DNA-binding activity of the transcription factor upstream stimulatory factor 1 (USF-1) is regulated by cyclin-dependent phosphorylation

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The ubiquitous transcription factor upstream stimulatory factor (USF) 1 is a member of the bzHLH (leucine zipper-basic-helix-loop-helix) family, which is structurally related to the Myc family of proteins. It plays a role in the regulation of many genes, including the cyclin B1 gene, which is active during the G2/M and M phases of the cell cycle and may also play a role in the regulation of cellular proliferation. We show that the affinity of recombinant USF-1 for DNA is greatly increased by treatment with active cyclin A2–p34^{cdc2} or cyclin B1–p34^{cdc2} complexes and that its interaction with DNA is dependent on p34^{cdc2}-mediated phosphorylation. We have localized the phosphorylation site(s) to a region that lies outside the minimal DNA-binding domain but overlaps with the previously identified USF-specific region. Deletion studies of USF-1 suggest that amino acids 143–197 regulate DNA-binding activity in a phosphorylation-dependent manner.

Key words: cdk1, cyclin, DNA-binding domain, USF, USR.

INTRODUCTION

The bzHLH (leucine zipper-basic-helix-loop-helix) transcription factor upstream stimulatory factor (USF) 1 was originally identified as an activator of the adenovirus major late promoter (AdMLP). It is distributed ubiquitously and is now known to play a role in the transcriptional regulation of many genes. Two gene products, the 43-kDa USF-1 and 44-kDa USF-2, have been identified. They are highly related except at the N-terminus ([1] and references therein). USF is structurally related to the Myc family of proteins and normally binds to an E-box as a heterodimer of USF-1 and USF-2 ([1] and references therein). USF is known to play an important role in a diverse array of cellular processes and in particular may play a role in the regulation of cellular proliferation, being involved in regulation of genes such as *p53* [2], *cdc2* (where cdc is cell division control) [3] and the cyclin B1 gene [4]. Additionally, USF is also involved in modulation of *ras* and c-*myc* transformation [5,6] and regulation of the HIV-1 long terminal repeat (LTR) promoter [7]. Both of these latter processes require physical interaction with other proteins via the DNA-binding domain. A recent report has shown that the activity of USF is modulated in a cell-specific manner [8]. This regulation depends on a short and unique sequence between the N-terminal transactivation domain and the DNA-binding domain known as the USF-specific region (USR), which plays an essential role in transactivation and nuclear localization [1].

USF has been implicated in regulation of the cyclin B1 gene, which is transcriptionally up-regulated prior to and during mitosis [4,9]. One report indicates that cell-cycle-specific regulation of the cyclin B1 promoter is dependent on a binding site for the transcription factor USF in its promoter and that the DNA-binding activity of USF is up-regulated at G2/M [4]. However, another report has shown that cell-cycle-regulated transcription of the cyclin B1 gene is mediated by a promoter deletion to position −90, which does not include the USF-binding site [9]. It is difficult to reconcile these reports, although obvious differences in methodology may account for the conflict. The USF-binding site becomes occupied by Max when cells enter G_0 [10]. Thus occupation of this site during G2/M may prevent other negatively acting factors from binding. We were interested in the mechanisms that regulate the ability of USF-1 to bind to DNA in a cell-cycle-specific manner. We demonstrate here that transcriptional activity of USF-1 is enhanced by mitotic cyclins and provide evidence that this is mediated by an increased affinity of USF-1 for DNA, which is regulated reversibly by phosphorylation. The phosphorylation site has been localized to a region encompassing the USR.

Progression through the cell cycle in all eukaryotes is driven by a family of kinases comprising a catalytic cyclin-dependent kinase (cdk) and regulatory cyclin ([11] and references therein). Levels of cdk subunits are constant throughout the cell cycle, whereas cyclin levels change periodically as a result of variations in gene transcription and by selective proteolysis. Progress through the S and M phases is regulated by different subsets of cyclin–cdk complexes in higher eukaryotes. Functional specificity of cyclin–cdks may be imparted through either altered substrate specificity of different cdk–cyclin pairs or by differences in subcellular localization of cyclins (reviewed in [12]).

Regulation of G1/S-specific transcription has been studied extensively, since this is the major point at which cell proliferation, differentiation and other environmental cues are integrated. During G2/M, which is less thoroughly characterized, it has been shown that RNA polymerase III transcription is repressed [13]. Mitotic repression of RNA polymerase III transcription has been shown to be due to the activity of a secondary kinase further down the hierarchy towards cyclin B1–p34^{cdc2} ([13]). The target for p34^{cdc2}-mediated phosphorylation was shown to be transcription factor TFIIIB [14] which, like the RNA polymerase II basal factor TFIID, is a complex of TATA-binding protein ('TBP') and TBP-associated factors ('TAFs'). Recent studies have shown that RNA polymerase II transcription

Abbreviations used: USF, upstream stimulatory factor; USR, USF-specific region; AdMLP, adenovirus major late promoter; LTR, long terminal repeat; cdk, cyclin-dependent kinase; NTA, nitrilotriacetic acid; DTT, dithiothreitol; USE, upstream sequence element.

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is globally repressed during mitosis in a similar manner by cdk-dependent phosphorylation of the TFIID complex [15].

Current knowledge of gene regulation during the G2 and M phases derives from the study of the cyclin A and *cdc25* genes, which are activated during S/G2 [16], and cyclin B1, which is activated during G2/M.

MATERIALS AND METHODS

Cell culture, synchronization and cell-cycle analysis

HeLa S3 cells were grown in Spinner medium (Gibco-BRL) containing 2 mM L-glutamine supplemented with 10 % foetal calf serum, streptomycin, penicillin and non-essential amino acids. Cells were incubated at 37 °C in 5 % CO_2 atmosphere. For synchronization, cells were treated with 400 μM mimosine for 16–24 h to obtain cells blocked at G1/S. Cells blocked in mitosis were obtained by treatment with 0.04 μg/ml nocodazole for 16–24 h. For cell-cycle analysis, cells were collected, washed in PBS, resuspended in 30 % PBS/70 % ethanol solution and stored at 4 °C until analysis. Prior to analysis, cells were treated with RNase A and stained with propidium iodide. Cell-cycle analysis was performed on a Becton-Dickinson FACScan cytofluorimeter and data were interpreted using the CellFIT cell-cycle-analysis program.

