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Mitotic silencing of human rRNA synthesis: inactivation of the promoter selectivity factor SL1 by cdc2/cyclin B-mediated phosphorylation

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We have used a reconstituted cell-free transcription system to investigate the molecular basis of mitotic repression of RNA polymerase I (pol I) transcription. We demonstrate that SL1, the TBP-containing promoter-binding factor, is inactivated by cdc2/cyclin B-directed phosphorylation, and reactivated by dephosphorylation. Transcriptional inactivation *in vitro* is accompanied by phosphorylation of two subunits, e.g. TBP and hTAF$_I$110. To distinguish whether transcriptional repression is due to phosphorylation of TBP, hTAF$_I$110 or both, SL1 was purified from two HeLa cell lines that express either full-length or the core domain of TBP only. Both TBP-TAF$_I$ complexes exhibit similar activity and both are repressed at mitosis, indicating that the variable N-terminal domain which contains multiple target sites for cdc2/cyclin B phosphorylation is dispensable for mitotic repression. Protein–protein interaction studies reveal that mitotic phosphorylation impairs the interaction of SL1 with UBF. The results suggest that phosphorylation of SL1 is used as a molecular switch to prevent pre-initiation complex formation and to shut down rDNA transcription at mitosis.

Keywords: cdc2/cyclin B/mitosis/phosphopeptide mapping/RNA polymerase I/TBP/TBP-associated factors

Introduction

In vertebrate cells, entry into mitosis is accompanied by a global inhibition of transcription. Cellular RNA synthesis stops by mid-prophase and resumes late in telophase (Taylor, 1960; Prescott and Bender, 1962). With respect to the molecular mechanisms underlying mitotic repression of transcription, substantial progress has been made using cell-free transcription systems that reproduce silencing of transcription *in vitro*. These studies revealed repressive mechanisms rather than changes in the chromatin structure being responsible for transcriptional shut-off during mitosis. Hartl *et al*. (1993) were the first to mimic mitotic repression of transcription of 5S and tRNA genes by RNA polymerase III (pol III) *in vitro* using extracts from cells arrested in mitosis with the microtubule polymerization inhibitor nocodazole. Consistent with cdc2/cyclin B being the protein kinase controlling entry of cells into mitosis, addition of cdc2/cyclin B kinase to extracts prepared from asynchronous cells or reconstituted transcription reactions caused a complete transcriptional shut-off (Gottesfeld *et al.*, 1994). The cyclin-dependent kinase inhibitor p21 could prevent the effect of cdc2/cyclin B, indicating that repression of transcription is due to protein phosphorylation. Subsequent studies demonstrated that repression of pol III transcription was due to a substantial decrease in the activity of TFIIIB (Gottesfeld *et al.*, 1994; White *et al.*, 1995), the basal factor which consists of the TATA-binding protein (TBP) and TBP-associated factors (TAFs) of 75 and 92 kDa (TAF$_{III}$90/Brf/TFIIIB90). Affinity-purified fractions containing the TBP-associated components of TFIIIB were capable of restoring transcription in both *Xenopus* and HeLa cell-free transcription systems, suggesting that a pol III-specific TAF(s) is a target(s) of mitotic repression. Indeed, phosphorylation of both TBP and TAF$_{III}$90 correlates with mitotic repression of pol III transcription (Leresche *et al.*, 1996).

Transcription by RNA polymerase II (pol II) can also be inhibited *in vitro* by cdc2/cyclin B and inhibition can be prevented by protein kinase inhibitors (Leresche *et al.*, 1996). Transcription rescue and inhibition experiments with each of the basal factors and pol II suggest that multiple components of the transcription machinery are inactivated by cdc2/cyclin B-directed phosphorylation. For an activated pol II promoter, targets of repressive phosphorylation are both TFIID and TFIIH, while for a basal promoter, TFIIH is the major target for mitotic inactivation (Segil *et al.*, 1996; Long *et al.*, 1998). Thus, multiple targets exist that are responsible for the global shutdown of pol II transcription in mitosis.

Little information is available on the molecular mechanisms that repress rRNA gene transcription during mitosis. Interestingly, major components of the pol I transcriptional machinery, including pol I (Scheer and Rose, 1984), UBF (Chan *et al.*, 1991; Zatsepina *et al.*, 1993; Roussel *et al.*, 1996), TIF-IB/SL1 (Jordan *et al.*, 1996; Roussel *et al.*, 1996) and topoisomerase I (Rose *et al.*, 1988), remain stably associated with the nucleolus organizing regions (NORs), the chromosomal sites of rRNA synthesis. The absence of rRNA synthesis during mitosis, despite the continued presence of the transcriptional machinery, suggests regulatory mechanisms that repress or retard transcription during mitosis.

Direct assessment of the mechanisms mediating transcriptional repression at mitosis requires *in vitro* systems that reflect the cellular transcription activity. In a previous communication we have used extracts from asynchronous and mitotic HeLa cells to analyze the molecular mechanisms underlying mitotic repression of pol I transcription (Kuhn *et al.*, 1998). Extracts prepared from mitotic HeLa cells were inactive in pol I transcription, but activity could

