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An Immediate Response of Ribosomal Transcription to Growth Factor Stimulation in Mammals Is Mediated by ERK Phosphorylation of UBF

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Summary

Ribosomal transcription in mammals is regulated in response to growth, differentiation, disease, and aging, but the mechanisms of this regulation have remained unresolved. We show that epidermal growth factor induces immediate, ERK1/2-dependent activation of endogenous ribosomal transcription, while inactivation of ERK1/2 causes an equally immediate reversion to the basal transcription level. ERK1/2 was found to phosphorylate the architectural transcription factor UBF at amino acids 117 and 201 within HMG boxes 1 and 2, preventing their interaction with DNA. Mutation of these sites inhibited transcription activation and abrogated the transcriptional response to ERK1/2. Thus, growth factor regulation of ribosomal transcription likely acts by a cyclic modulation of DNA architecture. The data suggest a central role for ribosome biogenesis in growth regulation.

Introduction

The primordial role of ribosomal RNA transcription as regulator of ribosome production makes it a key target for growth regulation (Warner, 1999). It has been shown that changes in growth and proliferation occurring during processes such as organ regeneration (Castle et al., 1978; Friedman et al., 1984; Loeb and Yeung, 1975; Nikolov et al., 1991), differentiation (Cavanaugh et al., 1995; Stoykova et al., 1983; Zahradka et al., 1991), hypertrophy (Hannan et al., 1996; Luyken et al., 1996), aging (Castle et al., 1978), and starvation (Grummt et al., 1976) are all correlated with changes in the rate of ribosomal transcription, and nucleolar size and number have for many years been used as a measure of tumor quality (e.g., Derenzini et al., 2000). Ribosomal transcription rates have been found to vary by up to a factor of four. Though such changes are small when compared to the regulation of many mRNA genes, they are very significant in the context of cell growth and proliferation rates. Given our present knowledge, it would seem reasonable to suppose that stimulators of cell growth and/or proliferation directly regulate transcription of ribosomal genes.

In addition to the dedicated RNA polymerase I (PolI), ribosomal transcription requires the so-called upstream binding factor (UBF), a multiple HMG box architectural factor, and the "selectivity" factor SL1, which contains the TATA box binding protein (TBP; see Paule, 1998). UBF catalyzes the recruitment and binding of SL1, or of the SL1-containing holopolymerase (Hannan et al., 1999a; Saez-Vasquez and Pikaard, 1997; Seither et al., 1998) to the PolI promoter, making it a potential regulator of preinitiation complex assembly and promotion. The minimal or core region of UBF necessary for transcription in vitro spans its N-terminal dimerization domain and the three HMG boxes constituting its DNA binding region (Jantzen et al., 1992; McStay et al., 1991). A dimer of this "core" UBF forms the ribosomal *enhancesome*, a structure in which about 140 bp of ribosomal DNA is looped into a single 360 degree turn (Bazett-Jones et al., 1994; Stefanovsky et al., 1996), suggesting that DNA architecture plays a key role in the promotion of transcription by PolI. Previous studies have suggested roles for both casein kinase II (CKII) (Kihm et al., 1998; O'Mahony et al., 1992a, 1992b; Tuan et al., 1999; Voit et al., 1992) and cyclin-dependent kinase 2/4 (CDK2/4; Voit et al., 1999) in regulating UBF activity. Differentiation, serum deprivation, and glucocorticoids have been found to induce a regulation of PolI activity, probably via the polymerase-associated RRN3 or TIF-1A factor (Bodem et al., 2000; Miller et al., 2001; Moorefield et al., 2000). These regulations occur over extended time periods, reinforcing the established view that ribosomal transcription responds indirectly to changes in cellular metabolism. In contrast, we show that activation of the classic MAP kinase (ERK) pathway induces an immediate upregulation of ribosomal transcription via UBF modification. As such, our data add yet another ERK-regulated transcription factor to a growing list (Sharrocks et al., 2000), but more importantly, demonstrate the existence of a direct link between growth factor signaling and ribosome biogenesis in mammalian cells.

Results

To better understand the relationship between growth stimulation and ribosomal gene transcription, we asked whether the treatment of mammalian cells with a typical

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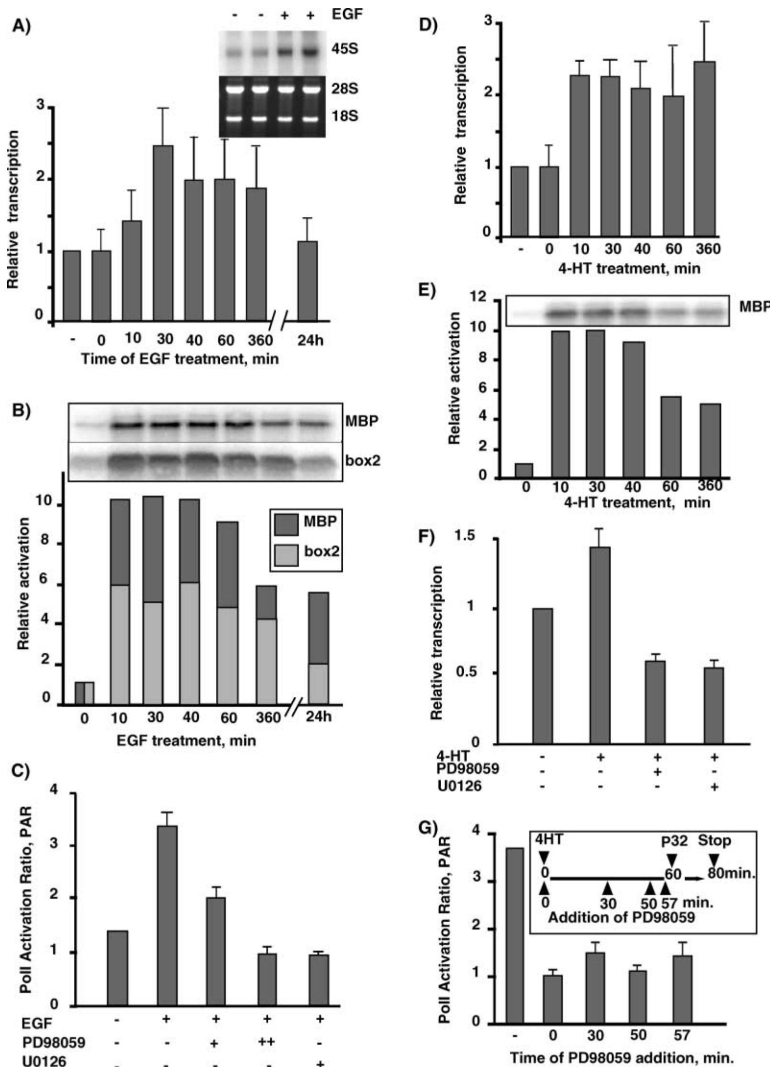

Figure 1. ERK Activation Leads to an Immediate Increase in Ribosomal RNA Transcription

(A) Time course of transcription activation by EGF in SKF-5 cells. The top inlayed panel shows ^{32}P pulse-labeled 45S precursor rRNA before and 1 hr after stimulation; the bottom panel shows EtBr-stained 28S and 18S rRNA. −, no EGF.

