The EMBO Journal vol.11 no.6 pp.2211 - 2218, 1992

The nucleolar transcription factor mUBF is phosphorylated by casein kinase II in the C-terminal hyperacidic tail which is essential for transactivation

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Communicated by I.Grummt

UBF is a DNA binding protein which interacts with both the promoter and the enhancer of various vertebrate ribosomal RNA genes and functions as a transcription initiation factor for RNA polymerase I (pol I). We have purified murine UBF to apparent molecular homogeneity and demonstrate that its transactivating potential, but not its DNA binding activity, is modulated in response to cell growth. *In vivo* labelling experiments demonstrate that UBF is a phosphoprotein and that the phosphorylation state is different in growing and quiescent cells. We show that UBF is phosphorylated *in vitro* by a cellular protein kinase which by several criteria closely resembles casein kinase II (CKII). A major modification involves serine phosphoesterifications in the carboxy terminal hyperacidic tail of UBF. Deletions of this C-terminal domain severely decreases the UBF directed activation of transcription. The data suggest that phosphorylation of UBF by CKII may play an important role in growth dependent control of rRNA synthesis.

Key words: casein kinase II/protein phosphorylation/RNA polymerase I/transcription initiation factors/UBF

Introduction

Eukaryotic rDNA transcription is mediated by the concerted action of at least four initiation factors which in the mouse system have been called TIF-IA, TIF-IB, TIF-IC and mUBF (Schnapp and Grummt, 1991). Whereas both the growth regulated activity TIF-IA (Buttgereit *et al.*, 1985; Schnapp *et al.*, 1990b) and factor TIF-IC (G.Heilegenthal, H.Rosenbauer and I.Grummt, manuscript in preparation) are associated with pol I, promoter recognition is brought about by the synergistic action of two DNA binding factors, TIF-IB and UBF. TIF-IB (Schnapp *et al.*, 1990a) which is responsible for the observed species specificity of rDNA transcription (Grummt *et al.*, 1982) forms a strong cooperative complex at the ribosomal gene promoter together with the upstream binding factor UBF. UBF is structurally and functionally conserved in vertebrates and exhibits similar DNA binding properties (Bell *et al.*, 1990; Pikaard *et al.*, 1990). UBF interacts both with the upstream control element (UCE) and the core promoter and has also been shown to bind to the 60 or 81 bp enhancer elements of *Xenopus laevis* and the functionally analogous 140 bp repeats in the mouse rDNA spacer (Bell *et al.*, 1990; Jantzen *et al.*, 1990; Pikaard *et al.*, 1990).

The recent cloning of human UBF (hUBF) has identified multiple domains of the protein which are involved in sequence specific DNA binding and which exhibit significant homology to the nuclear protein HMG1 (Jantzen *et al.*, 1990). Another striking feature of UBF is the primary structure of its carboxy terminus. Of the terminal 89 amino acids, 64% are acidic including two uninterrupted stretches of polyglutamic and aspartic acid residues of 21 and 18 amino acids, respectively. By analogy with various RNA polymerase II transcription factors where acidic domains have been shown to be involved in transcription activation (Ptashne, 1988), it may be assumed that this acidic tail interacts with TIF-IB and thus mediates transcription activation.

One of the most remarkable features of ribosomal gene transcription is the strict correlation between transcriptional activity and the growth rate of the cells. In order to elucidate the complex pleiotropic mechanisms which the cells initiate to regulate rRNA synthesis in response to mitogens and differentiating agents, we have investigated whether the activity of any of the auxiliary rDNA transcription initiation factors is subject to growth control. Previous experiments have demonstrated that growth dependent transcriptional regulation is mediated by TIF-IA. TIF-IA is a positively acting factor whose level or activity fluctuates in response to the physiological state of the cells (Buttgereit *et al.*, 1985; Schnapp *et al.*, 1990b). The mode of action of TIF-IA and its role in the chain of events by which extracellular signals are transmitted from the cell surface to the nucleus is not yet known. In this paper we demonstrate that not only TIF-IA, but also the activity of UBF, is modulated in response to extracellular signals. We show that UBF is phosphorylated within the acidic tail by casein kinase II and suggest that this post-translational modification may be important for transcriptional activity of UBF.

Results

Properties of UBF from growing and stationary cells

Previously we have demonstrated that the transcriptional activity of cell extracts mirrors the *in vivo* rDNA transcriptional activity, i.e. extracts derived from exponentially growing cells support high levels of transcription whereas extracts prepared from starved or stationary cells are virtually inactive (Buttgereit *et al.*, 1985; Schnapp *et al.*, 1990b). In view of the marked fluctuations of rDNA transcription in response to growth, we investigated whether in addition to alterations of the growth dependent factor TIF-IA, structural or functional changes in UBF are involved in this transcriptional control also. For this, UBF was prepared in parallel from equal amounts of extracts prepared from either growing or stationary cells, yielding UBF_a or