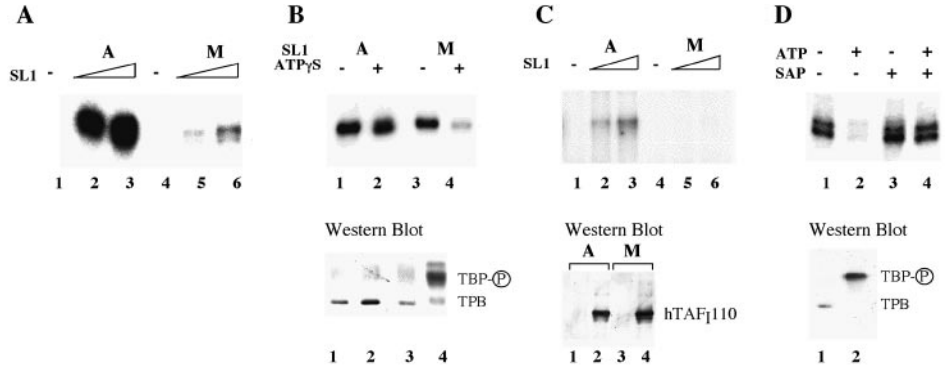

Fig. 1. SL1 is inactivated by mitotic phosphorylation. (**A**) SL1 immunoprecipitated from M-phase cell extracts is transcriptionally inactive. Identical amounts of extracts from asynchronous (A, lanes 1–3) and mitotic (M, lanes 4–6) HeLa cells were pre-incubated with 2.5 μM ATPγS for 30 min at 30°C. SL1 was immunoprecipitated with α-mTAF$_I$95 antibodies immobilized on sheep anti-rabbit IgG Dynabeads. After stringent washing, the activity of precipitates was monitored in a reconstituted transcription system that is inactive without exogenous SL1 (lanes 1 and 4). (**B**) Inactivation of SL1 requires phosphorylation by a mitotic kinase(s). Extracts prepared from asynchronous (lanes 1 and 2) and mitotic HeLa cells (lanes 3 and 4) were pre-incubated in the absence or presence of ATPγS as indicated. SL1 was immunoprecipitated with anti-mTAF$_I$95 antibodies and assayed for transcriptional activity (upper panel). To compare the relative amounts of SL1, aliquots of the immunoprecipitates were analyzed on immunoblots using α-TBP antibodies (3G3). (**C**) Mitotic SL1 is transcriptionally inactive. Interphase cells (A, lanes 1–3) or cells blocked in pro-metaphase (M, lanes 4–6) were lysed in buffer AM-600/0.5% NP-40 in the presence of 80 mM glycerophosphate to inhibit cellular phosphatases. One milligram of extract proteins was incubated with bead-bound immunoglobulins from pre-immune serum (lanes 1 and 4) or α-mTAF$_I$95 antibodies (lanes 2, 3, 5 and 6), washed extensively and assayed in the reconstituted transcription system (upper panel) and on immunoblots (lower panel). (**D**) Mitotic inhibition of SL1 activity is relieved by phosphatase treatment. M-phase HeLa cell extract was pre-incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of ATP, and SL1 was precipitated with α-mTAF$_I$95 antibodies. Half of each precipitate was incubated with shrimp alkaline phosphatase (SAP, lanes 3 and 4), the other half was incubated without phosphatase (lanes 1 and 2). After washing, aliquots of immobilized SL1 were assayed in the reconstituted transcription system (upper panel). Mitotic phosphorylation of the TBP moiety of SL1 was monitored on Western blots (lower panel) demonstrating marked differences in the electrophoretic mobility in the absence (lane 1) or presence (lane 2) of mitotic phosphorylation.

be restored by SL1, the human pol-I-specific TBP–TAF$_I$ complex (Comai *et al.*, 1992, 1994; Zomerdijk *et al.*, 1994). The results suggest that repression of transcription by all three classes of nuclear RNA polymerases is brought about by a common molecular mechanism involving reversible phosphorylation of components of the basal transcriptional machinery. In the present study we demonstrate that transcriptional inactivation of SL1 by cdc2/cyclin B *in vitro* correlates with phosphorylation of two subunits, e.g. TBP and hTAF$_I$110. Our data indicate that phosphorylation of hTAF$_I$110 rather than TBP accounts for the loss of SL1 activity. Moreover, protein–protein interaction studies reveal that mitotic phosphorylation impairs the capability of SL1 to interact with UBF. Since the cooperative binding of SL1 and UBF to the rDNA promoter is a prerequisite for pre-initiation complex formation, these studies provide a clue to the molecular mechanisms that shut-down rDNA transcription during mitosis.

Results

SL1 is inactivated by mitotic phosphorylation

We have demonstrated recently that human rDNA transcription is repressed in extracts from mitotic cells and that this repression can be overcome by addition of either kinase inhibitors or exogenous SL1 (Kuhn *et al.*, 1998). These studies suggest that SL1 is inactivated by cdc2/cyclin B-directed phosphorylation. If this is true, then SL1 from mitotic cells should display a significantly reduced activity. In the experiment shown in Figure 1A, extracts from asynchronous and mitotic HeLa cells were pre-incubated in the presence of ATPγS to phosphorylate the target protein(s), SL1 was immunoprecipitated by antibodies directed against the largest murine TAF$_I$, the immunoprecipitates were stringently washed and the transcriptional activity of bead-bound SL1 was assayed in a reconstituted transcription system. Consistent with SL1 being inactivated during mitosis, the activity of SL1 derived from mitotic extracts was strongly reduced (>10-fold).

In agreement with recent results demonstrating that the target protein(s) of the mitotic kinase(s) is dephosphorylated during extract preparation (Kuhn *et al.*, 1998), repression of transcription in whole-cell extracts was ATP-dependent, requiring *de novo* phosphorylation by a cellular mitotic kinase(s) (Figure 1B). However, if immunoprecipitation was performed in the presence of phosphatase inhibitors, SL1 from mitotic cells was inactive without prior incubation with ATP (Figure 1C), indicating that mitotic phosphorylation is counteracted by a cellular phosphatase(s).

If inactivation of SL1 is brought about by phosphorylation, phosphatase treatment of mitotic SL1 should restore transcriptional activity. To test this, SL1 was phosphorylated by pre-incubating mitotic extract in the presence of ATP and okadaic acid, and then SL1 was precipitated with bead-bound anti-mTAF$_I$95 antibodies and incubated in the absence or presence of shrimp alkaline phosphatase (SAP). Aliquots of immobilized SL1 were assayed for transcriptional activity. As shown in Figure 1D, phosphatase treatment fully restored SL1 activity (lane 4), demonstrating that dephosphorylation is sufficient for the reversal of SL1 inactivation.