(B) Corresponding time course of ERK2 activity in SKF-5 cells measured on MBP and UBF HMG box 2 (box2) substrates. Inlayed panel: the autoradiographs of typical assays.

(C) SKF-5 cells were mock treated or treated for 1 hr with EGF or a combination of EGF and 5 (+) or 50 (++) μM PD98059 or 1 μM U0126 and then ^{32}P pulse labeled as in (A).

(D and E) ^{32}P pulse labeling of the 45S rRNA precursor (D) and ERK2 (E) activity in mouse NIH3T3ΔRaf:ER cells before and after stimulation with 4-hydroxytamoxifen (4-HT).

(F) Pulse labeling of endogenous 45S precursor rRNA in the untreated and 4-HT or 4-HT plus 50 μM PD98059- or 4-HT plus 1 μM U0126-treated NIH3T3ΔRaf:ER cells. Pulse labeling was started 1 hr after addition of 4-HT or 4-HT plus inhibitor.

(G) ΔRaf:ER cells were stimulated with 4-HT and 1 hr later were pulse labeled to reveal the 45S precursor rRNA transcription rate as in (F). PD98059 was added at the indicated times before pulse labeling to inhibit ERK activation; see inset.

In (A), (C), (D), (F), and (G), labeling was for 20 min and ^{32}P incorporation into 45S RNA was normalized to bulk 18S RNA.

growth factor, in this case, human epidermal growth factor (EGF), activated ribosomal transcription, and if so, was this activation a slow or rapid event. Treatment of the human neuroepithelioma cell line SKF-5 (van Weering et al., 1995) with EGF resulted in an increase in endogenous 45S preribosomal RNA (45S pre-rRNA) transcription by 2.5 times (Figure 1A). This degree of activation is typical of ribosomal genes (see Introduction). Already 10 min after EGF addition, 45S pre-rRNA transcription had risen to 1.4 times the basal level and by 30 min had increased 2.5 times. Pre-rRNA transcription remained about 2 times the basal level for 360 min, but had nearly returned to the unstimulated level after 24 hr. Neither the changes in 45S half-life nor the uptake of ^{32}P-orthophosphate could explain these data. The 45S half-life was found to decrease by around 20%, while cellular uptake of ^{32}P-orthophosphate was reduced by less than 20% after EGF stimulation (see Experimental Procedures). Since both effects tended to mask an increase in transcription rate, our measurements somewhat underestimated the true degree of stimulation of 45S transcription by EGF. The extreme rapidity of the response to EGF was completely unexpected. It compared closely with the earliest nuclear responses to growth factors, such as the activation of the c-*fos*, c-*jun*, and actin genes, which occur within 10 to 30 min of growth factor addition (see Quantin and Breathnach, 1988 and references therein). On this basis, the ribosomal genes should, in fact, be reclassified as immediate response genes.

No significant changes in cell cycle distribution of the SKF-5 cells were observed during EGF treatment. The percentage of G1 cells remained unchanged, while a modest 8% increase in G2 cells and an equivalent decrease in S phase cells was noted after 6 hr of EGF treatment (see Experimental Procedures for details). These changes were far too small to account for the 2-fold increase in transcription either by an increased gene copy number or by mitotic regulation of the PolI machinery (see Experimental Procedures). Furthermore, measurements of CDK2 and CDK4 activities showed that the latter did not change, while the former activity decreased by 20%. Since CDK2 and/or CDK4 activity is required for ribosomal gene transcription (Voit et al., 1999), these changes again tended to mask its EGF activation.

ERK Activation Is Necessary and Sufficient to Activate Ribosomal Transcription in Mammalian Cells

The rapid response to EGF suggested a direct signaling mechanism via the MAP kinase (ERK1/2) cascade. In SKF-5 cells, ERK1/2 activity was rapidly elevated 1-fold by EGF treatment and remained more than 6-fold above the basal level for 6 hr, dropping to about 5-fold by 24 hr (Figure 1B). Thus, the endogenous ribosomal transcription rate qualitatively followed ERK1/2 activity. When ERK1/2 activation was specifically blocked by the MEK inhibitors PD98059 or U0126 (Dudley et al., 1995; Favata et al., 1998), EGF activation of 45S transcription was already significantly inhibited at 5 μM PD98059 and completely inhibited by 50 μM PD98059 or at 1 μM U0126 (Figure 1C). Both PD98059 and U0126 even suppressed basal ribosomal transcription, suggesting that a level of ERK activity was necessary for its maintenance. To facilitate comparison with subsequent data, the transcriptional response of the ribosomal genes to ERK1/2 activation, or PolI activation ratio (PAR), was defined as the ratio between transcription at maximal ERK activation (i.e., here with EGF) and at minimal ERK activation (i.e., with 50 μM PD98059). One hour after EGF addition, the PAR for endogenous transcription in SKF-5 cells was 3.3 (Figure 1C). Cellular uptake of ^{32}P-orthophosphate was unaffected by PD98059. PD98059 treatment (50 μM) somewhat inhibited endogenous CDK2/4 kinases, but to a very similar level as did EGF treatment, that is, less than 30% for PD98059 as compared with 20% for EGF. Thus, changes in CDK2/4 activities did not affect the PAR and could not have explained the inhibitory effects of PD98059. It was concluded that, in the EGF-stimulated human SKF-5 cells, the ribosomal transcription rate not only qualitatively followed, but was also dependent on ERK1/2 activation. Nuclear run-on experiments in SKF-5 cells treated as in Figure 1C repeatedly gave a PAR of 1.6 (see Experimental Procedures). Thus, EGF stimulated 45S transcription in part by increasing PolI loading onto the ribosomal genes, but also by another mechanism, presumably leading to an increase in elongation and termination rates.

ERK1/2 activation was clearly necessary for endogenous ribosomal transcription activation, but was it also sufficient? Treatment of NIH3T3ΔRaf:ER cells with 4-hydroxytamoxifen (4-HT) directly induces Raf activation, resulting in the activation of ERK1/2 (Pritchard et al., 1995). Already 10 min after 4-HT addition, 45S transcription had increased by about 2 times and remained at approximately this level for the next 6 hr (Figure 1D), closely following the activated levels of ERK1/2 (Figure 1E). Inhibition of MEK with 50 μM PD98059 or 1 μM U0126 not only prevented this activation but, as in the SKF-5 cells, very significantly repressed the basal transcription rate (40%; Figure 1F), giving an overall PAR of 2.3. As seen below, dominant-negative ERK2 also eliminated the response of PolI transcription to 4-HT (Figure 5B). Pulse-chase experiments showed that the rate of 45S pre-rRNA processing remained unchanged (±3%) during 4-HT or PD98059 treatments, while cellular uptake of ^{32}P-orthophosphate remained constant (data not shown). The ΔRaf:ER cells did not detectably change their cell cycle distribution during the 1 hr incubation used in Figure 1F. CDK2 activity remained constant during 4-HT treatment, while a 40% reduction in CDK4 activ-