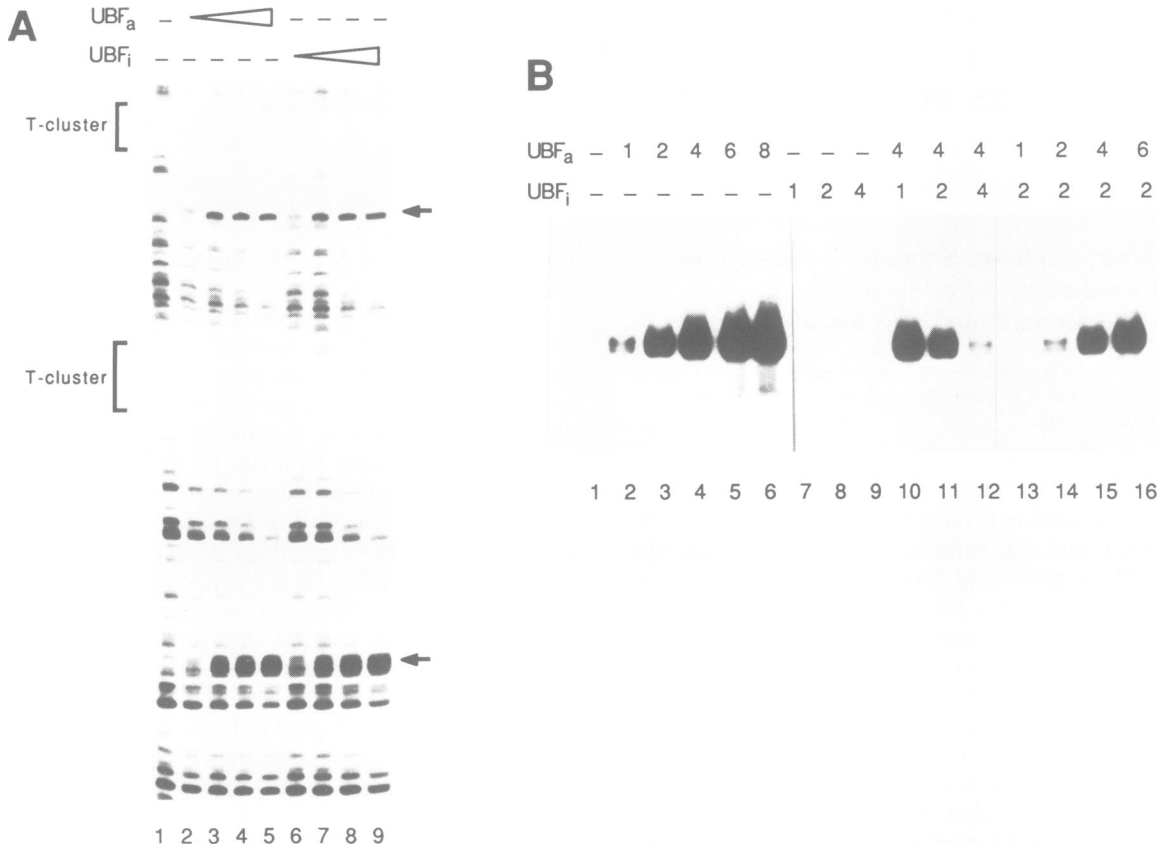

Fig. 1. DNA binding and transcriptional properties of UBF purified from exponentially growing and stationary cells. (**A**) DNase I footprinting of UBF prepared from exponentially growing cultured cells (UBF$_a$, lanes 2–5) or stationary cells (UBF$_i$, lanes 6–9). The reactions contained either no protein (lane 1), 2 μl of UBF (lanes 2 and 6), 4 μl of UBF (lanes 3 and 7), 6 μl of UBF (lanes 4 and 8), or 8 μl of UBF (lanes 5 and 9). (**B**) Transcriptional activity of UBF$_a$ and UBF$_i$. The template pMr600–EcoRI was transcribed in the reconstituted transcription system containing pol I, TIF-IA, TIF-IB and TIF-IC in a total volume of 7 μl, either in the absence of UBF (lane 1) or in the presence of increasing amounts of UBF prepared from growing cells (UBF$_a$) or from stationary cells (UBF$_i$). In each assay the total amount of fraction added to the reaction was brought up to 8 μl with buffer AM-100.

UBF$_i$, respectively. The chromatographic properties, yield and the polypeptide composition of the two UBF preparations were the same (data not shown). Also the DNA binding activities of UBF$_a$ or UBF$_i$ as measured in DNase footprinting experiments were indistinguishable (Figure 1A). In agreement with previous studies (Pikaard *et al.*, 1990), the most remarkable feature of the UBF footprints is the appearance of enhanced cleavage sites within the repeats (marked by arrows in Figure 1A) which are flanked by protected regions. Identical amounts of both factor preparations yielded quantitatively the same footprint. In contrast to DNA binding, there were remarkable differences in the capability of UBF$_a$ and UBF$_i$ to reconstitute transcription. In Figure 1B, the transcripts synthesized in the reconstituted system containing partially purified pol I, TIF-IA, TIF-IB, TIF-IC and either of the two UBF preparations are shown. As expected, increasing amounts of UBF derived from growing cells (UBF$_a$) gradually increased transcription (lanes 1–6). In striking contrast, addition of UBF from growth arrested cells (UBF$_i$) did not exert any stimulatory effect (lanes 7–9). This result suggests that UBF from stationary cells lacks either a component(s) or modification which is dispensable for binding but is required for transcription activation, or that the UBF$_i$ preparation contains an inhibitory component which was not detected by silver staining.

To distinguish between these two possibilities, varying ratios of UBF$_a$ and UBF$_i$ were added simultaneously to the reactions and the level of transcription was compared with the assays containing UBF$_a$ alone. Clearly, addition of UBF$_i$ resulted in a marked inhibition of transcription in the reconstituted system. The level of transcription observed was dependent on the relative amounts of UBF$_a$ and UBF$_i$ in the assays. For instance, a mixture of either 4 μl or 2 μl of both UBF$_a$ and UBF$_i$ directed transcription which was slightly lower than that obtained with 1 μl of UBF$_a$ alone (compare lanes 12 and 14 with lane 2). Similarly, the signal observed in the presence of 4 μl of UBF$_a$ and 2 μl of UBF$_i$ was comparable to that obtained with 1–2 μl of UBF$_a$ (compare lane 15 with lane 3). This result demonstrates that UBF$_i$ competes for the transactivating function of UBF$_a$, a finding which is compatible with either of the two possibilities: (i) UBF$_i$ is differently modified as compared with UBF$_a$ or (ii) UBF$_i$ copurifies with a repressing activity. Since there was no difference in the interaction with DNA, the functional difference between the abilities of both UBF preparations to promote transcription appears to be due to different abilities of the two factor preparations to interact with other components of the transcription apparatus.