TBP and TAF$_I$s are phosphorylated in prometaphase

To investigate which subunits of SL1 are phosphorylated *in vivo*, we compared the phosphorylation pattern of SL1 isolated from asynchronous and mitotic cells. HeLa cells were metabolically labeled with [^{32}P]orthophosphate and

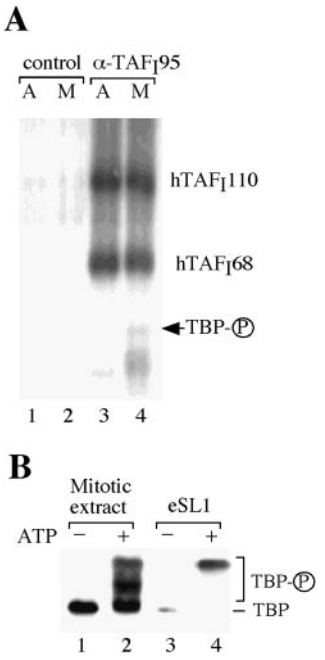

Fig. 2. Phosphorylation of SL1 *in vivo*. (**A**) Metabolic labeling of SL1 *in vivo*. Asynchronous (lanes 1 and 3) or mitotic (lanes 2 and 4) HeLa cells were metabolically labeled with [^{32}P]orthophosphate and SL1 was purified by precipitation with α-TBP antibodies, eluted with the epitope peptide and subsequently precipitated with control IgGs (lanes 1 and 2) or α-mTAF$_I$95 antibodies (lanes 3 and 4). Precipitated proteins were separated by SDS–PAGE and analyzed by autoradiography. (**B**) Mitotic SL1 contains hyperphosphorylated TBP. Mitotic extract was incubated in the absence or presence of ATP prior to immunoprecipitation of SL1 with immobilized α-mTAF$_I$95 antibodies. Aliquots of the extract and the immunoprecipitates were separated by SDS–PAGE and TBP was detected by Western blotting.

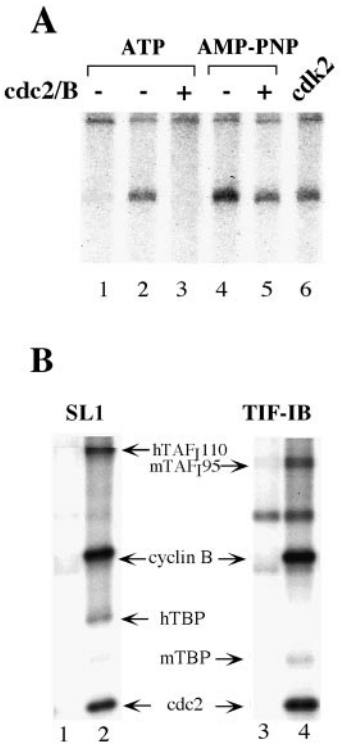

Fig. 3. Cdc2/cyclin B phosphorylates SL1 *in vitro*. (**A**) Affinity-purified SL1 is inactivated by cdc2/cyclin B. Cdc2/cyclin B and cdk2/cyclin complexes were immunoprecipitated from mitotic or asynchronous extracts by α-cyclin B1 or α-cdk2 antibodies. Affinity-purified SL1 was incubated with the bead-bound kinase in the presence of ATP (lanes 2, 3 and 6) or AMP-PNP (lanes 4 and 5). SL1 activity was assayed in the reconstituted transcription system containing 0.5 μM okadaic acid and DMAP. Lane 1 shows a control reaction in the absence of SL1. (**B**) The largest TAF$_I$ and TBP are phosphorylated by cdc2/cyclin B *in vitro*. SL1 (lanes 1 and 2) and TIF-IB (lanes 3 and 4) were incubated in the presence of [γ-^{32}P]ATP with bead-bound cdc2/cyclin B, and phosphorylated subunits were analyzed by SDS–PAGE and autoradiography. Cdc2/cyclin B complexes were immunoprecipitated from mitotic cells using immobilized monoclonal α-cyclin B1 antibodies. Control beads contained α-tubulin antibodies (lanes 1 and 3).

lysed in the presence of phosphatase inhibitors, and SL1 was purified by two successive immunopurification steps, using anti-TBP antibodies for the first immunoprecipitation and anti-mTAF$_I$95 antibodies for the second. Two TAF$_I$s, hTAF$_I$110 and hTAF$_I$68, were labeled both in asynchronous and mitotic cells, whereas no phosphorylation of hTAF$_I$48 was observed (Figure 2A, lanes 3 and 4). The fact that hTAF$_I$68 and hTAF$_I$110 were labeled in both asynchronous and mitotic cells suggests that constitutive phosphorylation may be required for SL1 function.

In contrast, the most apparent mitosis-specific phosphorylations were observed with TBP. The phosphorylated forms of TBP could be resolved into a series of tightly spaced bands (Figure 2A, lane 4). The mobility of the most slowly migrating TBP moiety (TBP-P) corresponds to an apparent molecular mass of ~50 kDa. To analyze hyperphosphorylation of TBP in mitotic SL1, M-phase extract was pre-incubated in the absence or presence of ATP, SL1 was immunoprecipitated and TBP was visualized on immunoblots. In the absence of ATP, a defined band of TBP was observed both in unfractionated mitotic extracts and in immunopurified SL1 (Figure 2B, lanes 1 and 3). After pre-incubation with ATP, a great portion of TBP underwent a mobility shift in SDS–PAGE (Figure 2B, lane 2), indicating that the heterogeneity was brought about by multiple phosphorylations. Remarkably, the most slowly migrating moiety of TBP is present in mitotic SL1 (Figure 2B, TBP-P, lane 4).