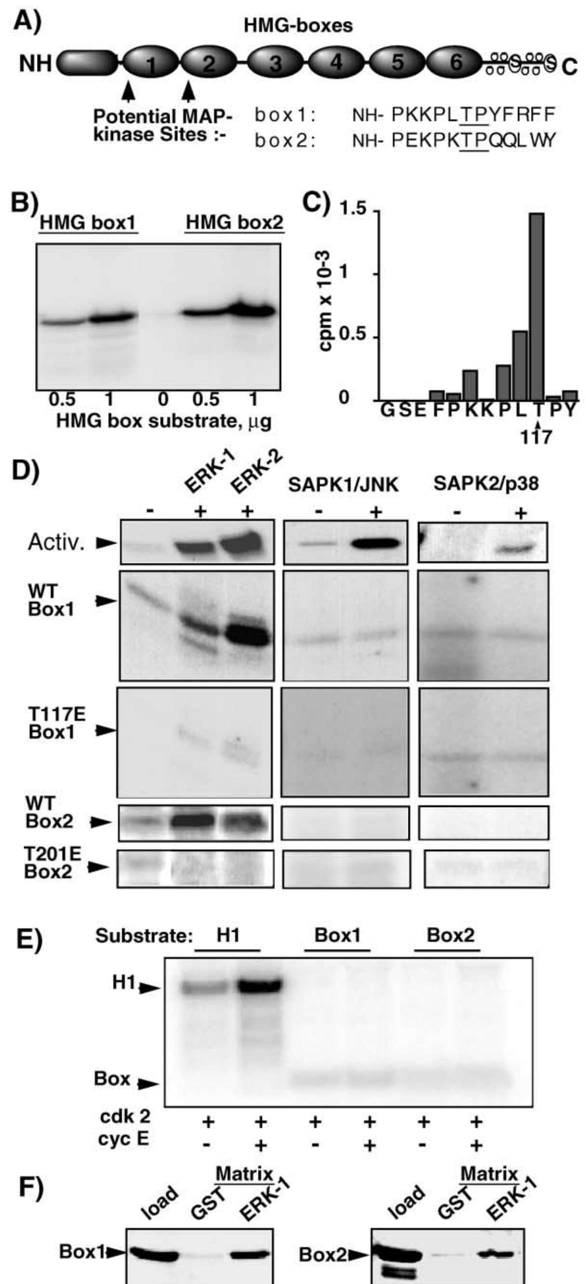

Figure 2. The MAP Kinase ERK Phosphorylates HMG Boxes 1 and 2 of UBF

(A) Schematic presentation of UBF1 and the positions of the putative MAP kinase sites in HMG boxes 1 and 2.

(B) Box 1 and box 2 were phosphorylated in vitro with [^{32}P]ATP and recombinant GST-ERK1.

(C) Box 1 was ERK phosphorylated and sequenced (see Experimental Procedures). The determined amino acid sequence is shown below a histogram of the corresponding released counts.

(D) ERK1, ERK2, SAPK1β/JNK2, and SAPK2/p38 expressed in NIH3T3 cells and activation by EGF (ERKs) or UV (SAPKs) were used to in vitro phosphorylate wt and T117E or T201E mutants of HMG boxes 1 and 2. The upper panel shows the control kinase assays, and the lower panels show the phosphorylation of the HMG box substrates.

(E) CDK2 and CDK2/cyclin E (cyc E) phosphorylation of H1 and HMG box substrates.

(F) Pull-down assays of HMG boxes 1 and 2 using ERK1 and control (GST) matrices. "Load" indicates the applied HMG box peptide.

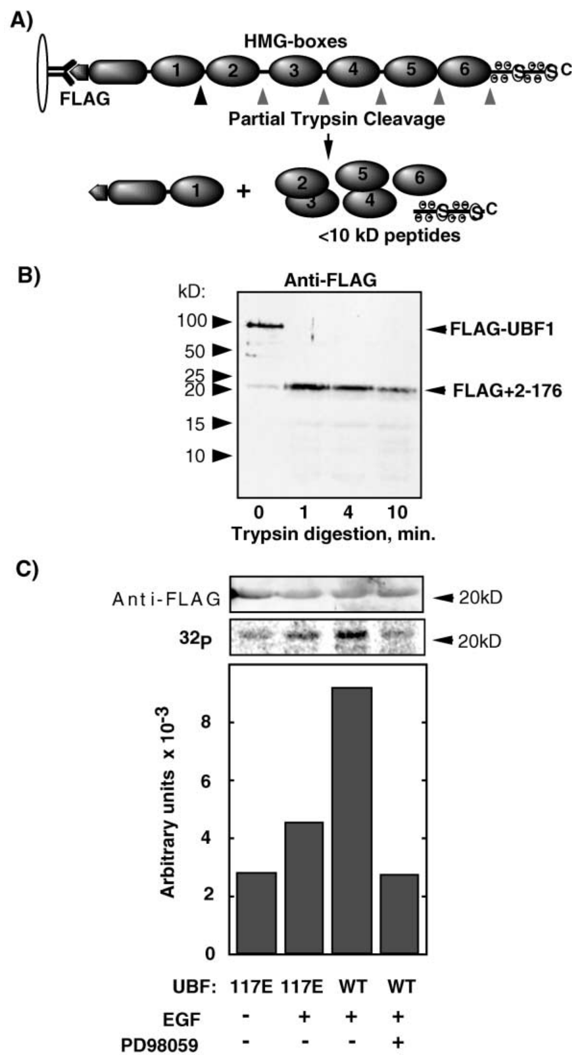

Figure 3. In Vivo Phosphorylation of UBF by ERK

(A) Schematic of experiment.

(B) Analytical time course of trypsin digestion of FLAG-UBF1. The N-terminal partial trypsin product of 22 kDa ($\pm$ 0.8 kDa) corresponded to amino acids 2–176 $\pm$ 6 of UBF1.

(C) Phosphorylation of peptide 2–176 from wt UBF1 or UBF1-T117E after treatment for 2 hr with EGF or EGF plus PD98059. The upper panel shows the FLAG-peptides, and the lower panel and histogram show the ^{32}P labeling observed by phosphoimaging.

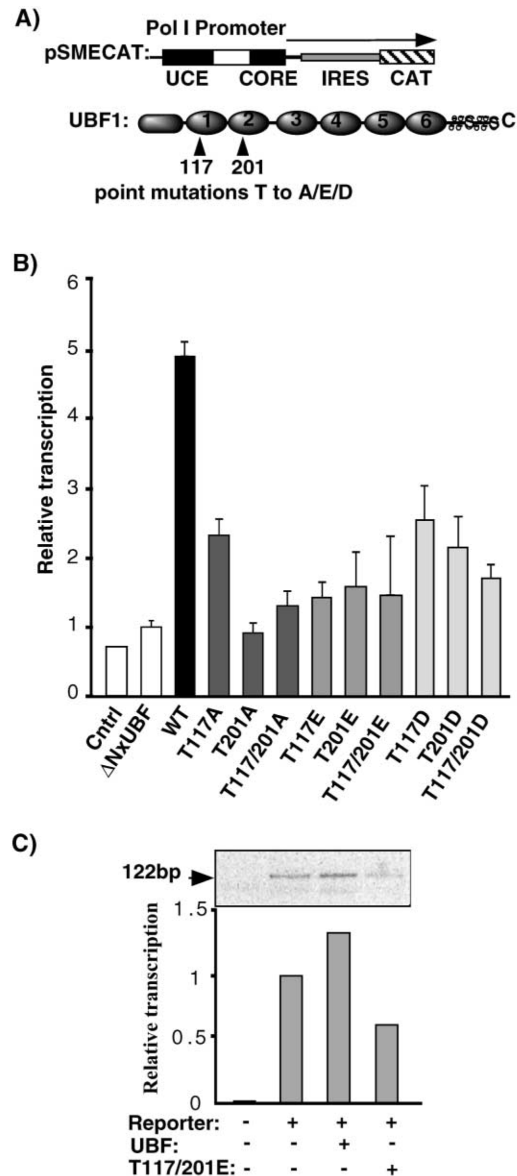

Figure 4. Transactivation of the Mouse and Human PolI Promoter by UBF1 and ERK Site Mutants

(A) Schematic of the pSMECAT reporter gene construct and the UBF1 mutants.