UBF is a phosphoprotein

One possible explanation for the changes of UBF activity in response to cell growth is that the activity of this factor is modulated by a post-translational modification. Since

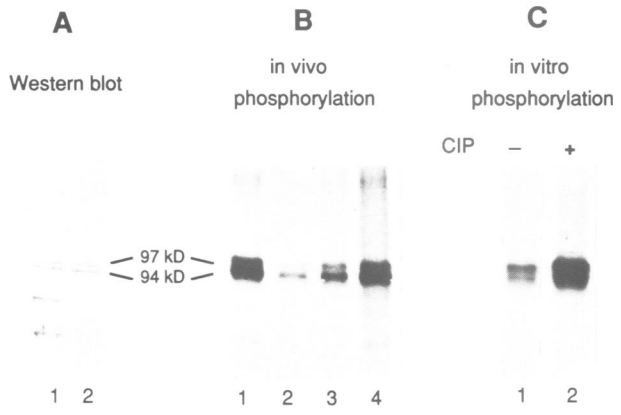

Fig. 2. Phosphorylation of UBF *in vivo* and *in vitro*. (**A**) Western blot of UBF derived from growing (lane 1) and serum starved (lane 2) 3T3 cells. Equal amounts (60 μg) of total protein derived from cells cultured in the presence of 10% or 0.3% fetal calf serum, respectively, were separated by SDS–PAGE, transferred to nitrocellulose and probed with anti-UBF antiserum. (**B**) Induction of UBF phosphorylation after mitogenic stimulation. 3T3 cells were cultured in the presence of 10% or 0.3% FCS, respectively and labelled for 3 h with [^{32}P]orthophosphate. Lane 1, immunoprecipitate from logarithmically growing cells. Lanes 2–4, immunoprecipitates from cells that were serum-starved for 24 h and were metabolically labelled either immediately (lane 2), after 8 (lane 3) or 16 h (lane 4) of mitogenic stimulation by 10% FCS. UBF was immunoprecipitated with anti-UBF antiserum from cell lysates, fractionated by SDS–PAGE and the degree of phosphorylation was determined by autoradiography. (**C**) *In vitro* labelling of UBF by a cellular protein kinase. Purified UBF (3 ng) was incubated in the presence of [γ-^{3}P]ATP, either without (lane 1) or after CIP treatment (lane 2). Both reactions contained 0.2 mM of Na_3VO_4 during the labelling period.

phosphorylation appears to be a common mechanism for reversibly modifying the activity of proteins, we tested whether UBF is phosphorylated *in vivo* and if so, whether the phosphorylation state changes during cell growth. When exponentially growing 3T3 cells were labelled for 3 h with [^{32}P]orthophosphate and immunoprecipitated UBF was analysed by gel electrophoresis and autoradiography, a strong labelling of the 97–94 kDa protein doublet was observed (Figure 2B, lane 1). The incorporation of phosphate was strongly reduced if the cells were starved of serum for 24 h (lane 2). When the starved cells were refed with complete medium for 8 or 16 h, respectively and labelled for 3 h, UBF phosphorylation recovered, finally reaching the level of that observed in logarithmically growing cells (lanes 3 and 4). The amount of UBF as quantified by Western blotting (Figure 2A) was approximately equal both in growing and quiescent cells indicating that there was not a significant alteration in the cellular concentration of UBF. We conclude from this result that the degree of UBF phosphorylation directly correlates with cell growth and appears to fluctuate during the cell cycle.

UBF is phosphorylated in vitro by CKII

To identify the protein kinase(s) responsible for UBF modification, we tried to phosphorylate UBF *in vitro*. Surprisingly, affinity purified UBF was labelled by an endogenous protein kinase which apparently copurifies with UBF (Figure 2C). The incorporation of phosphate was strongly increased if UBF was treated with phosphatase prior to the phosphorylation reaction (lane 2). This marked increase in labelling after phosphatase treatment suggests that a large portion of UBF was present in the phosphorylated form. Heating the UBF fraction for 10 min at 60°C inactivates the endogenous protein kinase (Figure 3A, lane 2). This heat sensitivity of the cellular protein kinase enabled us to search for known enzymes that could phosphorylate UBF *in vitro*. In the experiment shown in Figure 3A, we used heat treated dephosphorylated UBF and assayed several protein kinases for their ability to rephosphorylate the two UBF polypeptides. We found that of the kinases tested (protein kinase C, protein kinase A, protein kinase cdc2 and casein kinase II), only casein kinase II (CKII) efficiently labelled UBF in the presence of [γ-^{32}P]ATP. Both CKII holoenzyme and the recombinant catalytic α-subunit showed similar activities (lanes 3 and 4).

The next series of experiments was performed to find out whether the protein kinase present in the UBF fraction is identical or related to casein kinase II. CKII is known to use both GTP and ATP as substrate. When the phosphorylation reaction was performed in the presence of increasing amounts of nonradioactive ATP and GTP, both nucleoside triphosphates competed with almost the same efficiency for UBF phosphorylation indicating that both are used by the endogenous enzyme (data not shown). Furthermore, UBF phosphorylation was inhibited at identical concentrations of heparin as CKII. In addition, the ATP analogue DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole), an inhibitor of CKII, eliminated labelling of UBF by the endogenous kinase at similar concentrations to that of authentic CKII (unpublished data). To fortify these results, we used a CKII-specific synthetic peptide substrate (RRREEETEEE; Kuenzel *et al.*, 1987) and tested whether it was able to compete for UBF phosphorylation. Indeed, this specific substrate is a potent inhibitor of UBF phosphorylation, whereas a control peptide (AKAKTPKKAK) which is a substrate for protein kinase cdc2 showed no effect (Figure 3B).

These results indicate that the CKII related kinase which phosphorylates UBF is either associated with UBF or is an intrinsic property of this factor itself. To distinguish between these two possibilities, a photoreactive ATP analogue [α-^{32}P]8-azido-ATP was used. This ATP derivative is crosslinked to ATP binding proteins upon photolysis and therefore may be used to label the polypeptide which exerts the kinase activity (Haley, 1983). Using this technique, we reproducibly observed weak labelling of a 42 kDa protein in different preparations of purified UBF (Figure 3C, lane 3). The diffuse smear around 66 kDa was observed in all crosslinking experiments, irrespective of which protein was used (data not shown). When the UV exposure was omitted no labelling was observed (lane 4). Since the 97–94 kDa UBF doublet was not labelled at all, it suggests that the UBF polypeptides exhibit no ATP binding activity on their own. Interestingly, the 42 kDa protein which had been labelled with [α-^{32}P]8-azido-ATP had the same electrophoretic mobility as the α' subunit of purified CKII which has been autophosphorylated with [γ-^{32}P]ATP (lane 2). The heavily autophosphorylated β subunit of CKII cannot be detected by this technique, since the ATP binding domain of CKII resides in its α subunits (Pinna, 1990). Taken together, the results strongly suggest that the UBF preparations which were purified by at least four conventional chromatographic steps (including one FPLC column) followed by a site-specific DNA affinity chromatography contain a protein kinase activity which is identical with or closely related to CKII.