TBP and hTAF$_I$110 are phosphorylated by cdc2/cyclin B in vitro

The results presented so far demonstrate that phosphorylation by a mitotic kinase inactivates SL1. To investigate whether cdc2/cyclin B itself or a cdc2/cyclin B-dependent kinase targets SL1, the effect of purified cdc2/cyclin B on SL1 activity was assayed. SL1-dependent transcription was abolished if SL1 was phosphorylated with purified cdc2/cyclin B (Figure 3A, lane 3). Transcriptional repression was ATP-dependent, demonstrating the requirement of protein phosphorylation (Figure 3A, lane 5). Pre-incubation with cdk2/cyclin A/E complexes, on the other hand, had no effect (Figure 3A, lane 6), a finding which underscores the specificity of cdc2/cyclin B-mediated transcriptional repression.

To correlate transcriptional inactivation with phosphorylation of defined subunits of the TBP–TAF complex, both SL1 and the homologous murine factor TIF-IB were incubated with purified cdc2/cyclin B and [γ-^{32}P]ATP, and labeled polypeptides were analyzed by SDS–PAGE and autoradiography. Significantly, two subunits of SL1/TIF-IB were phosphorylated by cdc2/cyclin B, TBP and the largest TAF, e.g. hTAF$_I$110 in human and mTAF$_I$95 in

mouse (Figure 3B, lanes 2 and 4). Consistent with TBP and the largest TAF subunit being targeted by cdc2/cyclin B, recombinant TBP and hTAF$_I$110/mTAF$_I$95, but not TAF$_I$68 and TAF$_I$48, could be labeled *in vitro* by purified cdc2/cyclin B (data not shown).

Cdc2/cyclin B-directed phosphorylation of TBP does not inactivate SL1

Since inactivation of SL1/TIF-IB by cdc2/cyclin B correlates with phosphorylation of both TBP and TAF$_I$110/95, we wondered whether modification of either one or both proteins caused inactivation of SL1. To address this issue, we used two HeLa cell lines (LTRα3 and clone 20) which constitutively express epitope-tagged human TBP (pLTReTBP; Zhou *et al.*, 1992) or residues 155–339, i.e. the 'core', of human TBP (pLTReΔNTBP; Zhou *et al.*, 1993). In a previous study, we employed these cell lines to immunopurify SL1 containing full-length or N-terminally truncated TBP, respectively, and showed that the variable N-terminal part of TBP is dispensable for SL1 activity (Rudloff *et al.*, 1994). The rationale of the experiment shown in Figure 4 was to test whether deletion of the N-terminus which harbors all putative target sites for cdc2/cyclin B would prevent mitotic inactivation of SL1. For this, SL1 containing full-length TBP (eSL1) or truncated TBP (eΔNSL1) was immunopurified and incubated with cdc2/cyclin B or with mitotic extract in the presence of either ATPγS or the non-hydrolysable analog AMP-PNP. After washing, the activity of bead-bound eSL1 and eΔNSL1 was assayed in the reconstituted transcription system. As expected, in the absence of the kinase both complexes exhibited similar transcriptional activity (Figure 4B, lanes 1–4). However, after incubation with purified cdc2/cyclin B (Figure 4B, lanes 5–8) or mitotic extract (lanes 9–12) both eSL1 and eΔNSL1 were inactive. This inactivation was dependent on phosphorylation, because it was observed in the presence of ATPγS but not AMP-PNP. The fact that phosphorylation by cdc2/cyclin B abrogates the activity of both eSL1 and eΔNSL1 suggests that phosphorylation of the N-terminus of TBP is unlikely to be involved in mitotic inactivation of SL1.

To exclude the possibility that the core domain of TBP is targeted by cdc2/cyclin B, full-length and truncated TBP were incubated with cdc2/cyclin B in the presence of [γ-^{32}P]ATP and analyzed by autoradiography and Western blotting (Figure 4C). As expected, full-length TBP was efficiently phosphorylated by cdc2/cyclin B (Figure 4C, lane 1). In contrast, ~5-fold higher amounts of ΔNTBP (Figure 4C, lanes 3 and 4) were not labeled (lane 2), indicating that the core domain is not a substrate for cdc2/cyclin B.

