum,L.I., Haystead, A.J. and Owens,G.K. (1995) Angiotensin II-induced hypertrophy of rat vascular smooth muscle is associated with increased 18S rRNA synthesis and phosphorylation of the rRNA transcription factor, upstream binding factor. *J. Biol. Chem.*, **270**, 25096–25101.

Hinds,P.W., Mittnacht,S., Dulic,V., Arnold,A., Reed,S.I. and Weinberg, R.A. (1992) Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell*, **70**, 993–1006.

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.

Kass,S., Craig,N. and Sollner-Webb,B. (1987) Primary processing of mammalian rRNA involves two adjacent cleavages and is not species specific. *Mol. Cell. Biol.*, **7**, 2891–2898.

Kuhn,A., Voit,R., Stefanovsky,V., Evers,R., Bianchi,M. and Grummt,I. (1994) Functional differences between the two splice variants of the nucleolar transcription factor UBF: the second HMG box determines specificity of DNA binding and transcriptional activity. *EMBO J.*, **13**, 416–424.

Kuhn,A., Vente,A., Doree,M. and Grummt,I. (1998) Mitotic phosphorylation of the TBP-containing factor SL1 represses ribosomal transcription. *J. Mol. Biol.*, **284**, 1–5.

Mahajan,P.B. and Thompson,E.A. (1990) Hormonal regulation of transcription of rDNA. Purification and charactarization of the hormone-regulated transcription factor IC. *J. Biol. Chem.*, **265**, 16225–16233.

Matsushime,H., Quelle,D.E., Shurtleff,S.A., Shibuya,M., Sherr,C.J. and Kato,J.-Y. (1994) D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.*, **14**, 2066–2076.

Ohtsubo,M., Theodoras,A.M., Schumacher,J., Roberts,J.M. and Pagano,M. (1995) Human cyclin E, a nuclear protein esssential for G_1-to-S phase transition. *Mol. Cell. Biol.*, **15**, 2612–2624.

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.

Resnitzky,D. and Reed,S.I. (1995) Different roles for cyclins D1 and E in regulation of the G_1-to-S transition. *Mol. Cell. Biol.*, **15**, 3463–3469.

Schnapp,A. and Grummt,I. (1996) Purification, assay and properties of RNA polymerase I and class I-specific transcription factors. *Methods Enzymol.*, **273**, 346–359.

Schnapp,A., Pfleiderer,C., Rosenbauer,H. and Grummt,I. (1990) A growth dependent transcription initiation factor (TIF-IA) interacting with RNA polymerase I regulates mouse ribosomal RNA synthesis. *EMBO J.*, **9**, 2857–2863.

Schnapp,A., Schnapp,G., Erny,B. and Grummt,I. (1993) Function of growth regulated transcription factor TIF-IA in initiation complex formation at the ribosomal gene promoter. *Mol. Cell. Biol.*, **13**, 6723–6732.

Sherr,C.J. (1994) G_1 phase progression: cycling on cue. *Cell*, **79**, 551–555.

Tanaka,M. and Herr,W. (1990) Differential transcriptional activation by oct-1 and oct-2 interdependent domains induce oct-2 phosphorylation. *Cell*, **60**, 375–386.

Tower,J. and Sollner-Webb,B. (1987) Transcription of mouse rDNA is regulated by an activated subform of RNA polymerase I. *Cell*, **50**, 873–883.

Toyoshima,H. and Hunter,T. (1994) p27, a novel inhibitor of G_1 cyclin–cdk protein kinase activity, is related to p21. *Cell*, **78**, 67–74.

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.

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.

Voit,R., Schafer,K. and Grummt,I. (1997) Mechanism of repression of RNA polymerase I transcription by the retinoblastoma protein. *Mol. Cell. Biol.*, **17**, 4230–4237.

White,R.J., Gottlieb,T.M., Downes,C.S. and Jackson,S.P. (1995) Cell cycle regulation of RNA polymerase III transcription. *Mol. Cell. Biol.*, **15**, 6653–6662.

Received November 20, 1998; revised and accepted February 2, 1999