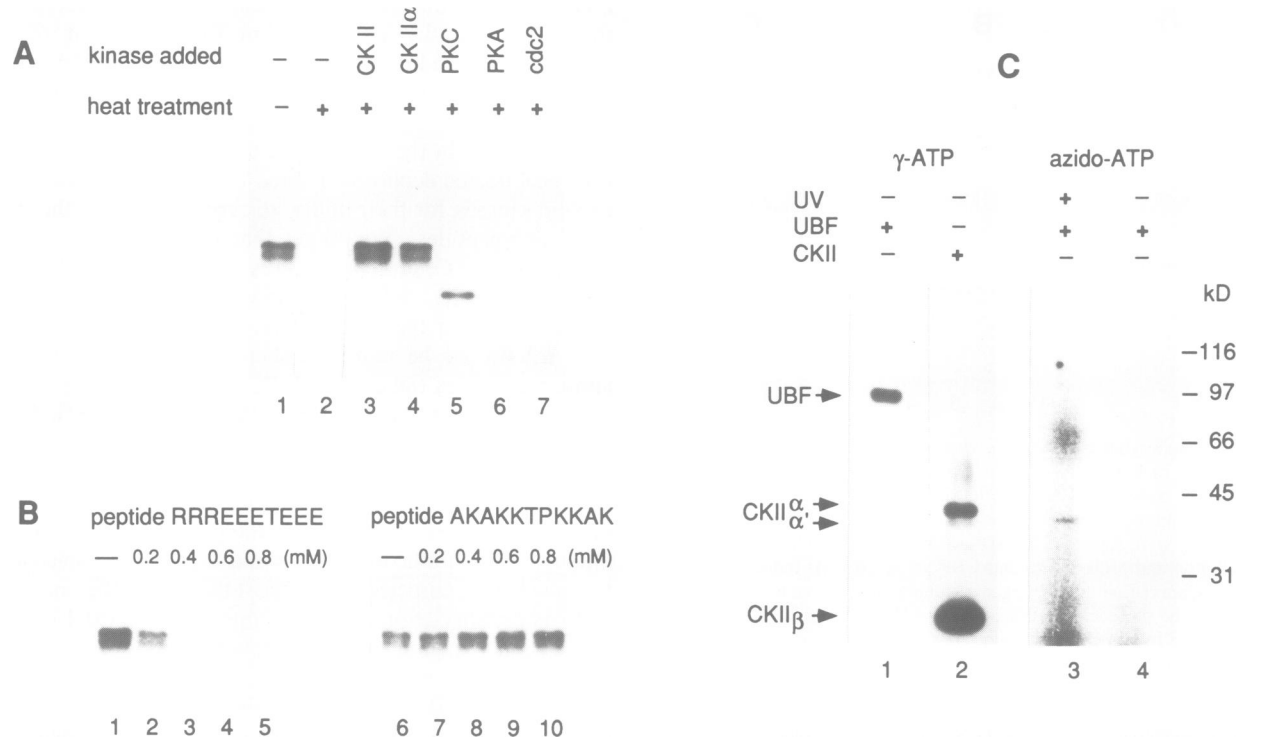

Fig. 3. UBF is phosphorylated by CKII. (**A**) Phosphorylation of UBF by CKII *in vitro*. Purified cellular UBF was dephosphorylated and either left on ice for 10 min (lane 1) or incubated for 10 min at 60°C (lanes 2 to 7) prior to the addition of [γ-^{32}P]ATP and exogenous protein kinases indicated above the lanes. Incorporation of labelled phosphate was analysed by 7.5% SDS−PAGE. The 77 kDa phosphoprotein visible in lane 5 represents autophosphorylated protein kinase C. (**B**) Competition of UBF phosphorylation by a CKII specific peptide substrate. CIP treated cellular UBF was phosphorylated by the endogenous protein kinase either in the presence of increasing concentrations of a CKII specific peptide substrate (lanes 1−5) or in the presence of increasing concentrations of a cdc2 kinase peptide substrate (lanes 6−10). (**C**) Affinity labelling of the copurifying protein kinase. UBF (lane 1) or CKII (lane 2) were autophosphorylated in the presence of [γ-^{32}P]ATP. In parallel reactions, UBF was incubated with the photoreactive ATP analogue [α-^{32}P]8-azido ATP (lanes 3 and 4). The reaction was performed either with (lane 3) or without UV exposure (lane 4) as described in Materials and methods. The labelled proteins were analysed by 7.5−15% SDS−PAGE, followed by autoradiography. The position of UBF as well as the position of CKII subunits are indicated at the left side. The sizes of marker proteins are indicated at the right. Lanes 1 and 2 were exposed for 9 h, lanes 3 and 4 for 160 h, respectively.

Fig. 4. Schematic diagram of the structural domains present in UBF. The position of the five HMG boxes is marked by hatched boxes, the filled boxes in the C-terminal region represent the two uninterrupted stretches of glutamic acid and aspartic acid. The putative casein kinase II phosphorylation target sites are marked by triangles.

CKII phosphorylates UBF at serine residues within the carboxy terminal acidic tail

Casein kinase II has been shown to phosphorylate serine and threonine residues at the amino terminal side of a string of 4−5 acidic residues (Kuenzel *et al.*, 1987). Examination of the amino acid sequence of UBF cDNA revealed several potential CKII recognition sites within the C-terminal hyperacidic tail of UBF. A diagrammatical sketch of the various domains of the UBF protein with the amino acid sequence of the C-terminal domain is shown in Figure 4. Clearly, there are several amino acid sequences which match the proposed recognition site for CKII.