(B) Activation of the mouse PolI promoter in NIH3T3 cells. Cells were transfected with 0.5 μg of pSMECAT and 0.5, 1, and 2 μg of appropriate UBF vector made up to a total of 3 μg DNA with the empty pCDNA3 plasmid. The data shown is for 2 μg of UBF vector only, this being representative. PolI transcription from the pSMECAT reporter was quantified by CAT assays as previously described (Hannan et al., 1996). See Experimental Procedures.

(C) Activation of the human PolI promoter in SKF-5 cells. Cells were transfected with 4 μg of the human PolI promoter construct (pHV3CAT) and 6 μg of either wt UBF1 or T117/201E-UBF1 expression vector. The upper panel shows the results of primer extension generating the expected 122 bp fragment. The signals were quantified by phosphoimaging.

ity was observed and treatment with 50 μM PD98059 did not inhibit CDK2 and inhibited CDK4 by less than 25%. Thus, the stimulatory effects of ERK activation were, if anything, underestimated in Figure 1F. It was concluded that ERK activation was both necessary and sufficient for the activation of ribosomal transcription in mouse NIH3T3 cells.

Sustained ERK Activity Is Required to Maintain the Activated Level of Ribosomal Transcription

ERK1/2 activity could regulate ribosomal transcription at gene activation or could be required to maintain stimulated transcription. In the former case, stimulation should be maintained for some time after ERK inactivation, while in the latter case, ERK inactivation should lead to a rapid decrease in transcription. Cells were treated as in Figure 1F, but the MEK inhibitor PD98059

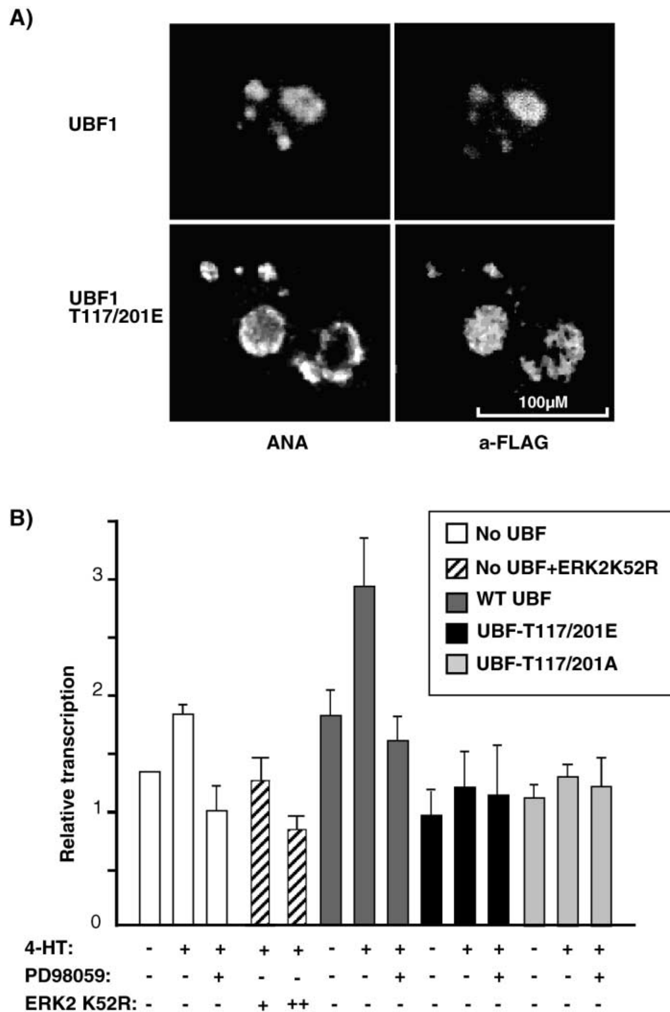

Figure 5. ERK Site Mutant UBFs Localize Correctly to the Nucleolus but Block MAP Kinase Activation of PolI

(A) Nucleoli were revealed using the ANA antibody (Sigma) while the wt and mutant UBFs were revealed using anti-FLAG antibody (a-FLAG).

(B) ΔRaf:ER cells were transfected with pSMECAT and with the empty pCDNA3 vector (No UBF), dominant-negative ERK2 (No UBF + ERK2K52R; +, 1 μg; ++, 1.5 μg), wt UBF1 (WT UBF), or the UBF1-T117/201E mutants. Each transfection was either mock treated, treated with 4-HT, or treated with both 4-HT and PD98059. The histogram presents the data from four independent experiments, each in duplicate. The expression levels of the wt and mutant UBFs were shown to be equivalent by Western blot.

was added at various times after 4-HT addition and before a 20 min pulse labeling was started (Figure 1G). Addition of the inhibitor as little as 3 min before pulse labeling reduced transcription to basal level. Identical results were obtained in EGF-stimulated human SKF-5 cells (data not shown). Thus, in both mouse and human cells, sustained ERK1/2 activity was required to maintain the activated state of the genes.

UBF Is a Target of ERK Phosphorylation Both In Vitro and In Vivo

UBF is limiting for transcription in vivo (Hannan et al., 1996, 1999b), and thus represents a potential target for regulation by ERK1/2. Inspection of the UBF sequence revealed only three possible sites for phosphorylation by ERK1/2 (PXS/TP; Aitken, 1999). One of these sites, (amino acids 482–487) is recognized by CDK2/4 (Voit et al., 1999). The other two novel sites, PLTP (amino acids 115–118) and PKTP (amino acids 199–202), are conserved in UBFs from *Xenopus* to man (Moss et al., 1998; Figure 2A). ERK1 efficiently phosphorylated HMG boxes 1 and 2 (Figure 2B). The in vitro ^{32}P-phosphorylated HMG box 1 was gas phase sequenced and the major peak of ^{32}P released with threonine 117, the predicted ERK site (Figure 2C). Both ERK1 and ERK2 were found to robustly phosphorylate HMG boxes 1 and 2, while mutation of the putative ERK sites from threonine to glutamic acid (T117E and T201E) eliminated this phosphorylation (Figure 2D). Neither stress-activated protein kinase 1/Jun N-terminal kinase (SAPK1/JNK), SAPK2/p38/Hog, nor activated CDK2/cyclin E detectably phosphorylated the HMG boxes of UBF (Figures 2D and 2E).