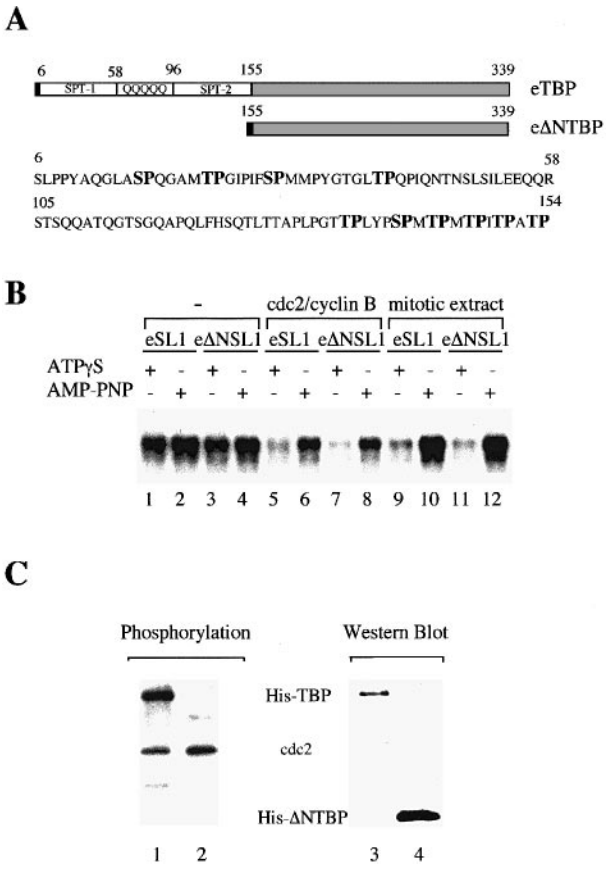

Fig. 4. The N-terminal part of TBP is dispensable for cdc2/cyclin B-mediated inactivation of SL1. (**A**) Schematic illustration of TBP and ΔNTBP. The SP/TP-rich sequences and the run of glutamines (Q) are indicated. The hemagglutinin (HA)-tag is marked by a black box. The sequence of the two SPT regions is shown below. Putative target sites for cdc2/cyclin B are marked by bold letters. (**B**) Both eSL1 and eΔNSL1 are inactivated by mitotic phosphorylation. HA-tagged TBP-TAF complexes were immunoprecipitated from 250 μl extracts from LTRα3 or clone 20 cells, respectively, to yield eSL1 or eΔNSL1. Bead-bound eSL1 and eΔNSL1 were incubated for 30 min at 30°C with purified cdc2/cyclin B (lanes 5–8) or mitotic extract (lanes 9–12) in the presence of AMP-PNP or ATPγS as indicated. After washing, SL1 was assayed in the reconstituted transcription system. (**C**) His-tagged full-length TBP (lanes 1 and 3) and ΔNTBP (lanes 2 and 4) were phosphorylated with purified cdc2/cyclin B and [γ-^{32}P]ATP, subjected to 12% SDS–PAGE, transferred onto a nitrocellulose filter and visualized by autoradiography (lanes 1 and 2). The filters were subsequently probed with a monoclonal α-TBP antibody (mAb58C9) to localize the positions of full-length His-TBP (40 kDa) and His-ΔNTBP (22 kDa).

Tryptic peptide maps of hTAF$_I$110 labeled in vivo and in vitro

To assess whether hTAF$_I$110 is phosphorylated at multiple sites by cdc2/cyclin B and to compare the pattern of phosphopeptides that are labeled *in vivo* and *in vitro*, tryptic peptide mapping experiments were performed. In the experiment shown in Figure 5, Sf9 cells were infected with baculoviruses containing the four subunits of SL1, i.e. TBP and the three TAF$_I$s, in either the absence or presence of baculoviruses expressing cdc2 and cyclin B. The infected cells were labeled with [^{32}P]orthophosphate and epitope-tagged TAFs were isolated by immunoprecipitation with anti-FLAG antibodies. The subunits were separated by electrophoresis and blotted onto nitrocellulose. hTAF$_I$110 was digested with trypsin and subjected to two-dimensional peptide analysis. In the absence of exogenous kinases two hTAF$_I$110-specific tryptic phosphopeptides (labeled **a** and **b**) were observed (Figure 5A). Coinfection with baculoviruses encoding human cdc2 and cyclin B yielded one additional phosphopeptide, spot **c** (Figure 5B). A parallel analysis of the phosphopeptide pattern of recombinant hTAF$_I$110 that has been phosphorylated with purified cdc2/cyclin B and [γ-^{32}P]ATP revealed exclusive labeling of spot **c** (Figure 5C). The identity of peptide **c** phosphorylated *in vivo* and *in vitro* was revealed by mixing the tryptic peptides of hTAF$_I$110 labeled in Sf9 cells with those labeled *in vitro*. Indeed,

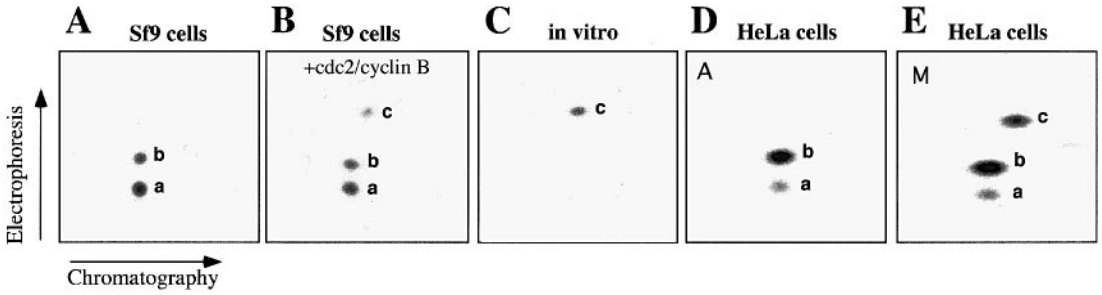

Fig. 5. Tryptic phosphopeptide maps of $hTAF_I110$ phosphorylated *in vivo* and *in vitro*. (**A**) Tryptic peptides of $hTAF_I110$ phosphorylated in Sf9 cells. Sf9 cells co-infected with baculoviruses expressing all four subunits of SL1 were metabolically labeled with [^{32}P]orthophosphate. $hTAF_I110$ was immunoprecipitated, digested with trypsin and subjected to two-dimensional tryptic peptide mapping. The individual phosphopeptides are designated **a**–**c**. (**B**) Tryptic peptides of $hTAF_I110$ phosphorylated in Sf9 cells overexpressing cdc2/cyclin B. Sf9 cells were co-infected with baculoviruses expressing the four components of SL1 as well as human cdc2 and cyclin B. (**C**) Tryptic peptides of $hTAF_I110$ phosphorylated *in vitro*. Recombinant $hTAF_I110$ phosphorylated *in vitro* with purified cdc2/cyclin B was subjected to tryptic peptide analysis. (**D** and **E**) Phosphopeptides of $hTAF_I110$ labeled in HeLa cells. Asynchronous (A) or M-phase (M) cells that overexpress FLAG-tagged $hTAF_I110$ were labeled with [^{32}P]orthophosphate. $hTAF_I110$ was immunoprecipitated with α-FLAG antibodies and subjected to two-dimensional tryptic peptide mapping.

the tryptic peptides precisely coincided, indicating that the same sites of $hTAF_I110$ were labeled *in vivo* and *in vitro* (data not shown).