Recombinant USF

Recombinant full-length USF-1, used for initial experiments, was purified from *Escherichia coli* essentially as described by Pognonec and Roeder [17], except a further purification step on heparin Ultrogel (IBF) was included after ammonium sulphate precipitation. Recombinant USF-1 was judged to be 90–95 % pure after SDS/PAGE analysis.

USF deletions

Deletions of USF (as detailed in Figure 5, see below), were constructed by PCR amplification of USF-1 cDNA (a generous gift from Dr. R. Roeder, Rockefeller Institute, New York, NY, U.S.A.) using appropriate primers, then cloned into the expression vector pET30a (Novagen), which contains an N-terminal polyhistidine tag. Recombinant proteins were overexpressed and affinity purified on Ni-NTA (nitrilotriacetic acid)–agarose as described in the manufacturer's (Qiagen) supplied protocols. Recombinant USF-1 was judged to be 90–95 % pure after SDS/PAGE analysis.

In vitro transcription assay

Interphase *Xenopus* extracts were prepared as described in [18]. Templates for the *in vitro* transcription reactions, pTATAG-(393) and $pMLC_2AT$, were obtained from Dr. Hinrich Gronemeyer (IGBMC, Strasbourg, France) and Dr. R. Roeder respectively. *In vitro* transcription reactions were performed as described in [19]. Briefly, 100 μg of extract were pre-incubated at 21 °C with 200 ng of template in a final volume of 25 μl containing 50 mM Hepes/KOH (pH 7.8), 50 mM KCl, 0.5 mM EDTA, 10 % glycerol and 1 mM dithiothreitol (DTT). To convert interphase extracts to a mitotic state, 1 μg of purified recombinant sea urchin cyclin B(Δ90) or bovine cyclin A2 (generous gifts from Dr. Paul Clarke, University of Dundee, Dundee, U.K.) was added to extracts for 1 h prior to beginning the transcription reaction. Reactions were pre-incubated for 10 min and transcription was initiated by addition of 5 mM $MgCl_2$/0.4 mM ATP/0.4 mM CTP/10 μCi of [α-^{32}P]rUTP (800 Ci/mmol, 10 mCi/ml; NEN)/4 mM phosphoenolpyruvate/0.2 units of pyruvate kinase (Boehringer Mannheim)/4 units of RNasin (Boehringer Mannheim). Transcription was carried out at 21 °C for 30 min and terminated by addition of 40 units of RNase T1 (Boehringer Mannheim) in 200 μl of 10 mM Tris/HCl (pH 7.5)/5 mM EDTA/300 mM NaCl for 20 min at 25 °C, followed by treatment with 200 μg of Proteinase-K (Boehringer Mannheim) in 0.05 % SDS for 30 min at 30 °C. Labelled transcripts were precipitated in ethanol and separated by electrophoresis in a 6.5 % polyacrylamide/urea gel. Gels were dried and autoradiographed.

DNase I footprinting

HeLa nuclear extracts, prepared from either synchronized or unsynchronized cultures, were prepared as described in [20]. Probes for DNase I footprinting were prepared by PCR with a ^{32}P-end-labelled oligonucleotide corresponding to regions of the human cyclin B1 promoter (as detailed in Figure 2, see below). A typical reaction contained 50000 c.p.m. of end-labelled probe incubated with 50 μg of nuclear extract in a final concentration of 20 mM Hepes/KOH (pH 7.9)/50 mM KCl/1.5 mM $MgCl_2$/5 % glycerol/0.2 mM EDTA/0.5 mM DTT/2 μg of poly(dI-dC)·poly(dI-dC) (Pharmacia)/0.5 mM PMSF. Following 30 min of incubation at 20 °C, the samples were treated with 0.025–1.6 units of DNase I (Boehringer Mannheim) for exactly 2 min and then stopped with addition of Stop buffer [0.1 % SDS/150 mM NaCl/10 mM Hepes/KOH (pH 7.9)/5 μg of tRNA]. The samples were extracted with phenol/chloroform, and DNA was precipitated with 2 vols. of 100 % ethanol, washed with 70 % ethanol and air dried. The pellets were resuspended in sequencing buffer and analysed by electrophoresis on an 8 % denaturing gel alongside a dideoxy sequence of the probe, generated using the same oligonucleotide that was labelled in the PCR as a primer. The gel was exposed to X-ray film with an intensifying screen at −80 °C overnight.

Gel mobility-shift assays

Each reaction containing either 5 μg of nuclear extract or 10 ng of pure USF-1 in 10 mM Tris/HCl (pH 7.5)/50 mM NaCl/5 % (v/v) glycerol/0.1 mM EDTA/1 mM DTT/1 μg of poly(dI-dC) was incubated on ice for 10 min and then equilibrated to room temperature. ^{32}P-End-labelled oligonucleotide probe (10000 c.p.m.) was added and incubation was continued for a further 20 min. Samples were subjected to electrophoresis on a 6 % non-denaturing polyacrylamide gel run in 0.5 × TBE (where 1 × TBE = 45 mM Tris/borate/1 mM EDTA). The gel was dried and then autoradiographed for visualization of protein–DNA complexes. The following oligonucleotides were used as probes: cyclin B1 E-box, 5′-gatcAGAGGCAGACCACGTGAGAGCCTGG-3′; AdMLP-USE (upstream sequence element), 5′-gatcGGTGTAGGCCACGTGACCGGGTGTTCCTGA-3′; and Sp1, 5′-GTCACCGAGGCCCCGCCCCTCCGGCGCGA-3′, where lower-case sequence indicates non-complimentary single-stranded 'sticky' ends to facilitate cloning.