The MAP kinases interact with their substrates via docking sites, D domains, and F-X-F-P motifs (Sharrocks et al., 2000). A search of UBF revealed six potential D domain homologies including K-H-P-E-L-N-I, in the C terminus of HMG box 2. Three imperfect matches to the F-X-F-P motif were found, F-R-F and P-D-F-P both flanking the ERK site of HMG box 1. Pull-down assays showed that both recombinant HMG box 1 and box 2 were selectively retained on immobilized recombinant ERK1 (Figure 2F). This interaction was not inhibited by prebinding the HMG boxes with an excess of a natural UBF DNA binding sequence (data not shown).

We next asked whether UBF was also phosphorylated by ERK1/2 in vivo. SKF-5 cells, transfected with FLAG-tagged wild-type or mutant (T117/201E) UBF1, were stimulated with EGF and labeled with ^{32}P-orthophosphate, and FLAG-UBF was isolated. Tryptic peptides

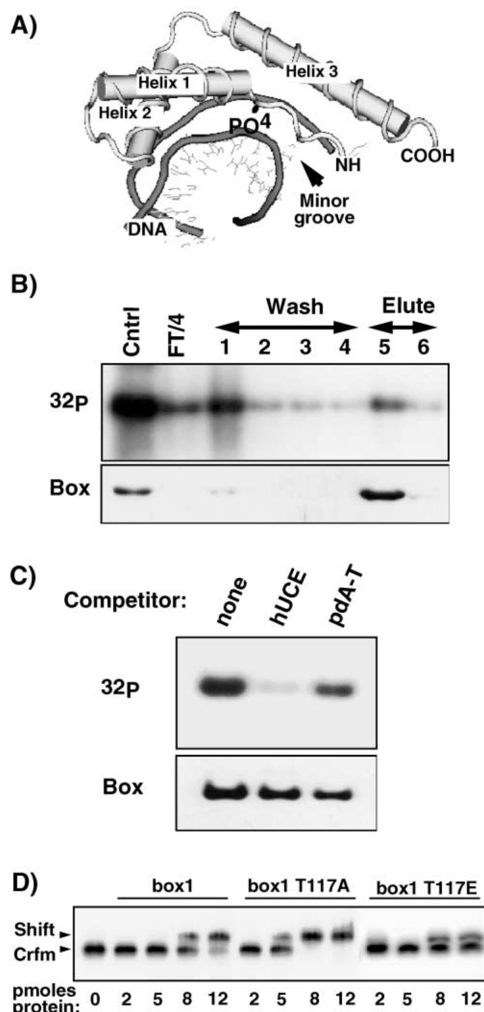

Figure 6. ERK Phosphorylation of HMG Box 1 Diminishes Its Interaction with DNA

(A) The binary structure of HMG-D (Murphy et al., 1999), indicating that ERK phosphorylation of UBF (PO_4) would lie in the minor DNA groove. The peptide chain is indicated in light gray while the DNA backbone is shown in dark gray.

(B) Phosphorylation of HMG box 1 by ERK1 prevents its binding to the human UCE promoter duplex. Cntrl, total protein load; FT/4, one quarter of the flow through; tracks 1–6, wash and elution.

(C) HMG box 1 was phosphorylated with ERK1 in the absence of DNA, none, the presence of 2 μg of hUCE duplex, or of 2 μg of poly dA-T. In both (B) and (C), ^{32}P autoradiograph is shown in the upper panel, and the Western blot box in the lower panel.

(D) DNA mobility shifts of wt and mutant (T117E/A) HMG box 1 on 100 fmoles of ^{32}P-labeled cruciform (Crfm).

of the gel-purified, endogenously labeled wild-type (wt) FLAG-UBF were fractionated by HPLC on a C18 column and peaks containing ^{32}P analyzed by MALDI-TOF (Castro et al., 1992). The HMG box ERK site tryptic peptides KPLTPYFR (amino acids 114–121) and TPQQLWYTHEK (amino acids 201–211; see Figure 2A) were both identified. To follow ERK phosphorylation of UBF after various cell treatments, homogeneous, endogenously ^{32}P-labeled wt or T117E mutant FLAG-UBFs were subjected to partial trypsin digestion to release amino acids 2–176 ± 6 of wt UBF while reducing the rest of UBF to peptides of 10 kDa and smaller (Figures 3A and 3B). This peptide, containing a unique ERK site, was separated from the other digestion products on SDS-PAGE and subjected to Western blotting and phosphoimaging. EGF-stimulated phosphorylation of this peptide was found to be reduced some 4-fold by the MEK inhibitor PD98059 and by greater than 2-fold when the threonine of the ERK site was mutated to glutamic acid (T117E; Figure 3C). Since the exogenous T117E mutant UBF heterodimerized with endogenous wt UBF (Hannan et al., 1999b), recovery of the N-terminal polypeptide from the endogenous UBF could not be avoided, explaining the residual phosphorylated peptide with T117E-FLAG-UBF (Figure 3C). Interestingly, the ratio of UBF phosphorylation with and without MEK inhibitor (Figure 3C) equaled the PAR for these cells (see Figure 1C). That is, the level of ribosomal transcription was proportional to the level of ERK-phosphorylated UBF.

Mutation of the ERK Phosphorylation Sites on UBF Inhibits Transcription Activation

PolI transcription from the pSMECAT reporter, as measured by chloramphenicol acetyltransferase (CAT) levels, faithfully reflects regulation of the endogenous ribosomal genes (Hannan et al., 1996). The ERK sites of UBF were mutated to glutamic (T117E and T201E) or aspartic acid (T117D and T201D), simulating phosphorylation, or to alanine (T117A and T201A), preventing phosphorylation. UBF1 was found to activate PolI transcription of pSMECAT some 5-fold relative to ΔN-xUBF, a control for possible squelching (Pelletier et al., 2000), while T to A mutation of HMG boxes 1 and 2 significantly reduced or abrogated this activation (0.9–2.4 times; Figure 4B). Surprisingly, the T to E and T to D mutations also severely reduce activation (1.4–2.5 times).

Overexpression of wt UBF gave only weak activation of the human PolI reporter in SKF-5 cells as estimated by primer extension (Figure 4C). However, when both ERK phosphorylation sites of UBF were mutated (T117/201E), activation was not only abolished but transcription was suppressed well below the basal level. Thus, mutation of the ERK phosphorylation sites of UBF1 abrogated or significantly reduced activation of PolI transcription both in mouse and in human cells.