To demonstrate that the phosphopeptide pattern of $hTAF_I110$ in cellular SL1 compares to that expressed in insect cells, a HeLa cell line was established which stably expresses FLAG-tagged $hTAF_I110$. Asynchronous and mitotic cells were labeled with [^{32}P]orthophosphate, $hTAF_I110$ was immunoprecipitated and subjected to two-dimensional phosphopeptide mapping. Consistent with the fingerprints of $hTAF_I110$ expressed in insect cells, phosphopeptides **a** and **b** were phosphorylated both in asynchronous and mitotic HeLa cells (Figure 5D). Peptide **c**, on the other hand, was exclusively labeled in mitotic cells (Figure 5E). Although this experimental approach does not unambiguously prove that $hTAF_I110$ has been correctly assembled into SL1, immunoprecipitation experiments demonstrate that in the stable cell line used, a significant amount of FLAG-tagged $hTAF_I110$ is contained in active SL1 (data not shown).

Mitotic phosphorylation impairs the interaction of SL1 with UBF

Next we addressed the question of which function of SL1 is affected by mitotic phosphorylation. SL1 has been known to associate with the ribosomal gene promoter through protein–protein interactions with UBF (Learned *et al.*, 1985; Bell *et al.*, 1988; Jantzen *et al.*, 1992; Hempel *et al.*, 1996). To investigate whether the interaction between UBF and mitotic SL1 was impaired, agarose beads containing FLAG-tagged UBF or the FLAG-peptide alone were incubated with extracts prepared from asynchronous and mitotic HeLa cells. Prior to binding, the extracts were pre-incubated in the presence of ATP to maintain SL1 in the phosphorylated state. After incubation with the extracts, the beads were washed and assayed for the presence of $hTAF_I110$ on Western blots. As shown in Figure 6A, very low amounts of SL1 were retained on the FLAG-peptide control beads (M2, lanes 3 and 5). However, consistent with UBF interacting with SL1 in solution (Hempel *et al.*, 1996), significant amounts of SL1 from asynchronous cells associated with bead-bound UBF (Figure 6A, lane 4). Importantly, the Western blot signal of $hTAF_I110$ retained at the UBF resin was almost one order of magnitude lower for SL1 from mitotic as compared with asynchronous extracts (Figure 6A, lanes 4 and 6), indicating that the interaction of mitotic SL1 with UBF was severely impaired.

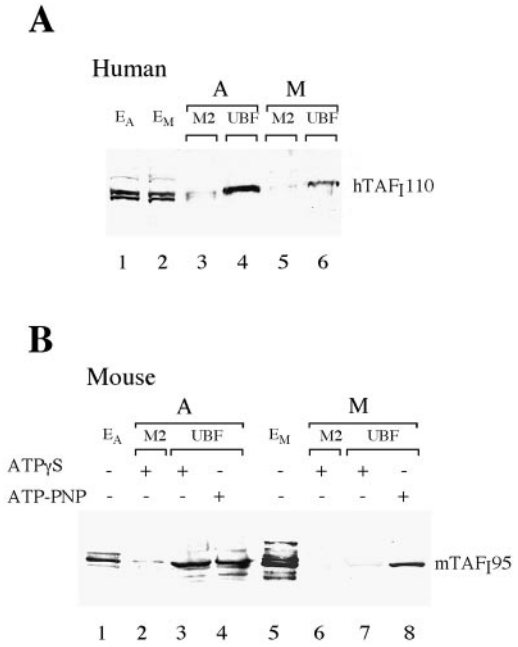

Fig. 6. The interaction between mitotic SL1/TIF-IB and UBF is impaired. (**A**) UBF/SL1 interaction. Extracts from asynchronous (A, lanes 1, 3 and 4) and mitotic (M, lanes 2, 5, 6) HeLa cells were pre-incubated for 20 min at 30°C with 2 mM of ATP and then incubated for 30 min at room temperature with either control beads containing the FLAG-epitope peptide (M2) or bead-bound FLAG-tagged UBF as indicated. The beads were washed and bound proteins were analyzed on immunoblots using α-$mTAF_I95$ antibodies. Lanes 1 and 2 show the amounts of $hTAF_I110$ that were present in 10% of asynchronous (E_A) and mitotic (E_M) extracts. The lower band is a degradation product of $hTAF_I110$ that does not interact with UBF. (**B**) UBF/TIF-IB interaction. Extracts from asynchronous (A, lanes 1–4) and mitotic (M, lanes 5–8) FT210 cells were pre-incubated for 20 min at 30°C with 2 mM of either ATPγS or AMP-PNP as indicated and incubated with beads coated with the FLAG epitope peptide (M2, lanes 2 and 6) or UBF (lanes 3, 4, 7 and 8). TIF-IB binding was monitored on immunoblots with α-$mTAF_I95$ antibodies. Lanes 1 and 5 show the amounts of $mTAF_I95$ that were present in 10% of asynchronous (E_A) and mitotic (E_M) extracts.

An even more pronounced effect was observed when this 'pull-down' experiment was performed with extracts from murine cells to measure interactions of the murine

counterpart of SL1, e.g. TIF-IB (Figure 6B). To examine phosphorylation-dependent changes in interactions between TIF-IB and UBF, extracts from asynchronous or mitotic mouse cells were pre-incubated with ATPγS or AMP-PNP either to facilitate or prevent *de novo* phosphorylation. As with SL1, TIF-IB from asynchronous extracts efficiently interacted with UBF, irrespective of whether or not phosphorylation was allowed to occur (Figure 6B, lanes 3 and 4), indicating that kinases present in the asynchronous extract did not affect binding. In contrast, phosphorylation by the kinase(s) present in the mitotic extract rendered TIF-IB incapable of interacting with UBF, as revealed by the marked decrease of the $mTAF_I95$ signal in M-phase extract that had been pre-incubated in the presence of ATPγS (Figure 6B, lane 7). However, if pre-incubation of the mitotic extract was performed in the presence of AMP-PNP, the capability of TIF-IB to interact with UBF was partially restored (Figure 6B, lane 8). This result emphasizes that mitotic phosphorylation is counteracted by a cellular phosphatase(s) that dephosphorylates SL1/TIF-IB, and implies that in cell extracts, the interaction-incompetent state of SL1 requires phosphorylation by a kinase(s) that is present in the mitotic but not asynchronous extract.