In vitro phosphorylation assay

Cdc2 kinases were prepared and purified by incubation of either *Xenopus* glutathione S-transferase–cyclin B1 or A2 with *Xenopus* egg extracts as described in [21] and purified by glutathione S-transferase-affinity chromatography [22]. We verified, using monoclonal antibodies specific for cdc2 and cdk2, that the cyclin A2 kinase was 80 % cdc2 and 20 % cdk2 (results not shown).

USF-1 (1 μg) was phosphorylated *in vitro* by cdc2 kinases (around 6–8 pmol of PO_4^{3-} transferred to histone H1/μl per min) for 15 min at 37 °C in 80 mM β-glycerophosphate (pH 7.3)/20 mM EGTA/15 mM $MgCl_2$/1 mM DTT/1 mM ATP/0.5 μCi [γ-^{32}P]ATP (4500 Ci/mmol; ICN)/0.5 mM PMSF. The reaction mix was either used immediately for mobility-shift analysis or separated on an SDS/PAGE gel and exposed to a Fuji PhosphorImager plate-type BAS III. The data were read and analysed on a Fuji BAS 2000 PhosphorImager.

Acid phosphatase treatment

Potato acid phosphatase (Boehringer Mannheim) was dialysed against two changes of 500 vols. of 50 mM Pipes/KOH (pH 6.5). For phosphatase treatment of 2 μg of mitotic HeLa nuclear extracts, 0.1 units was used at 37 °C for 60 min. Samples were used subsequently for gel-retardation assays except that end-filled DNA probes were used. In all cases, experiments were performed at least three times and representative Figures are shown.

RESULTS

USF transcriptional activity *in vitro* is enhanced in mitotic extracts

Initially, we sought to find out whether activated RNA polymerase II transcription could be modulated *in vitro* by mitotic kinases. Crude extracts from interphase *Xenopus* oocytes have been used previously to study cell-cycle-regulated transcription from both RNA polymerase II and RNA polymerase III promoters [23]. We have found that crude S100 *Xenopus* oocyte extracts can support RNA polymerase II-specific transcription, although basal levels of transcription were very low (Figures 1A and 1B, lanes 1, 2, 5 and 6). Transcription from all RNA polymerase II templates is stimulated efficiently by addition of recombinant USF-1 in interphase extracts (Figures 1A and 1B, compare lanes 1, 2, 5 and 6 with lanes 3, 4, 7 and 8), including that from a TATA-containing promoter with no USE. This may be due to the interaction of USF with the Inr (initiation) element, contained in all templates used [24]. Stimulation of transcription by recombinant USF-1 was enhanced in *Xenopus* extracts treated with recombinant cyclins A or B1, by 2.1- and 3.2-fold respectively, from only USE-containing templates and not from an internal control template lacking a USE site (Figures 1A and 1B, compare lanes 3 and 4). The effect of the cyclins correlated with repression of RNA polymerase III transcription from the yeast $tRNA^{leu}$ promoter (Figure 1A, lanes 9 and 10) and phosphorylation of histone H1 kinase in the same treated extracts (results not shown). Thus whereas USF generally stimulated transcription from all templates in *Xenopus* extracts, enhanced stimulation in mitotic extracts was only seen in USE-containing templates.

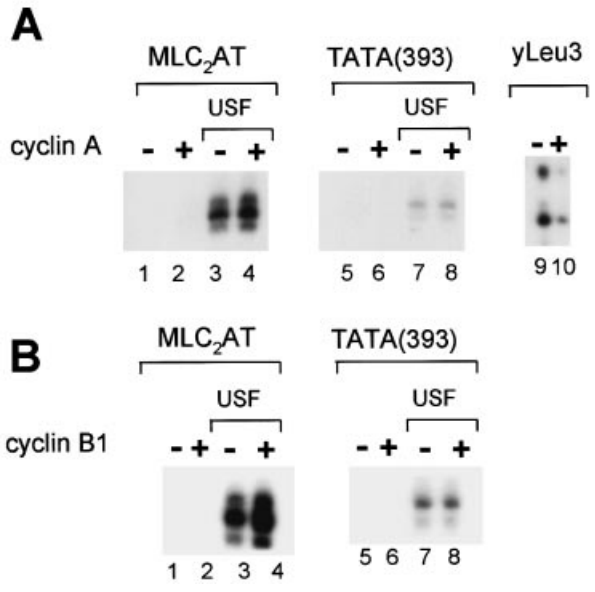

Figure 1 USF-1 activates transcription *in vitro* in a cell-cycle-regulated manner

In vitro transcription from G-less cassette TATA-G (−393) and AdMLP-containing pC_2AT templates in *Xenopus* interphase extracts, with added recombinant USF-1 (100 ng) where indicated, after activation with (**A**) cyclin A or (**B**) cyclin B1. yLeu3, yeast leu3 tRNA gene promoter upstream of a conventional run-off template.

USF binds to DNA in a cell-cycle-specific manner

It was reported that the cyclin B1 gene promoter contains an upstream E-box capable of binding USF, and that 5′ deletions of the promoter that do not contain this E-box are not regulated in a cell-cycle-specific manner [4]. USF-1 may therefore play a role in regulation of the cyclin B1 gene, which is at its most active during G2/M. We therefore set out to investigate further the manner in which the activity of USF-1 is regulated during the cell cycle.

Using nuclear extracts made from synchronized HeLa cells, DNase I footprinting studies demonstrated protection of a proximal E-box-containing motif between residues −160 and −178 with mitosis-synchronized extracts (Figure 2A, lane 4), but not with G1/S-phase or unsynchronized extracts (Figure 2A, lanes 2 and 3). Recombinant USF-1 also protected the same motif, but significant quantities of the protein were required to bind to this site (Figure 2A, lane 6). This may indicate that affinity of bacterially expressed recombinant USF-1 for DNA is relatively low for binding to this site under conditions used in our assay, or that additional factors are required for efficient binding.