Having recombinant UBF expressed in the vaccinia virus system allowed us to test whether or not the C-terminal part of UBF is the target site for phosphorylation. Wild type UBF and the deletion mutant ΔC552, which lacks the acidic tail and the fifth HMG box, were assayed for phosphorylation both by the endogenous protein kinase and by addition of CKII. As shown in Figure 5C, full-length recombinant UBF was efficiently phosphorylated both by the endogenous kinase

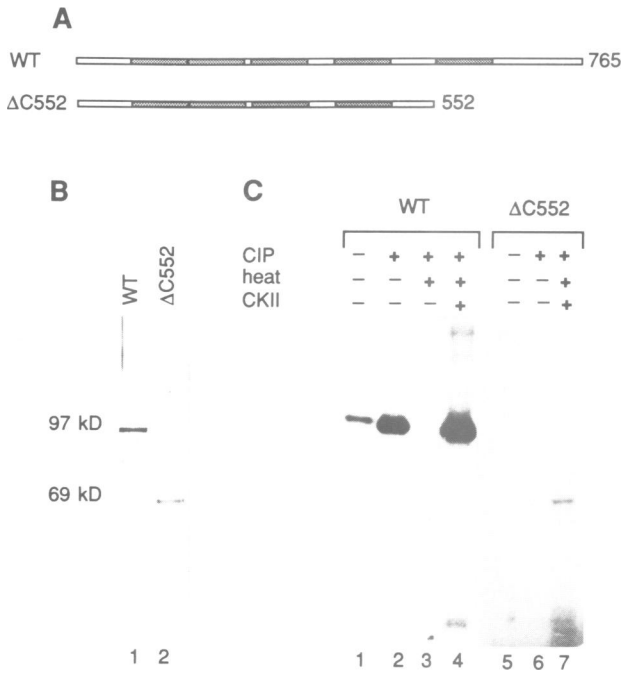

Fig. 5. The phosphorylation resides within the C-terminal domain of UBF. (**A**) Schematic representation of recombinant UBF constructs used for analysing the phosphorylation sites *in vitro*. The hatched regions mark the positions of the individual HMG boxes. (**B**) SDS−PAGE and siver staining of wild type UBF (WT) and mutant ΔC552 expressed in the vaccinia virus system. (**C**) The carboxy terminal region of the UBF is the target site for phosphorylation. Recombinant UBF (3 ng) was either treated with alkaline phosphatase (CIP) prior to the kinase reaction or left untreated as indicated above the lanes. The phosphorylation reactions were carried out by either the endogenous kinase or, following heat inactivation of endogenous kinase, by the addition of purified CKII. The labelled polypeptides were analysed by autoradiography. Exposure times were 30 min (lanes 1−4) and 4 h (lanes 5−7), respectively.

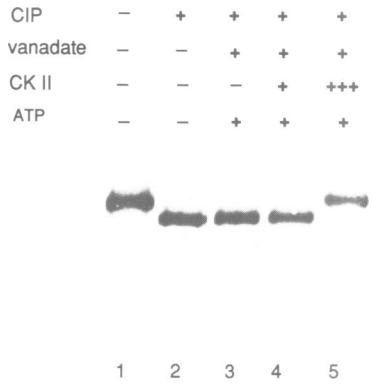

Fig. 6. The phosphatase induced shift in electrophoretic mobility of UBF is reversed by CKII. Lane 1, 30 ng of untreated recombinant UBF; lane 2, UBF treated with CIP; lanes 3−5, UBF was treated with CIP as in lane 2, then the phosphatase was inhibited by sodium vanadate before ATP (1 mM) was added, and UBF was incubated for 20 min without exogenous kinase (lane 3) or with two different concentrations of purified CKII (the sample in lane 5 contains a 20-fold more CKII than that in lane 4). After electrophoresis the proteins were transferred to nitrocellulose filters, and UBF was detected by Western blotting.

and by exogenous CKII (lanes 1,2 and 4). For efficient labelling UBF had to be dephosphorylated by prior CIP treatment. However, when the deletion mutant was assayed, only

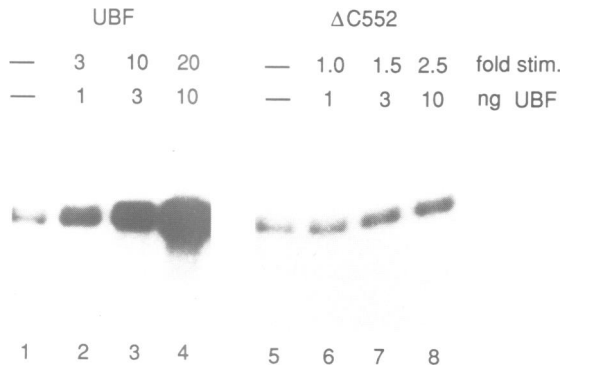

Fig. 7. Transcriptional activity of recombinant UBF and the deletion mutant ΔC552. 5 ng of template DNA pMrWT/NdeI were transcribed in the reconstituted transcription system containing a mixture of partially purified pol I and the transcription initiation factors TIF-IA, TIF-IB and TIF-IC. Transcriptions were carried out in the absence of UBF (lanes 1 and 6) or in the presence of increasing amounts of either form of UBF as indicated above the lanes.

a very weak, perhaps non-specific, labelling of the 69 kDa protein was observed (lanes 5−7). This failure of the C-terminally truncated UBF to be phosphorylated strongly suggests that the potential CKII recognition sequences present in the acidic tail are sites of UBF phosphorylation.

The presence or absence of phosphate groups often alters the mobility of a protein on denaturing SDS−polyacrylamide gels. We therefore investigated whether dephosphorylation and rephosphorylation would shift the electrophoretic mobility of UBF. In the experiment shown in Figure 6, UBF expressed in the vaccinia system was dephosphorylated by CIP (lane 2) and subsequently phosphorylated by either the endogenous kinase (lane 3) or by two concentrations of CKII (lanes 4 and 5). The reactions were subjected to SDS−PAGE and UBF was detected by Western blotting. The result demonstrates clearly that there is an increase in electrophoretic mobility after dephosphorylation which is reversed by rephosphorylation. This finding supports the assumption that most or all of UBF phosphorylation is brought about by CKII.