Discussion

During mitosis, cell nuclei undergo extensive structural changes and, concomitantly, transiently arrest transcriptional activity. With regard to transcription by RNA pol I, nucleolar structure undergoes extensive changes, and pol I transcriptional activity ceases almost completely. Although most nucleolar proteins disperse throughout the mitotic cell, all known basal factors required for transcription initiation are maintained on metaphase chromosomes (Zatsepina *et al.*, 1993; Jordan *et al.*, 1996; Roussel *et al.*, 1996). The selective retention of the pol I transcription apparatus could be a regulatory mechanism that marks these genes for rapid assembly into pre-initiation complexes when cells re-enter the G_1 phase of the cell cycle. The mechanism of pol I arrest during mitosis is poorly characterized. Early studies suggested that mitotic repression occurs during elongation. This conclusion was based on biochemical experiments showing that in mitotic HeLa cells pol I transcription could be reactivated by treatment of condensed chromosomes with Sarkosyl or heparin (Gariglio *et al.*, 1974; Matsui and Sandberg, 1985), indicating that transcription complexes with their nascent pre-rRNA chains may become transiently arrested during mitosis and resume elongation once the mitotic block is relieved. A careful re-examination of this model using fluorescence *in situ* hybridization with a variety of antisense riboprobes failed to detect any nascent pre-rRNA molecules at the chromosomal NORs during mitosis (Weisenberger and Scheer, 1995; Beven *et al.*, 1996; Lazdins *et al.*, 1997). Two different models have been postulated to explain the absence of 'frozen Christmas trees' on mitotic chromosomes. One model assumes that prematurely terminated transcripts are detached from stalled pol I which remains bound to the template. Another model postulates pausing of initiated pol I complexes 210 bp downstream from the promoter with short nascent transcripts hidden within the transcription complex and thus escaping detection by *in situ* hybridization. In both cases, elongating pol I complexes persist bound to the rDNA template throughout mitosis.

Based on the following lines of experimental evidence, we postulate that phosphorylation of SL1 is causally involved in mitotic silencing of pol I transcription. First, we found that the activity of SL1 purified from cells blocked in prometaphase was more than one order of magnitude lower than SL1 isolated from asynchronous cells. Secondly, when mitotic SL1 was treated *in vitro* with phosphatase, it regained transcriptional activity. Thirdly, *in vitro* phosphorylation of affinity-purified active SL1 by cdc2/cyclin B abrogated transcriptional activity. Finally, the interaction of mitotic SL1 with UBF was impaired indicating that phosphorylation of SL1 interferes with pre-initiation complex assembly.

Inactivation of SL1 by cdc2/cyclin B *in vitro* was accompanied by phosphorylation of two subunits, TBP and $hTAF_I110$. With regard to TBP, it was noted before that TBP from mitotic cells is hyperphosphorylated, but no function could yet be ascribed to mitotic phosphorylation of TBP. Some indirect experimental evidence suggested that the phosphorylation state of TBP is not responsible for the impaired activity of TFIIIB and TFIID. Affinity-purified TAF_{III} fractions stimulated pol III transcription in M-phase extracts, indicating that the TAF_{III} components were limiting or inactive (White *et al.*, 1995). Likewise, the TBP moiety of TFIID, although specifically phosphorylated during mitosis, appears to be irrelevant for inactivation of pol II transcription, because mitotic inactivation of TFIID affected activator-dependent but not basal pol II transcription (Segil *et al.*, 1996). Thus, the TBP moiety of TFIID and TFIIIB, although specifically phosphorylated during mitosis, does not appear to be the relevant target for inactivation of pol II and pol III transcription.

Our results suggest that the same is true for pol I transcription. Analysis of the electrophoretic mobility by SDS–PAGE revealed that mitotic SL1 contains the most highly phosphorylated, i.e. the most slowly migrating form of TBP. All putative target sites for cdc2/cyclin B reside within the variable N-terminal part of TBP, and consistent with this, *in vitro* phosphorylation experiments showed that the core domain of TBP is not phosphorylated by cdc2/cyclin B. To investigate whether or not phosphorylation of the Ser/Pro and Thr/Pro sites within the variable N-terminal part of human TBP plays a role in the shut-down of pol I transcription during mitosis, we compared the activity of SL1 containing full-length or N-terminally truncated TBP prepared from asynchronous or mitotic cells. We have previously found that the N-terminal part of human TBP can be deleted without affecting SL1 activity (Rudloff *et al.*, 1994). We now demonstrate that the respective TBP–TAF_I complexes, e.g. eSL1 and eΔNSL1, which exert the same transcriptional activity when isolated from asynchronous cultures, can both be inactivated by cdc2/cyclin B-directed phosphorylation. Moreover, SL1 activity was abrogated in mitotic cells, irrespective of whether the TBP–TAF complexes were isolated from cells expressing full-length or truncated TBP. The lack of a direct correlation between the phosphorylation state of TBP and SL1 activity suggests that, by analogy to TFIID and TFIIIB, it is the phosphorylation

of a TAF subunit(s) rather than of TBP that is responsible for pol I transcriptional inhibition. Nevertheless, we cannot exclude the possibility that hyperphosphorylation of the N-terminal domain of TBP plays a role in regulating SL1 activity. When phosphorylated during mitosis, the N-terminal domain could have a repressor function either by masking a region of the core domain required for interaction with another transcription factor(s) or by conferring an inactive conformation on the C-terminal domain. In this context, it is noteworthy that the electrophoretic mobility of TBP present in mitotic SL1 was lower than TBP phosphorylated *in vitro* by cdc2/cyclin B (compare Figures 1, 2 and 4).