These observations were confirmed by gel-shift analysis of protein binding to this site (Figure 2B). A single retarded band was seen when an oligonucleotide representing the E-box motif in the cyclin B1 promoter was incubated with nuclear extracts made from HeLa cells blocked in mitosis, but no retarded bands were visible when G1/S or unsynchronized extracts were used (Figure 2B, lanes 1–3). In parallel with this we carried out binding studies using the AdMLP USE element as a probe (Figure 2B, lanes 4–6). Essentially these data confirmed those from the previous experiment, in that a factor bound to the E-box with the same cell-cycle specificity and mobility. Protein binding to a probe containing the Sp1-binding site did not change according to the cell-cycle stage of the nuclear extracts (Figure 2B, lanes 7–9), verifying that the effect of cell-cycle stage on USF-1 binding was specific. To verify the identity of protein binding to either the AdMLP or cyclin B1 E-box in crude mitotic extracts, we performed a supershift assay using two different antibodies specific for USF-1, confirming that USF-1 occupies the cyclin B1 gene E-box during mitosis (results not shown). This is in agreement with previous published results [4].

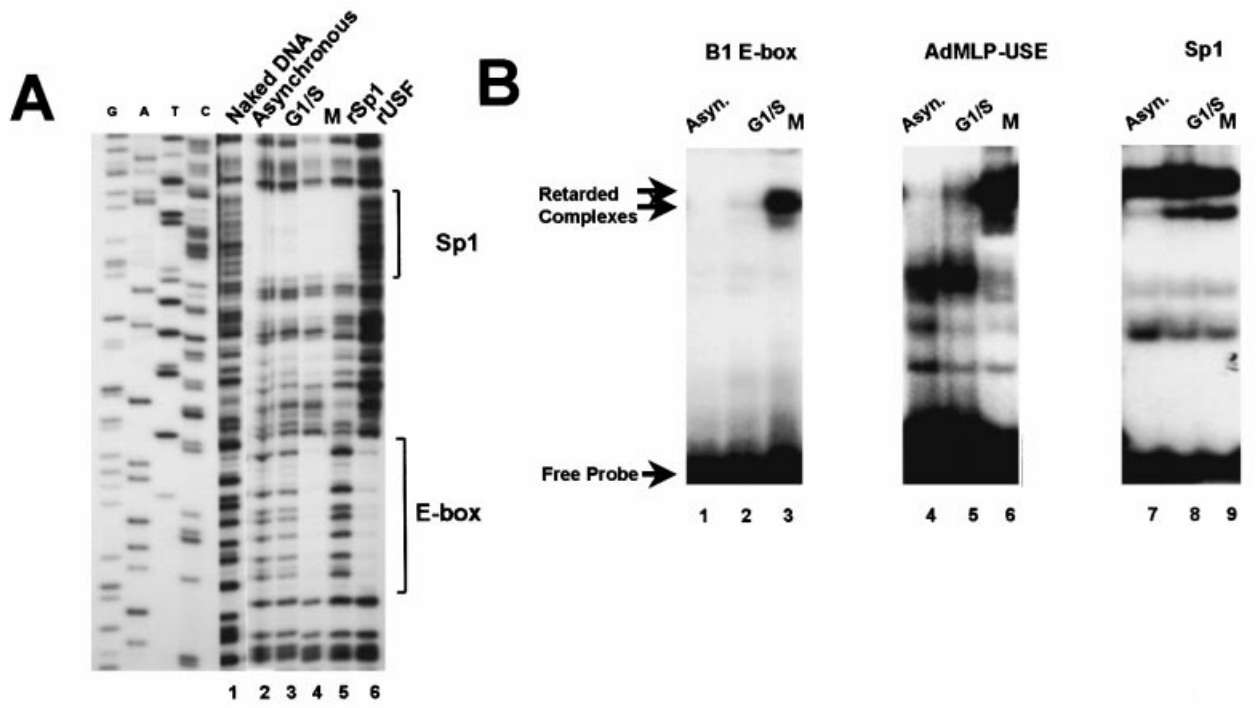

Figure 2 DNA binding of USF-1 is cell-cycle-regulated

Synchronized nuclear extracts were prepared from HeLa S3 cells and USF-1-binding activity was measured by DNase I footprint and mobility-shift assays. (**A**) DNase I footprinting of the human cyclin B1 promoter was carried out in the absence (lane 1) or in the presence of unsynchronized (lane 2), G1/S (lane 3) or mitotic (M; lane 4) nuclear extracts, 10 ng of recombinant (r) Sp1 (lane 5) or 100 ng of recombinant USF-1 (lane 6). (**B**) Mobility-shift assays were carried out in unsynchronized (Asyn.), G1/S or mitotic HeLa S3 nuclear extracts with oligonucleotide probes representing cyclin B1 E-box (lanes 1–3), AdMLP-USE (lanes 4–6) and a canonical Sp1-binding site (lanes 7–9).

USF-1 binding to DNA is regulated by cdc2-dependent phosphorylation

A logical mechanism whereby the DNA-binding activity of USF-1 could be altered in a cell-cycle-dependent fashion is by phosphorylation, most probably by cyclin–p34^{cdc2} complexes themselves. To test this hypothesis we attempted to label recombinant USF-1 with ^{32}P by incubation with active cyclin A2– or cyclin B1–p34^{cdc2} complexes (Figure 3, lanes 1 and 2 and lanes 3 and 4 respectively). This experiment showed that USF-1 is a good substrate for p34^{cdc2}-dependent phosphorylation, at least comparable to an equal amount of histone H1 (for comparison see Figure 6D, below).