The C-terminal domain is important for the transcriptional activity of UBF

The data presented so far have demonstrated that phosphorylation of UBF resides predominantly or exclusively within the C-terminal part of UBF. If *in vivo* modification of UBF by phosphorylation plays a functional role in modulating its activity, then this part of the UBF molecule should serve an essential function. To investigate the function of the C-terminal domain in UBF directed transcriptional activation, we compared the activity of full-length UBF with that of the deletion mutant ΔC552. Both the wild type and the mutant were expressed in the vaccinia virus system and purified by three chromatographic steps as described in Materials and methods. Equal amounts of both forms of UBF were added to the reconstituted transcription system containing partially purified pol I as well as TIF-IA, TIF-IB and TIF-IC. In the absence of UBF, a very weak transcription signal was observed (Figure 7, lanes 1 and 5). Addition of increasing amounts of full-length UBF stimulated transcription. In the presence of 10 ng of UBF a 20-fold stimulation was observed (lane 4). Interestingly, the mutant ΔC552 was virtually inactive. This failure of the mutant to

activate transcription was not due to a decreased DNA binding activity. Both wild type UBF and the mutant ΔC552 bind to their target sequence with about the same affinity (Jantzen *et al.*, 1990; and our own data). Therefore the transcriptional inactivity of ΔC552 very likely reflects some failure of the mutant to interact with some other component(s) of the transcription machinery. The slight transcriptional stimulation in the presence of ΔC552 (lanes 5–8) may be attributed to the presence of traces of HeLa UBF which is difficult to separate completely from the mutant protein.

Discussion

Very little is known about the molecular mechanisms which shut down pol I transcription as growth slows and essentially nothing is known about the signalling pathway that regulates this process. In this communication we report two observations which suggest a way by which rRNA synthesis is linked to the proliferation rate of the cells. First, we present evidence which suggests that the pol I specific transcription factor UBF may play a role in growth dependent rDNA transcription regulation. Secondly, we demonstrate that UBF is a phosphoprotein and casein kinase II is very probably the enzyme which modifies UBF post-translationally. Although definite proof is still lacking, the data suggest that phosphorylation may contribute to control the transactivating function of UBF.

Post-translational modification of RNA polymerase II transcription factors by phosphorylation appears to be a common molecular mechanism to link cell growth and gene activity either by modifying DNA binding capability or modulating protein–protein interactions. Since rRNA synthesis is intimately coupled to cell growth, it is tempting to speculate that hierarchical phosphorylation reactions are also involved to adapt rDNA transcription to the proliferation rate of the cells. Previously we have shown that the activity of an essential initiation factor, TIF-IA, fluctuates according to external signals (Buttgereit *et al.*, 1985; Schnapp *et al.*, 1990b). In this communication we demonstrate that a second factor, UBF, is also subject to growth control. In a reconstituted transcription system a strong response to UBF prepared from exponentially growing cells was observed whereas UBF derived from stationary cells did not activate transcription. The approximate amount of the factor, the chromatographic properties and the DNA binding activities of both UBF preparations were about the same. Any of the following hypotheses could explain the functional inactivity of UBF from quiescent cells: (i) there could be a UBF associated protein that mediates interactions between the individual transcription initiation factors which is present only in growing cells; (ii) there could be an 'anti-UBF' protein whose negative effect is alleviated in proliferating cells; and (iii) UBF might undergo a modification that is necessary for transcription activation.

Although our results do not rigorously exclude any of these possibilities, they are most consistent with the last hypothesis. The experiments reported here demonstrate that UBF is a phosphoprotein and that the phosphorylation state changes during cell growth. The phosphorylation of UBF is strongly reduced in serum starved 3T3 cells as compared with exponentially growing cells. When the starved cells are refed with serum, the extent of UBF phosphorylation reaches that of growing cells. This result demonstrates that the degree of phosphorylation of UBF fluctuates according to cell growth. Similar findings have been reported recently by O'Mahony *et al.* (1982).

Furthermore, we show that this phosphorylation is catalysed by a protein kinase which is identical or closely related to casein kinase II (CKII). The identification of the cellular UBF kinase as CKII is based on phosphoamino acid analysis (not shown here), inhibition of kinase activity by heparin and DRB, the ability to use both ATP and GTP as a phosphate donor and on specific substrate competitions.

Although we cannot exclude that *in vivo* a different protein kinase is responsible for the phosporylation of UBF, the following results strongly suggest that CKII is probably the enzyme which modifies cellular UBF. First, the transactivating domain of UBF contains amino acid sequences which are ideal CKII phosphorylation sites. Secondly, the kinase is not an intrinsic property of UBF as shown by crosslinking experiments with the photoreactive ATP analogue 8-azido-ATP. Using this method we failed to detect any ATP binding capacity of the 97 and 94 kDa UBF prolypeptides but identified a 42 kDa protein which most likely represents the α or α' subunit of CKII which copurified with UBF. Thirdly, among several protein kinases tested (PKA, PKC$\alpha\beta$, PKCδ, cdc2, CKII holoenzyme and CKII recombinant α subunit) only the two CKII enzymes were able to phosphorylate UBF *in vitro*. The incorporation of labelled phosphate was greatly stimulated by prior treatment of UBF with alkaline phosphatase, indicating that in the cell UBF is present in the phosphorylated form. After dephosphorylation, the electrophoretic mobility of UBF was increased and this shift in electrophoretic mobility could be reversed by CKII. Finally, the assumption that CKII is the cellular kinase which phosphorylates UBF is supported by the fact that UBF phosphorylation changes by at least one order of magnitude in response to the growth rate of the cells. CKII activity has been reported to increase in a time dependent manner after mitogenic stimulation by growth factors (Sommercorn *et al.*, 1987; Carroll *et al.*, 1988; Klarlund and Czech, 1988). On the basis of homology with yeast cell division control proteins, it has been suggested that CKII may be involved in some aspect of the control of cell proliferation (Chen-Wu *et al.*, 1987; Takio *et al.*, 1987). The 6-fold elevation in CKII activity after serum stimulation was transient, showing two more activation cycles after 12 and 24 h (Carroll and Marshak, 1989). These oscillations in CKII activity are largely independent of protein synthesis and thus are likely to reflect cycles of post-translational activation and inhibition of the cellular kinase pool. Interestingly, our data on the phosphorylation of UBF after serum stimulation agree well with these observations. Complete recovery of UBF phosphorylation was achieved within 16–19 h after serum stimulation, correlating with induced CKII activity during the transition from G_1 to S phase. On the other hand, the low level of UBF phosphorylation 8 h after mitogenic stimulation correlates with the low CKII activity present during transition from G_0 to G_1 (Carroll and Marshak, 1989). In addition, CKII has been shown to be localized in the nucleolus of proliferating cells and to be a limiting factor for rDNA transcription in nuclei from confluent cells which show an 85% reduction of rDNA transcription (Belenguer *et al.*, 1989). Furthermore, administration of dexamethasone to rats was found to

increase the levels of liver nucleolar CKII (Suzuki *et al.*, 1987). These data suggest that CKII plays a vital role in signal transduction pathways which modulate rDNA transcription in response to cell growth.