UBF Phosphorylation Site Mutants Act as Dominant Negatives, Preventing the Activation of PolI Transcription by the ERK Signaling Cascade

Given that PolI transcription is exclusively nucleolar, it was possible that ERK phosphorylation was necessary for UBF targeting. However, the mutant UBFs colocalized with the nucleolar marker ANA (Sigma); see examples in Figure 5A. If, then, UBF was a direct major target for ERK1/2-dependent regulation of the PolI transcription machinery, its ERK site mutants should act as dominant negatives, rendering PolI transcription nonresponsive to ERK1/2. Mouse NIH3T3ΔRaf:ER cells were cotransfected with the wt or T117/201E and T117/201A UBFs and PolI reporter gene (pSMECAT) transcription was monitored (see Figure 5B). In the absence of transfected UBF, reporter gene transcription was activated ~1.4 times by the addition of 4-HT and inhibited to below basal level (~0.7 times) by PD98059, giving a PAR of 2,

similar to endogenous ribosomal transcription in these cells (2.3; Figure 1F). Cotransfection of an expression vector for dominant-negative ERK2 (ERK2-K52R; Frost et al., 1994; Figure 5B) and dominant-negative MEK1 (MEK1-S218/222V; data not shown) abrogated the transcriptional response of pSMECAT to 4-HT as effectively as did the MEK inhibitor PD98059.

Cotransfection of wt UBF activated PolI transcription of pSMECAT in the ΔRaf:ER cells by approximately 1.4 times (Figure 5B). This was a weaker response than for the parent NIH3T3 line (see Figure 4B), possibly due to the elevated basal level of ERK activity (e.g., see Figure 1E), or an elevated endogenous UBF level (Glibetic et al., 1995). However, addition of 4-HT caused a further activation of transcription of 1.6 times and addition of PD98059 reduced this to below the unstimulated level. The PAR after cotransfection of wt UBF was then essentially the same as that in uncotransfected cells (1.8 compared with 2 times) and again very similar to that for the endogenous genes. When the double mutants UBF-T117/201E or UBF-T117/201A were cotransfected, transcription of the reporter gene was found to be below that observed in the uncotransfected cells. In fact, the transcription level more closely resembled transcription in the uncotransfected cells treated with PD98059. Most importantly, transcription no longer responded to 4-HT nor was it repressed by PD98059. This was especially clear when the PAR values were compared. After cotransfection of UBF-T117/201E or UBF-T117/201A, the PAR was <1.1. Again, here, treatment with 4-HT or PD98059 did not induce significant changes in cell cycle distribution nor in CDK2/4 levels (see Experimental Procedures). Thus, the activation of PolI transcription via the MAP kinase cascade required the direct phosphorylation of HMG boxes 1 and 2 of UBF.

ERK Phosphorylation of the HMG Boxes of UBF Diminishes Their Interaction with DNA

The ERK phosphorylation sites of UBF lie within the N-terminal peptides of HMG boxes 1 and 2. By analogy with other HMG boxes, these peptides are probably implicated in DNA binding and lie in the minor DNA groove (Read et al., 1995; see Figure 6A). Since the sites in the HMG boxes of UBF were accessible to ERK, their phosphorylation might be expected to regulate DNA binding. To test whether this was the case, recombinant HMG box 1 of UBF was partially phosphorylated (1%–2%) with ERK1 and allowed to bind the immobilized human UCE duplex. The unphosphorylated box bound the UCE and was efficiently eluted only in 0.6 M NaCl (see lower panel in Figure 6B), while the phosphorylated box did not significantly bind to the UCE, >90% being found in the flow through (see upper panel in Figure 6B). A minor phosphorylated fraction (<10%), consistently retained by the UCE, may have represented retention via HMG box homodimerization (Bianchi et al., 1992) or nonspecific interaction with the column matrix. In vitro, phosphorylation of the HMG box was inhibited by prebinding to the UCE duplex but not to poly dA-T, to which it bound poorly (Figure 6C). Thus, phosphorylation of the ERK site of HMG box 1 abrogated interaction with DNA, while prior binding of the box to DNA inhibited ERK phosphorylation. HMG box 1 bound cruciform DNA with a K_D of 1 μM, and as expected, the T to A mutant had the same affinity (Figure 6D). However, the T to E mutation reduced the affinity for cruciform DNA by 2 to 4 times, suggesting that it partially simulated the phosphorylation event.

Discussion

Our data demonstrate that ribosomal transcription responds immediately to EGF signaling via ERK activation. The rapidity of this response is surprising and suggests a direct parallel with the stringent response in microorganisms (Nomura, 1999; Warner, 1999). ERK phosphorylation of UBF at two novel sites was found to be a necessary step in the stimulation of ribosomal transcription via the ERK pathway. Interestingly, both ERK and UBF activation of transcription was inhibited whether the ERK sites of UBF were mutated to alanine, to prevent their phosphorylation, or to glutamic or aspartic acid, to simulate their phosphorylation. Furthermore, ERK phosphorylation of UBF prevented the interaction of its two essential N-terminal HMG boxes with DNA. Thus, stimulation of ribosomal transcription by ERK would seem to require a cyclic phosphorylation event. Intriguingly, ERK phosphorylates the same two HMG boxes of UBF that recruit CBP (Pelletier et al., 2000), while the activity of CBP is enhanced by its ERK phosphorylation (Ait-Si-Ali et al., 1999; Espinos et al., 1999; Liu et al., 1999), suggesting a coordinate action of ERK and CBP.

Increased ERK activity might lead to an increase in the number of active ribosomal genes. However, preliminary experiments using differential psoralen accessibility (Conconi et al., 1989) tend to exclude this as a possibility (T.M., M. Muller, and J.M. Sogo, unpublished data). A more plausible hypothesis is that ERK phosphorylation of UBF plays a role in the initiation of PolI transcription. Two adjacent dimers of UBF are believed to bind at the PolI promoter, suggesting that they juxtapose the UCE and core elements on the surface of two consecutive *enhancesomes* (Bazett-Jones et al., 1994; Moss et al., 1998; Stefanovsky et al., 1996). While this model accounts for what is known of preinitiation complex formation, it appears incompatible with promoter clearance by the polymerase. For example, it is difficult to see how the polymerase could transcribe the first 60 or so bases downstream of the initiation site that are bound by UBF (Leblanc et al., 1993). ERK phosphorylation of UBF might, however, transiently release DNA from the grip of individual UBF HMG boxes, thereby enhancing promoter clearance. This model predicts that UBF phosphorylation would be dynamic and less than stoichiometric. Indeed, we found that on average, UBF was phosphorylated at less than one of its two ERK sites (see Experimental Procedures for details). Further, it was recently shown in vitro that PolI transcription is limited at the level of promoter clearance (Panov et al., 2001). Clearly, elucidation of the precise mechanism by which the ERK phosphorylation of UBF leads to increased ribosomal gene transcription will require significant further work. However, the present study places ribosomal transcription under the direct control of extracellular growth signaling.

Experimental Procedures

Plasmids and Constructs

Rat UBF1 and ΔNxUBF1 mammalian expression vectors and the pSMECAT reporter construct have been described elsewhere (Hannan et al., 1996, 1999b). Point mutations were introduced by PCR using mutated primers and were each sequenced. UBF HMG box 1 (amino acids 110–184 or 110–189) and box 2 (amino acids 194–266) as well as the box 1-T117E and -T117A and the box 2-T201E and -T201A mutants were expressed in *E. coli* after subcloning from *Xenopus* UBF2 into the BamHI/EcoRI site of pGEX2T (Amersham Pharmacia Biotech). Mammalian expression vectors for the MAP kinases pEXV-ERK2tag, pCDNA-HA-p38, and pMT2-HA-SAPK1β were a gift from J. Landry, CDK2 (E. Harlow) and cyclin E (R. Weinberg) were gifts from S. Meloche, pERK2-K52R (Frost et al., 1994) was a gift from N. Colburn and M. Young, and pMEK1VV (R. Erikson) and pGEX-ERK1 were gifts from S. Pelech. The pHV3CAT was a gift from L. Comai.