In order to analyse the effect of cdc2-mediated phosphorylation on DNA binding, the phosphorylated USF-1 was tested in a gel mobility-shift assay (Figure 4A), which shows that the DNA-binding affinity of USF-1 phosphorylated by both cyclin A2–p34^{cdc2} (lanes 1 and 2) and cyclin B1–p34^{cdc2} (lanes 3 and 4) is increased dramatically compared with untreated USF-1. To further demonstrate that USF-1 DNA binding is mediated by phosphorylation, mitotic HeLa nuclear extracts were treated with potato acid phosphatase and then subjected to gel mobility-shift assay with an end-filled probe spanning the cyclin B1 E-box region. As shown in Figure 4(B), a potato acid phosphatase-treated extract lost its ability to bind to the probe compared with non-treated extracts. Together, these results show clearly that the DNA-binding activity of USF-1 is regulated in a cell-cycle-dependent manner, and also that the protein is phosphorylated *in vivo*. The possibility that cell-cycle-dependent DNA-binding activity is regulated by cdc2-dependent phosphorylation would be consistent with our observations thus far, but would not rule out other indirect means of regulation.

The USR is the target for cyclin-specific phosphorylation

We attempted to determine sites of cdc2-mediated phosphorylation in USF-1 by examining the capacity of a series of N- and C-terminal deletion mutants (Figure 5) to bind to DNA and to act as substrates for cdc2-mediated phosphorylation. Deletion mutants of USF were prepared as His-tag fusions (Figures 5A and 5B). In all cases, activity of the mutants was compared with a His-tagged fusion of full-length USF-1 to control for any effect of the tag. Deletions were incubated with both cyclin A2–cdc2 and cyclin B1–cdc2. For both kinases, efficient phosphorylation of the full-length and the 143–310 mutant (his$_6$-USF$\Delta^{143–310}$) was observed consistently (Figures 5C and 5D, lanes 1 and 4), whereas the 1–143 mutant (his$_6$-USF$\Delta^{1–143}$) was not phosphorylated by either kinase (Figures 5C and 5D, lane 3). The effect of cyclin–cdc2-mediated phosphorylation on the 1–196 (his$_6$-USF$\Delta^{1–196}$) and 197–310 (his$_6$-USF$\Delta^{197–310}$) mutants was dependent on the cyclin used. his$_6$-USF$\Delta^{1–196}$ was a poor substrate for cyclin A2–cdc2, whereas cyclin B1–cdc2 phosphorylated it as efficiently as full-length USF-1 or his$_6$-USF$\Delta^{144–310}$. The reasons for this discrepancy may be due to differences in the ability of the cyclin–cdc2 kinases to access phosphorylation sites in mutant USF molecules. his$_6$-USF$\Delta^{197–310}$ was not phosphorylated by cyclin A2–cdc2 kinase. However, it was a substrate for cyclin B1–cdc2 kinase, albeit at a level lower than that observed for full-length USF or his$_6$-USF$\Delta^{1–196}$. This difference may also be due to

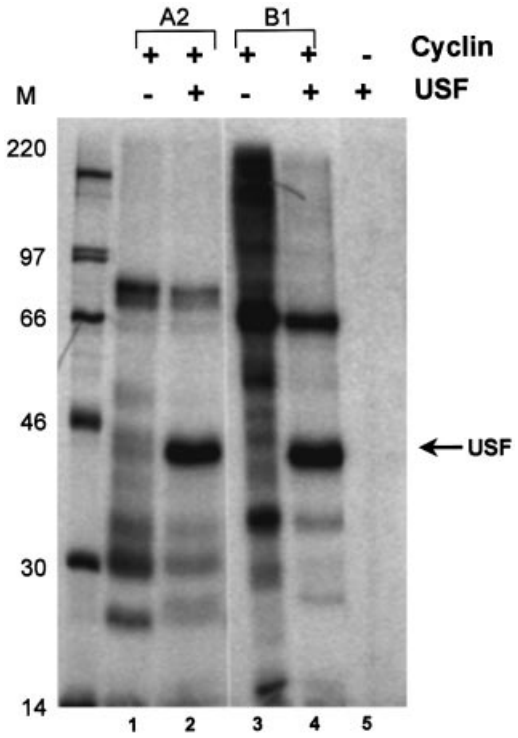

Figure 3 ***In vitro*** **phosphorylation of purified USF-1 by cdc2 kinases**

Purified *Xenopus laevis* cyclin A2–p34^{cdc2} (lanes 1 and 2) or cyclin B1–p34^{cdc2} (lanes 3 and 4) kinases were used to phosphorylate purified USF-1 with or without the addition of the indicated kinases, as denoted in the Figure. Lane 5, USF only. Note that both cyclin A2 and cyclin B1 are themselves autophosphorylated.

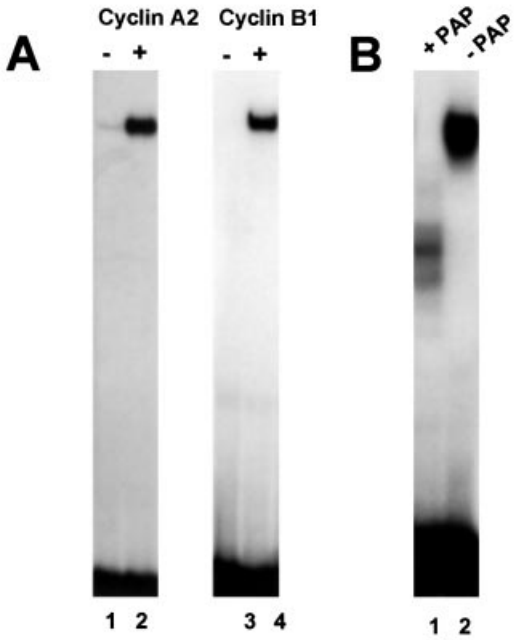

Figure 4 USF-1 binding to DNA is regulated by phosphorylation

(**A**) DNA-binding activity of USF-1 in the presence of cdc2 kinases. Gel-shift assays were as described in Figure 1. USF-1 was incubated in either the absence or presence of cyclin A–cdc2 (lanes 1 and 2) or cyclin B–cdc2 (lanes 3 and 4). (**B**) Mitotic HeLa S3 nuclear extract was treated in the absence (lane 1) or presence (lane 2) of potato acid phosphatase (PAP). The treated extracts were then used in a gel mobility-retardation assay with the ^{32}P-labelled cyclin B1 E-box probe.

differences in the ability of each kinase to access phosphorylation sites or, more likely, because there are no consensus p34^{cdc2} phosphorylation sites in USFΔ^{1-196}, that cyclin B1–cdc2 preparations contain low levels of non-cdk contaminating kinases (discussed below; see Figure 6).