Experiments are in progress to map the phosphorylation sites within UBF precisely and to determine which phosphate residues are changed in cells grown under different physiological conditions. In addition, site directed mutagenesis of the phosphorylated amino acids is being carried out to determine whether or not there is a causal relationship between the phosphorylation state of UBF and its transactivating function. Until this analysis has been completed, we will be unable to link UBF phosphorylation with rDNA transcription. We have performed a number of experiments to demonstrate a correlation between the activity of UBF in the reconstituted transcription system and the degree of phosphorylation. The results of such de- and rephosphorylation experiments were highly variable and did not permit a definite conclusion. One possible explanation for this variability of the results is that of several phosphorylation states of UBF both the hyperphosphorylated and the fully dephosphorylated forms are transcriptionally active, whereas a defined hypophoshorylated form of UBF is inactive. A similar situation has recently been described for the Rel associated pp40 protein. The activity of pp40 to inhibit the DNA binding of Rel and NF-$\varkappa$B depends on its degree of phosphorylation. The hypophosphorylation form of pp40 prevents DNA binding of Rel, but dephosphorylated or hyperphosphorylated forms have been shown not to manifest any inhibitory effect (Kerr *et al.*, 1991). Although other plausible interpretations are also possible, the hypothesis that only a defined hypophosphorylated form of UBF is transcriptionally inactive, could explain why we have failed until now to functionally reactivate either UBF from quiescent cells or dephosphorylated UBF by exogenous CKII. In addition, although CKII appears to be responsible for the majority of phosphate groups incorporated into the C-terminus, we cannot exclude the possibility that yet to be identified phosphorylation site(s) in other regions of UBF, which are recognized by other kinases, may either be functionally more important or act in concert with CKII to regulate UBF activity. In any case, if the transactivating function of UBF is regulated by a phosphorylation–dephosphorylation mechanism, then both specific kinase(s) and specific phosphatase(s) should act in concert to adapt the rate of rRNA synthesis to cell growth. We envision that the equilibrium between active and inactive factors varies depending on the cellular environment and that as a result of growth stimulation by external signals this equilibrium is shifted markedly in favour of the active molecules. The availability of cloned cDNA encoding mUBF sets the stage for a future mutational analysis aimed at elucidating the role of phosphorylation in regulating UBF function and rDNA transcriptional activity.

Materials and methods

Cultivation of cells and extract preparation

Transcriptionally active extracts were obtained from cultured cells which were harvested in the exponential phase of growth as described before (Schnapp *et al.*, 1990b). Transcriptionally inactive extracts were prepared from stationary phase cells, which were taken either directly from the cavity of mice or were grown to maximal density ($1.5-2 \times 10^6$ cells/ml) in culture medium and incubated for an additional 24 h before harvesting.

In vitro transcription assays

25 μl reactions contained 10–50 ng of template DNA and 15 μl of a mixture of pol I, TIF-IA, TIF-IB, TIF-IC and UBF (Schnapp and Grummt, 1991). The templates used were either pMr600 (containing rDNA sequences from −324–+292) truncated with *Eco*RI or mMrWT (containing rDNA sequences from −170–+155) truncated with *Nde*I which yielded 297 or 371 nucleotide run-off transcripts, respectively.

Purification of transcription factors and RNA polymerase I

TIF-IA, TIF-IB and TIF-IC were purified from cultured Ehrlich ascites cells as described previously (Schnapp and Grummt, 1991). Extracts (100–200 ml of a mixture of nuclear and cytoplasmic extracts) were chromatographed on a DEAE–Sepharose CL-6B column, followed by fractionation on Heparin Ultrogel A4-R by step elution with buffer AM (20 mM Tris–HCl, pH 7.9, 0.1 mM EDTA, 20% glycerol, 5 mM $MgCl_2$) containing different salt concentrations. TIF-IA and TIF-IC eluted at 200 mM KCl, RNA pol I at 400 mM KCl and TIF-IB at 600 mM KCl. The experiments described in this paper were performed with this relatively crude pol I fraction (H-400) which showed a significantly higher stimulation by UBF than more purified preparations. UBF was purified from the fractions eluting at 1 M KCl which contained ~30% of cellular UBF activity. The UBF containing fractions were dialysed and applied to MonoQ FPLC column. UBF eluted at ~450 mM KCl and was subsequently purified on a sequence specific DNA affinity column as described by Bell *et al.* (1988).

DNase protection

Footprinting was performed as described previously (Learned *et al.*, 1986). The enhancer probe used contained the *StuI*–*SalI* −640 to −168 fragment from mouse rDNA labelled at the *StuI* site.

Phosphorylation of UBF in vitro

To achieve maximal labelling, UBF was dephosphorylated prior to phosphorylation. Usually 3 ng of UBF were incubated for 15 min at 30°C with 0.2 U of calf intestinal alkaline phosphatase (CIP, Boehringer Mannheim). The phosphatase was inactivated by the addition of 0.2 mM of Na_3VO_4. Phosphorylation was performed for 15 min at 30°C in 15 μl of kinase buffer (20 mM Tris–HCl, pH 7.4; 80 mM KCl; 5 mM $MgCl_2$; 12% glycerol; 0.5 mM DTE; 0.5 mM PMSF) containing 5 μM ATP and 5 μCi of [γ-^{32}P]ATP. To assay protein kinase C, the phosphorylation reaction was supplemented with TPA (1 μM), $CaCl_2$ (500 μM) and sonicated phosphatidylserine (0.25 mg/ml).