Tissue Culture and Transfection

NIH3T3 cells (ATCC) and neuroepithelioma SKF-5 cells were grown in Dulbecco's modified essential medium (DMEM; BRL) containing 10% fetal bovine serum (Wisent). NIH3T3ΔRaf:ER cells were grown in DMEM without phenol red. NIH3T3 cells (1×10^6) were transfected with a constant amount of 3 μg total plasmid DNA by lipofectamine (BRL) as described elsewhere (Hannan et al., 1999b; Pelletier et al., 2000). SKF-5 cells (1×10^6) were transfected by the standard $CaPO_4$/chloroquine method with a constant amount of 10 μg total plasmid DNA. Where indicated, SKF cells were treated with 50 ng/ml human EGF (BRL), and NIH3T3ΔRaf:ER cells with 1 μM 4-hydroxytamoxifen (4-HT; Sigma). The concentrations of the MEK inhibitors were 5 or 50 μM (PD98059) and 1 μM (U0126; Calbiochem). The chloramphenicol acetyltranferase (CAT) assays of pSMECAT reporter plasmid expression were performed as described (Ausubel et al., 1991; Gorman et al., 1982). Primer extension assays of pHV3CAT reporter transcription in SKF-5 cells assays were performed as described elsewhere (Kingston, 1991; Zhai and Comai, 2000).

In Vivo Pulse Labeling, RNA Extraction, and Analysis

SKF-5 cells (1×10^6 cells per petri dish) were grown for 24 hr, starved for 18 hr in serum-free medium, and then washed twice with phosphate-free medium and treated for different periods of time with human EGF. NIH3T3ΔRaf:ER cells were treated as were the SKF cells but 4-HT was added in place of EGF. Where indicated, MEK inhibitor PD98059 or U0126 was added. Labeling was performed for 20 min immediately preceding harvesting by the addition of 0.5 or 1 mCi ^{32}P-orthophosphate (NEN). The uptake of ^{32}P-orthophosphate was measured as the total ^{32}P per cell, (Granick, 1975). The RNA was extracted by the acidic phenol method (Chomczynski and Sacchi, 1987). Ten micrograms of total RNA was resolved on a 1% agarose gel, EtBr stained, and digitally recorded, and then dried and subjected to phosphoimaging using a STORM 860 (Molecular Dynamics). The 28S and 18S RNAs served as an internal control for the total amount of RNA loaded. 45S half-lives were estimated from the ratios of 45S to 32S and to 18S after a 1, 2, and 3 hr treatment of SKF-5 or ΔRaf:ER cells, respectively with EGF or 4-HT. Cells were pulse labeled as above for 20 min and then chased by replacing the medium with DMEM containing unlabeled phosphate and EGF or 4-HT.

Nuclear Run-On

The relative loading of PolI onto the ribosomal genes of SKF-5 cells was determined 1 hr after their stimulation with EGF in the presence or absence of the inhibitor PD98059 following a previously described procedure (Affolter and Ruiz-Carrillo, 1986). The RNA recovered from the run-on reaction was then hybridized in duplicate to the immobilized human 45S coding sequence DNA fragment spanning nucleotides +2086 to +4244 at 42°C in 5× SSC, 50% formamide, 0.1% SDS, and washed to a final stringency of 0.3× SSC or 0.1× SSC, at 65°C.

Flow Cytometry

Cells were stained with propidium iodide and the DNA content was estimated in an EPICS Elite ESP (Beckman Coulter) using standard techniques (Dressler, 1990). The data were analyzed with the EXPO2 Cytometer Software (Applied Cytometry Systems) imposing no constraints on any of the three cell cycle phases. The proportion of tetraploid SKF cells in G2 increased by only 8% during EGF treatment while the proportion of S phase cells decreased in proportion, with typical G1:S:G2 ratios before, 2 hr after, and 6 hr after addition of EGF being 54:45:1, 60:34:6, and 54:37:9, respectively. Eighteen hour treatment of the NIH3T3ΔRaf:ER cells with 4-HT only led to a 10% increase of cells in G1 at the expense of those in G2, while treatment with 4-HT in combination with PD98059 led to a 10% increase of cells in S phase at the expense of those G1. Typical G1:S:G2 ratios for untreated cells and cells treated with 4-HT or 4-HT plus PD98059 were 34:27:39, 46:25:29, and 24:39:37, respectively after 18 hr of treatment. These variations and the concomitant changes in gene copy number would lead to an underestimate rather than an overestimate of the effects of ERK.

Endogenous Kinase Assays and In Vitro UBF Phosphorylation Reactions

Endogenous ERK2 activities were determined as follows: cells were extracted in 100 μl MGE (20 mM MOPS [pH 7.0], 10% glycerol, 1 mM Na_3VO_4, 1 mM EDTA, 10 mM EGTA, 5 mM sodium pyrophosphate, 50 mM NaF, 80 mM β-glycerophosphate, 1% Triton X-100, 1 mM benzamidine, 1 mM DTT, 1 mM PMSF) and centrifuged at 12000 g for 10 min. Immunoprecipitations were performed on 15 μl of cell extract to which was added 45 μl MIKI (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM Na_3VO_4, 1% Triton X-100, 1 mM PMSF) and excess anti-ERK2 antibody (J. Grose). After 1 hr shaking at 4°C, 20 μl of 50% protein A-Sepharose in MIKI was added and incubated for 30 min at 4°C. The Sepharose beads were washed three times with 0.5 ml MIKI and then used for kinase assays. The 20 μl final reaction volume contained 5 μg MBP (BRL) or 2 μg homogeneous recombinant UBF HMG box 2, 150 μM [γ-^{32}P]ATP (3 μCi), 0.5× MPM_{30} (30 mM MOPS [pH 7.0], 10% glycerol, 120 mM paranitrophenyl phosphate, 30 mM $MgCl_2$, 2 mM DTT, 0.2 mM PMSF). Kinase reactions were incubated for 30 min at 30°C, SDS gel fractionated, and subjected to phosphoimaging.

Assays of endogenous CDK2/4 were performed as follows. The kinases were immunoprecipitated from cell extracts as described above using anti-CDK2 (M2) and anti-CDK4 (C22; Santa Cruz Biotechnology). The immunoprecipitated kinase was incubated for 30 min at 30°C in a 40 μl final volume with 5 μg histone H1 (Calbiochem) for CDK2 or 2 μg pRb(769) (Santa Cruz Biotechnology) for CDK4 in 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 0.5 mM DTT, 5 mM paranitrophenyl phosphate (ICN), 1 mM γ[-^{32}P] ATP (2 μCi). The reactions were gel fractionated and subjected to phosphoimaging.