To test the effect of cdc2-mediated phosphorylation on DNA binding of USF mutants, the ability of the USF deletion mutants to bind to DNA in phosphorylated or unphosphorylated states was compared (Figures 5E and 5F). In this instance results obtained using both cyclin A2 and cyclin B1 were identical. In all cases the ability of either full-length his$_6$-USF-1 or his$_6$-USF$\Delta^{144-310}$ to bind to DNA was enhanced by cyclin-dependent phosphorylation. In contrast, his$_6$-USF$\Delta^{197-310}$ bound to DNA very well in the unphosphorylated form and incubation with cyclin–cdc2 had no further effect on DNA-binding activity (see Figures 5E and 5F, lanes 5 and 6). In comparison with the behaviour of full-length USF-1 or his$_6$USF$\Delta^{144-310}$ this suggests that the region between residues 144 and 196 is responsible for regulation of DNA binding by cdk-dependent phosphorylation. This region spans the USR [1] and contains three putative cdc2 phosphorylation sites at positions 153, 165 and 186.

We also performed experiments to determine whether phosphorylation of USF-1 was specifically by cyclin–cdc2 kinases by examining the effects of three known specific or selective inhibitors of these kinases: olomucin, roscovitin and p21^{CIP1} (Figure 6). Olomucin and roscovitin both inhibited USF phosphorylation in a dose-dependent manner (Figure 6A), paralleling their effect on cyclin–cdc2-mediated histone H1 phosphorylation (results not shown). p21^{CIP1} in particular is a specific inhibitor of a number of cdks and efficiently inhibited phosphorylation of full-length USF-1 (Figures 6B and 6C, lanes 5 and 6) by both cyclin B1–cdc2 and cyclin A2–cdc2, in parallel with its effects on histone H1 phosphorylation (Figure 6D). p21 also partially inhibited phosphorylation of his$_6$-USF$\Delta^{197-310}$ by the same kinases (Figures 6B and 6C, lanes 8 and 9). This mutant is predicted not to contain any cdc2 phosphorylation sites. We conclude, from the partial effect of p21, that low levels of phosphorylation by cyclin B1–cdc2 seen here and in a previous experiment (Figure 5C) are more likely to be a consequence of a contaminant in the p34^{cdc2} kinase preparation rather than being due to the action of cdc2 kinase itself. In any case, weak phosphorylation of his$_6$-USF$\Delta^{197-310}$ has no effect on its ability to bind to DNA (see Figures 5E and 5F, lanes 5 and 6).

DISCUSSION

In this report, we provide direct evidence that the activity of USF-1 is regulated by its phosphorylation by cdks. USF-1-dependent transcription of the AdMLP was enhanced selectively from USE-containing templates in mitotic *Xenopus* extracts, indicating that modification of the activity of USF probably did not affect its interaction with the Inr. This result was unexpected because previous reports have shown that transcription is repressed in mitotic *Xenopus* extracts [23]. However, the enhancement of transcription seen in our mitotic extracts correlated with repression of yeast tRNA^{leu} transcription. USF-1 has also been shown to regulate the promoter of human cyclin B1 in a cell-cycle-specific manner [4]. DNA-binding studies showed