Photoaffinity labelling with 8-azido-ATP

Photoaffinity labelling with [α-^{32}P]8-azido-ATP (ICN) was performed on ice in a 96-well tissue culture plate. Activation of the azido group was achieved by irradiation with UV light (254 nm) for 3 min at a distance of ~3 cm between sample and lamp. Reactions (final volume 30 μl) were carried out in labelling buffer (25 mM Tris–HCl, pH 7.2, 10 mM $MgCl_2$, 40 mM KCl, 8% glycerol and 0.3 μCi [α-^{32}P]8-azido-ATP (5.3 Ci/mmol) in the absence of DTE. Affinity labelled proteins were subjected to SDS–PAGE (7.5–15%) and visualized by autoradiography.

Recombinant plasmids encoding UBF cDNA

A cDNA covering the coding region of mUBF as well as 51 bp and 371 bp of the 5′ and 3′ untranslated region, respectively, was cloned into pBluescript^{KS}, yielding pKSmUBF–SE. For expression in the vaccinia virus system, the mUBF cDNA was inserted between the *Bam*HI and the *Eco*RI site of the vaccinia recombination vector pgpt-delta-6, which yielded the construct pVACmUBF–SE. To create the C-terminal deletion pVACmUBF–ΔC552, a 1.7 kb *Pvu*II fragment from pKSmUBF–SE was cloned into the *Bal*I site of the vaccinia recombination vector pATAgpt-STOP3 (de Magistris and Stunnenberg, 1988; Stunnenberg *et al.*, 1988).

Preparation of UBF from recombinant vaccinia virus infected cells

Recombinant vaccinia virus expressing full-length UBF or the deletion mutant ΔC552 was amplified using standard procedures (de Magistris and Stunnenberg, 1988). For extract preparation, the pellet derived from 1.5 l of infected HeLa cells was homogenized in 5 vol of a buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTE and 0.5 mM PMSF and centrifuged for 5 min at 1500 r.p.m. The supernatant was mixed with 0.1 vol of 0.3 M HEPES, pH 7.9, 1.4 M KCl, 30 mM $MgCl_2$, 0.5 mM DTE and 0.5 mM PMSF, incubated on ice for at least 10 min and centrifuged for 1 h at 35 000 r.p.m. Nuclear extracts were prepared according to Dignam *et al.* (1983).

Both cytoplasmic and nuclear extracts were fractionated on DEAE–Sepharose followed by chromatography on Biorex 70. UBF was

recovered at 480–600 mM KCl and then chromatographed on Q-Sepharose. At this resin the deletion mutant ΔC552 eluted at 100 mM KCl and wild type UBF at 500 mM KCl.

Expression of recombinant UBF in Escherichia coli and antibody production

A cDNA fragment, encoding amino acids 1–346 of mUBF was cloned between the *Nde*I and *Eco*RI sites of the T7 expression vector pJC20 (Clos *et al.*, 1990) after generating an *Nde*I site in mUBF cDNA adjacent to the translation initiation codon by oligonucleotide directed mutagenesis using the Amersham Kit. The recombinant protein was expressed in *E.coli* BL21 (DE3)pLysS (Studier *et al.*, 1990) and was isolated by SDS–PAGE and electroelution. Rabbits were immunized with 500 μg of purified recombinant UBF in Freund's complete adjuvant. At 4 week intervals the rabbits were boosted twice by injection of 500 μg of protein in incomplete Freund's adjuvant and serum was collected 2 weeks later.

In vivo labelling of 3T3 cells with [^{32}P]orthophosphate and immunoprecipitation

Cells were plated at a density of 1.8×10^5 per 3.5 cm dish and grown for 24 h in DMEM medium, supplemented with 10% FCS. For *in vivo* labelling of proteins with ^{32}P inorganic phosphate cells were then washed with phosphate free DMEM (NEN) and incubated for 3 h in 1 ml phosphate free DMEM containing 10% dialysed FCS and 0.8 mCi/ml [^{32}P]orthophosphate. For serum deprivation studies cells were grown as described above and then starved of serum in DMEM–0.3% FCS for 24 h prior to *in vivo* labelling. Where indicated serum starved cultures were refed in DMEM containing 10% FCS for 8 or 16 h, before labelling with [^{32}P]orthophosphate was done as specified for logarithmically growing cells.

Immunochemical techniques

For immunoprecipitation, cells from a 3.5 cm dish were washed in ice cold STET (150 mM NaCl, 10 mM Tris–HCl, pH 7.4 and 1 mM EDTA) and lysed by incubating for 30 min on ice in 1 ml RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 20 mM sodium phosphate, pH 7.2, 100 mM NaCl, 20 mM KF and 0.3 mM sodium orthovanadate) supplemented with 1 mM PMSF, 50 μg/ml peptstatin A and 1% aprotinin. After centrifugation, the supernatants were precleared by incubation with protein A–Sepharose beads. UBF was immunoprecipitated by a 1:100 dilution of polyclonal rabbit anti-UBF antiserum (K8) and protein A–Sepharose beads. Immunoprecipitates were washed four times in RIPA buffer before resuspending in SDS sample buffer and analysing by SDS–PAGE.

For immunoblots proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature with PBS, pH 7.5, 2.5% milk powder and 0.2% Tween 20. The filters wre incubated with anti-UBF antiserum (1:1000 dilution) followed by incubation with an anti-rabbit horseradish–peroxidase conjugated secondary antibody (Promega). Protein–antibody complexes were visualized by an enhanced chemiluminescence (ECL) Western blotting detection system according to the specifications of the manufacturer (Amersham).

Acknowledgements

We thank S.Traub for help in cell culture, extract preparation and factor purification and C.Kurth for technical assistance. The two overlapping recombinant plasmids encoding mUBF cDNA were generously provided by H.-M.Jantzen. We are indebted to W.Pyerin, P.Belenguer, F.Marks, U.Walter and G.Draetta for the supply of the recombinant α-subunit of casein kinase II, for purified casein kinase II, protein kinase C, protein kinase A and cdc2 kinase, respectively. This work was supported by the Deutsche Forschungsgemeinschaft (Leibnitz-Programm and SFB 229), the Fond der Chemischen Industrie and by a grant of the Science Program of the Commission of the European community (SCI*-0259-C).

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Received on January 16, 1992; revised on March 16, 1992