A typical in vitro UBF phosphorylation reaction contained 2 μg of HMG box 1 or box 2 in 50 μl of MAP kinase buffer (12 mM MOPS [pH 7.2], 10 mM $MgCl_2$, 1 mM DTT), 10 μCi [γ-^{32}P]ATP, 50 μM ATP, and either 10 μl GST-ERK1 agarose suspension (Kinetek Biotechnology) or 20 μl of the appropriate immunoprecipitated kinase as a 50% protein A-Sepharose (Pharmacia) suspension in MIKI (see below). The reaction was incubated at 30°C for 30 min before gel fractionation and subsequent quantification by phosphoimaging.

In order to obtain the various kinases in activated form, NIH3T3 cells were transfected with the MAP kinase or with CDK2 and cyclin E or control expression vectors. After 18 hr, the kinases were activated in the cells by either (1) overnight serum deprivation followed by serum addition for 10 min for ERK1 and ERK2, (2) UV irradiation (UVP UVGL-25; 100 J/m^2) for SAPK2/p38 and SAPK1/JNK, or (3) no further treatment for CDK2/cyclin E. The cells were then extracted and immunoprecipitated as for ERK2 above except for SAPK1, where Sepharose-immobilized GST-c-Jun was added instead of protein A-Sepharose. The Sepharose beads were washed three times with 0.5 ml MIKI and then used for kinase assays with specific substrates. For SAPK2: 20 μl final reaction volume containing 1 μg GST-ATF-2, 75 μM [γ-^{32}P]ATP (3 μCi), 0.5× MPM_{45} (as MPM_{30} but 45 mM $MgCl_2$); for SAPK1: 20 μl final reaction volume containing 150 μM [γ-^{32}P]ATP (3 μCi), 0.5× MPM_{30}; for ERK-1 and ERK-2: 20 μl final reaction volume containing 5 μg MBP (BRL), 150 μM [γ-^{32}P]ATP (3 μCi), 0.5× MPM_{30}; for CDK2/cyclin E: 40 μl final volume containing 5 μg histone H1 (Calbiochem) or 2 μg UBF HMG box 1 or box 2 and 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 0.5 mM DTT,

5 mM paranitrophenyl phosphate (ICN), 1 mM [γ-^{32}P]ATP (2 μCi). All kinase reactions were incubated for 30 min at 30°C, gel fractionated, transferred to nitrocellulose, and subjected to phosphoimaging.

Phosphopolypeptide Sequencing
The phosphorylated recombinant HMG box was covalently attached to Sequelon-AA (Millipore) by carbodiimide coupling. The sequencing was performed on an Applied Biosystems 470A protein sequencer using 90% aqueous acetonitrile as the anilinothiazolinone extraction solvent (Shively et al., 1987; Xu and Shively, 1988). Each total cycle product was used for Cerenkov cpm measurement and then PTH-amino acid analysis.

Pull-Down Assays
Recombinant GST-ERK1 was expressed in *E. coli* and bound to G-Sepharose. Recombinant UBF HMG box 1 or box 2 was applied to 5 μg of immobilized GST-ERK-1 or GST in HBS (50 mM HEPES [pH 7.9], 150 mM NaCl) and incubated for 1 hr at 4°C. After three washes with HBS, the matrix-bound proteins were resolved on 18% Tris-Tricine SDS-PAGE, and then gel transferred to nitrocellulose and stained with SYPRO Ruby protein blot stain (Molecular Probes) according to the manufacturer's protocol. The membrane was then analyzed by blue fluorescent imaging (STORM 860; Molecular Dynamics).

In Vivo Phosphorylation of UBF1
UBF1 and UBF1-T117/201E were expressed in SKF-5 cells (see above). The cells were labeled with 3 mCi ^{32}P-orthophosphate (NEN) in phosphate-free medium for 3 hr. Crude nuclear extracts were prepared from cell pellets by homogenization in 10 mM Tris-HCl [pH 7.9], 0.3 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1% NP-40, 0.1% Triton X-100, 1 mM DTT, 0.1 mM EDTA, and the inhibitors 100 μM Na_3VO_4, 50 mM NaF, and 1 μg/ml each leupeptin and pepstatin (Roche). After a brief centrifugation, the nuclear pellets were resuspended in 0.42 M NaCl, 50 mM Tris-HCl (pH 7.9), 12.5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, plus inhibitors. After the addition of 1/8 volume 8 M urea, the samples were briefly sonicated and centrifuged in an SW 50.1 rotor (Beckman) for 30 min at 30000 rpm. The supernatants were incubated with 50 μl of M2 anti-FLAG affinity matrix (Sigma), and the FLAG-UBF1 protein was recovered according to the manufacturer's protocol. The labeled UBF1 was then analyzed by one of two approaches: (1) after standard SDS-PAGE fractionation, it was subjected in gel to total tryptic digestion. The peptides were then fractionated by reverse phase HPLC on a C18 microbore column and radioactive fractions were analyzed by MALDI-TOF mass spectrometry (Eastern Québec Proteomic Service), or (2) a limited digest of the labeled UBF protein with 10 μg of trypsin (Sigma) was performed on ice for 3–5 min. The products were then separated by 5%–15% Tris-Tricine SDS-PAGE, transferred to a nitrocellulose filter, subjected to phosphoimaging, and probed with anti-FLAG antibody.

It was estimated by phosphoimaging that no more than 2% of the total ^{32}P counts incorporated in vivo into FLAG-UBF were recovered with the N-terminal peptide. UBF has 21 potential CKII sites in the C-terminal acidic domain and several other phosphopeptides have been detected (O'Mahony et al., 1992a; Voit et al., 1992, 1999). A gross upper limit for the number of UBF phosphorylation sites, including the ERK sites, therefore lies at around 24. Assuming all sites were equally modified, each site should have accounted for 4% of UBF counts, while if fewer than 24 sites were actually modified, or some sites were only partially modified, the percentage of counts per stoichiometrically modified site should increase in inverse proportion. On this basis, our measurements showed that the ERK site of HMG box 1, accounting as it did for only 2% of the total counts, was at most only 50% phosphorylated even when ERK was maximally activated.

Immunocytochemistry
NIH3T3 cells were transfected with UBF1, UBF1-T117E, UBF1-T201E, or UBF1-T117/201E in 4 mm^2 chamber slide wells and immunostained as described elsewhere (Hannan et al., 1999b). Specimens were viewed on a Bio-Rad MRC-1024 confocal imaging system and treated with CytoFish and EDIT3D software developed at RFMQ, Grenoble (see Panse et al., 1999).

DNA Binding Experiments
The human UCE was affinity labeled with Klenow polymerase and biotin-7 dATP (BRL) and bound to streptavidin-agarose (BRL). Typically, 0.5 μg of in vitro-labeled protein was then incubated with 50 μl of this streptavidin-agarose-hUCE affinity matrix in 100 μl binding buffer (80 mM NaCl, 50 mM Tris [pH 7.9]) for 2 hr at 4°C. After three washes in binding buffer, the protein was recovered with 0.6 M NaCl and gel fractionated, transferred to a nitrocellulose membrane, autoradiographed, and probed with anti-HMG box 1 antibody. Cruciform DNA mobility shift assays were performed as previously described (Pöhler et al., 1998).

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