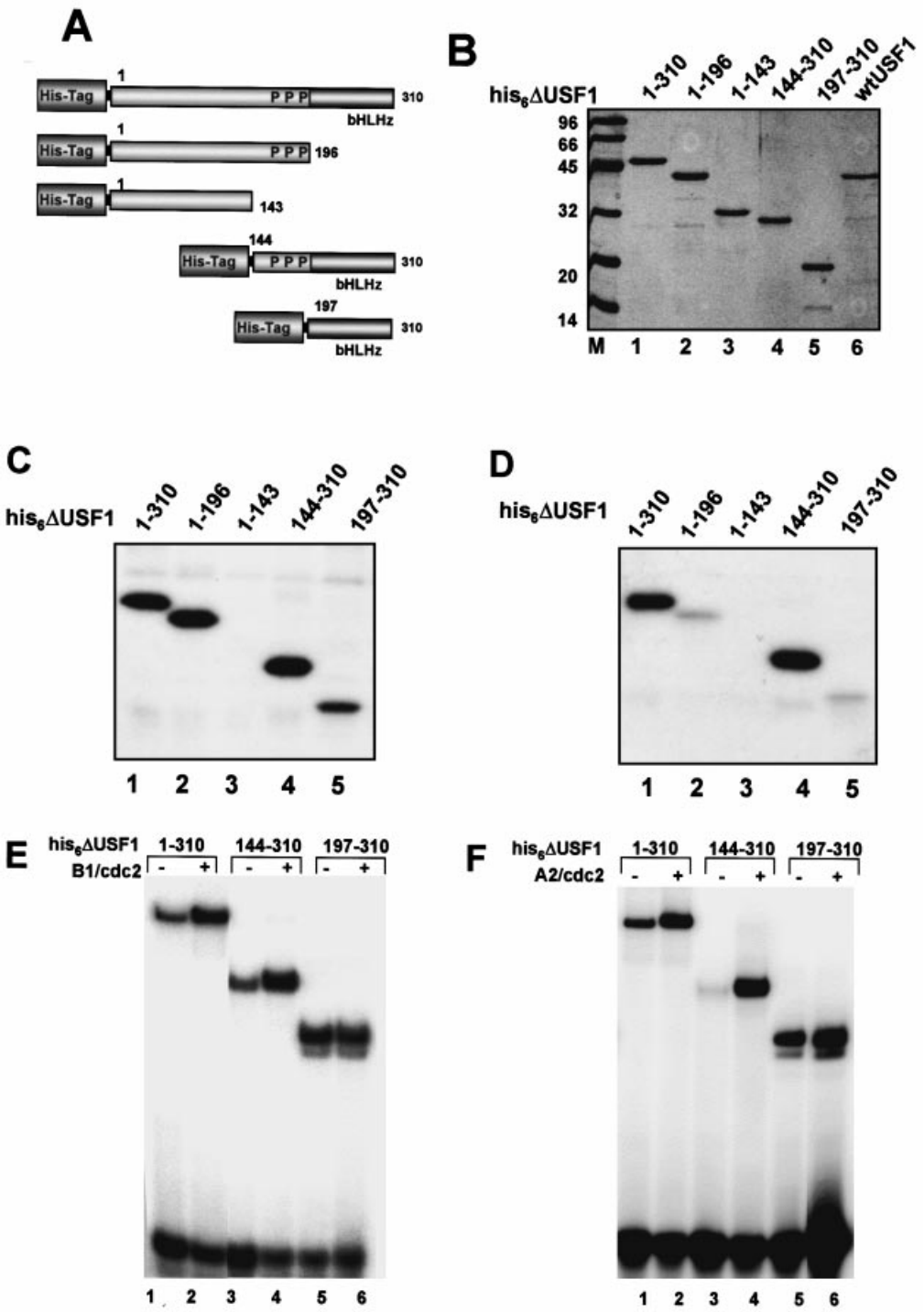

Figure 5 The USR is the site of cdc2-specific phosphorylation

(**A**) Construction and purification of his$_6$ΔUSF-1 proteins. Diagram showing the different USF-1 deletion constructs. Putative cdc2 phosphorylation sites within the USR are indicated by 'P'. (**B**) Coomassie Brilliant Blue-stained SDS/PAGE showing the five his$_6$ΔUSF-1 proteins after purification on an Ni-NTA column. Lanes 1–5 correspond to his$_6$USFΔ$^{1–310}$, his$_6$USFΔ$^{1–196}$, his$_6$USFΔ$^{1–143}$, his$_6$USFΔ$^{144–310}$ and his$_6$USFΔ$^{197–310}$ respectively. Purified wild-type (wt) USF-1 is shown in lane 6. (**C**) SDS/PAGE gel of a phosphorylation assay *in vitro* with cyclin B1–cdc2 of his$_6$ΔUSF-1 mutants, as detailed for (**B**). (**D**) Phosphorylation of his$_6$ΔUSF-1 proteins with cyclin A2–cdc2: details as in (**C**). (**E**) Effect of cyclin B1–cdc2 phosphorylation on his$_6$ΔUSF-1 DNA-binding activity. His-tagged USF-1 deletion proteins, as indicated, were incubated either in the presence or absence of cyclin B1–cdc2 and then subjected to a gel mobility-shift assay, as described in the Materials and methods section. (**F**) Effect of cyclin A2–cdc2 phosphorylation on his$_6$ΔUSF-1 DNA-binding activity. As for (**E**) except USF deletions were incubated with cyclin A2–cdc2.

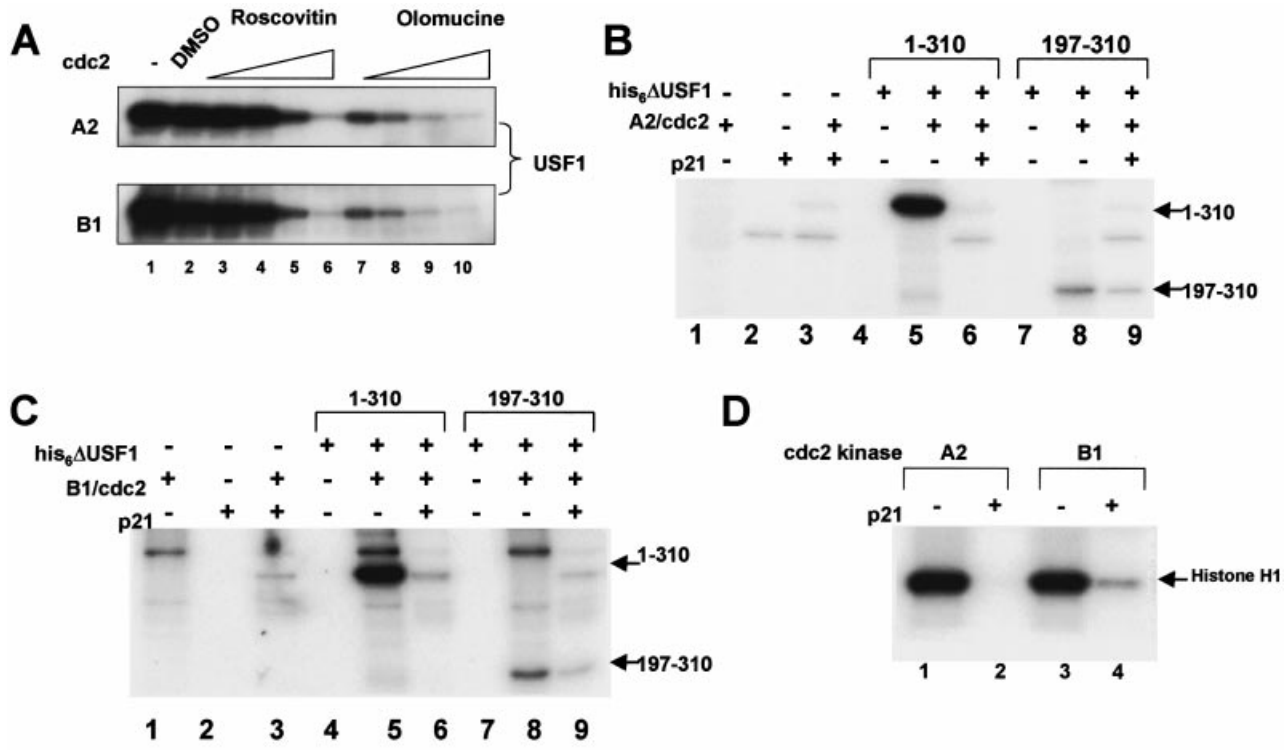

Figure 6 Inhibitors of cdks inhibit USF-1 phosphorylation

(**A**) Treatment of mitotic kinases with chemical inhibitors. DMSO (lane 2), or increasing amounts of roscovitin (lanes 3–6) or olomucine (lanes 7–10) were pre-incubated with purified USF-1 for 5 min prior to addition of mitotic kinases cyclin A2–cdc2 and cyclin B1–cdc2. (**B** and **C**) Effects of $p21^{CIP1}$ on phosphorylation of USF by (**B**) cyclin A2–cdc2 and (**C**) cyclin B1–cdc2. $His_6USF\text{-}1\Delta^{1-310}$ and $his_6USF\text{-}1\Delta^{197-310}$ were incubated for 15 min in the absence (lanes 5 and 8) or presence (lanes 6 and 9) of 3 μg of $p21^{CIP1}$ prior to a phosphorylation reaction *in vitro* with cyclin–cdc2. Control reactions included kinase only (lane 1), p21 only (lane 2), kinase and p21 (lane 3), $his_6USF\text{-}1\Delta^{1-310}$ only (lane 4) and $his_6USF\text{-}1\Delta^{197-310}$ only (lane 7). (**D**) A control experiment comparing the effect of p21 on mitotic kinase activity in our preparations using histone H1 as the substrate. Histone H1 was either treated in the absence or presence of p21 prior to addition of mitotic kinases cyclin A2–cdc2 (lanes 1 and 2) or cyclin B1–cdc2 (lanes 3 and 4).

clearly that USF-1 bound to an E-box with a higher affinity in synchronized mitotic HeLa nuclear extracts compared with extracts in G1/S. This is in general agreement with previous published work [4].

We have demonstrated here that this increase in binding affinity is due to phosphorylation of USF-1 by either cyclin A2–$p34^{cdc2}$ or cyclin B1–$p34^{cdc2}$. A region which corresponds to the previously identified USR [1] is the most likely target for cdk-mediated phosphorylation. We deduce, from our observations, that the USR inhibits DNA binding unless it is phosphorylated. Phosphorylation of USF-1 within the USR may induce a conformational change resulting in unmasking of the DNA-binding domain. The USR lies between the DNA-binding and transactivation domains and may be acting as a phosphorylation-dependent hinge. Thus from our results, *in vitro* phosphorylation of USF-1 by cyclin A2– or cyclin B1–$p34^{cdc2}$ mimics the enhancement of USF-1 DNA binding observed in extracts from G2/M cells and the enhanced transcription in *Xenopus* mitotic extracts.

The USR plays an important role in many of the functions of USF. It contributes to transcriptional activity of the protein, especially on Inr-dependent promoters [1], and also is necessary, but not sufficient, for nuclear localization [1]. This activity is context-dependent, since the USR cannot function as an activation domain when transposed to another part of the molecule. This lends further support to the notion that the USR may have a structural role. A recent report has also shown that the USR plays an essential role in modulating cell-specific transcriptional activity of USF-1 [8]. Qyang and colleagues [8] hypothesize that the USR may recruit either cell-specific co-activators or repressors. We have not determined whether USR phosphorylation plays a direct role in transactivation, but we do have preliminary evidence that it may also regulate nuclear localization since we have detected increased levels of USF-1 protein in mitotic nuclei (E. Cheung, unpublished work), although levels of USF-1 protein [25] or mRNA [26] did not change over time. Regulation of USF-2 nuclear localization by interleukin-3 has been observed in mast cells, where it appears to be essential for cell survival [27]. This observation supports our own and points to the possibility that nuclear localization of USF family members can be regulated by phosphorylation and that this regulation is of physiological importance.

USF-1 belongs to the basic helix-loop-helix leucine-zipper family of transcription factors that also includes the c-Myc proto-oncogene product. c-Myc has been shown to be hyperphosphorylated during mitosis [28] and to be a substrate for mitogen-activated protein kinases, casein II kinase and cyclin B–$p34^{cdc2}$ [29–31]. The first evidence to show that USF-1 might be a phosphoprotein came from the identification of a 39-kDa USF-1-like factor (HIV-TF1) binding to the E-box region in the HIV-1 LTR region [32]. Phosphatase treatment of this USF-like factor with potato acid phosphatase greatly decreased its binding affinity, thus implicating the importance of phosphorylation for its DNA binding.

Further evidence exists indicating that USF-1 is a phosphoprotein. Galibert and colleagues [33] have shown that USF-1 is a phosphoprotein *in vivo* and that the phosphorylated form of USF-1 binds preferentially to DNA. A later report demonstrated that treatment of Swiss 3T3 fibroblasts with sphingosylphosphocholine can enhance the DNA-binding activity of USF-1 [26]. Sphingosylphosphocholine can activate a host of kinases, including mitogen-activated protein kinases and casein kinase II. There is a casein kinase II phosphorylation site within the USR that may be the target for sphingosylphosphocholine-mediated phosphorylation. Taken together, this evidence points to the possibility that the activity of USF proteins may be modulated by multiple pathways.

In this article we provide the first direct evidence that USF-1 can be phosphorylated by specific kinases that are essential for G2/M progression of the cell cycle. Taken together, this means that USF-1 and c-Myc share more than just structural similarities, in that both can be phosphorylated by the same kinases. However, in contrast to mitotically phosphorylated c-Myc, whose DNA-binding activity is decreased, our results show that phosphorylation of USF-1 by cdc2 kinases has an opposite effect on DNA binding. This may be important in the regulation of USF activity and its relationship with c-Myc. It has been proposed that USF may act as an anti-proliferative influence [6], perhaps counterbalancing the effects of c-Myc. In this context co-ordinate but reciprocal control of the activity of the two proteins would be logical.

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