Inactivation of SL1 *in vitro* by cdc2/cyclin B was accompanied by phosphorylation of $hTAF_I110$ and TBP. *In vivo*, phosphorylation of a third subunit, $hTAF_I68$, was observed. Both $hTAF_I110$ and $hTAF_I68$ were also phosphorylated in asynchronous cells, and the functional significance of this constitutive phosphorylation of these TAF_Is is not yet known. Consistent with $hTAF_I110$ being phosphorylated in interphase cells, tryptic digests and phosphopeptide mapping revealed two $hTAF_I110$ phosphopeptides that were labeled both in HeLa and Sf9 cells. Significantly, one additional spot (spot **c**) was phosphorylated both in mitotic HeLa cells expressing tagged $hTAF_I110$, and after co-infection of Sf9 cells with baculoviruses encoding TAF_Is, TBP, cdc2 and cyclin B. Spot **c** precisely coincided with a tryptic peptide derived from recombinant $hTAF_I110$ that was phosphorylated *in vitro* by cdc2/cyclin B, and therefore is likely to be targeted by cdc2/cyclin B *in vivo* and *in vitro*. However, a final answer has to await the identification of specific phosphorylation sites within the TAF_I(s) and the elucidation of the consequences of site-directed mutagenesis on SL1 function.

Genetic and biochemical data have suggested that TAF_{II} subunits of TFIID are required for progression through G_1 (Hisatake *et al.*, 1993; Ruppert *et al.*, 1993; Walker *et al.*, 1997), indicating a link between the cell's cycle and its transcriptional machinery. However, the previous studies did not address the question of how the transcriptional apparatus is altered by mitotic phosphorylation. Our results demonstrate that reversible phosphorylation of SL1 during the cell cycle affects an early step in pre-initiation complex assembly, and therefore has dramatic functional consequences. Human SL1 has no detectable sequence-specific DNA-binding activity on its own, but it associates with the human rDNA promoter through specific interactions with UBF (Bell *et al.*, 1988, 1990). Given that the interaction between SL1 and UBF is a prerequisite for the formation of a productive pol I pre-initiation complex, our finding that mitotic phosphorylation impairs the association of SL1 with UBF provides a molecular explanation for the mechanism underlying repression of pol I transcription during mitosis. We could show a direct correlation between phosphorylation-dependent inactivation of SL1 in M-phase extracts and a decrease of SL1–UBF interactions. Similarly, pre-incubation of mitotic extract from mouse cells with ATP, but not with AMP-PNP, abolished the interaction of the murine factor TIF-IB with UBF. TIF-IB from asynchronous cell extracts, on the other hand, efficiently bound to UBF irrespective whether or not it was pre-incubated in the presence of ATP or the ATP analog. This finding underscores the interference of mitotic phosphorylation on the interaction between UBF and SL1/TIF-IB, and suggests that despite differences in the primary structure of human and mouse pol-I-specific TAF_Is which account for the species specificity of pol I transcription (Heix and Grummt, 1995; Heix *et al.*, 1997), the network of protein–protein contacts is conserved between human and mouse.

Although gaps still remain, our studies uncover an intricate connection between cell-cycle regulation and transcriptional control. Apparently, regulators of cell-cycle progression, the cyclins and the cyclin-dependent kinases, regulate transcription of all eukaryotic nuclear RNA polymerases. Moreover, not only is TBP a common component of all transcription initiation complexes and the TAFs impart selectivity and regulatory potential to the respective RNA polymerase, but the individual TBP–TAF complexes, i.e. SL1/TIF-IB, TFIID and TFIIIB, are central targets of mitotic repression. The functional dissection of the molecular mechanisms that regulate rDNA transcription during cell cycle progression will reveal which components of the pol I transcription machinery are also modified and contribute to cell-cycle dependent fluctuations of rRNA synthesis.

Materials and methods

Cell culture, synchronization and extract preparation

HeLa cells or the two stable cell lines that express epitope-tagged human TBP (LTRα3; Zhou *et al.*, 1992) or a tagged TBP mutant (eΔNTBP, clone 20; Zhou *et al.*, 1993) were grown at a density of $4–8\times10^5$ cells/ml in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). Whole-cell extracts (Manley *et al.*, 1980) were prepared from either logarithmically growing cells or from cells that were treated for 25 h with 2.5 mM thymidine, released from the S-phase block for 5 h, and then blocked in G_2–M phase by incubation for 8 h in the presence of 40 ng/ml of nocodazole (Pines and Hunter, 1991). Murine FT210 cells (Th'ng *et al.*, 1990) were maintained at 32°C in RPMI supplemented with 10% newborn calf serum (NCS). For synchronization, FT210 cells were arrested in G_2 by incubation for 18 h at the nonpermissive temperature (39°C), and then cultured for 1 h at 32°C to start progression through M-phase. For FACS analysis, 1×10^6 cells were fixed and stained with propidium iodide. Whole-cell extracts were dialyzed against buffer AM-100 (100 mM KCl, 20 mM Tris–HCl pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, 0.5 mM DTE, 1 mM PMSF), clarified by centrifugation at 12 000 *g* for 10 min, frozen in liquid nitrogen and stored at –80°C.

Purification of transcription factors

The fractionation scheme for purification of murine pol I and pol-I-specific transcription factors has been described previously (Schnapp and Grummt, 1996). To separate proteins on phosphocellulose, 80 ml of nuclear extract (750 mg protein) from log phase HeLa cells were applied to 80 ml phosphocellulose resin in buffer AM-100 and step-eluted with buffer A (same as AM-100 without $MgCl_2$) containing 350, 600 and 1000 mM KCl, respectively. SL1 activity was recovered in the 1 M KCl step. For affinity-purification of SL1, anti-TBP (Brou *et al.*, 1993) or anti-$mTAF_I95$ (Heix *et al.*, 1997) antibodies were covalently coupled to Dynabeads containing sheep anti-mouse IgG or sheep anti-rabbit IgG (Dynal), and incubated in buffer AM-100 supplemented with 2 mg/ml BSA and 2 mg/ml phosphatidylcholine to block nonspecific interactions. Packed beads (7.5 μl) were incubated with 45 μl of HeLa cell extracts (450 μg protein) in buffer AM-300 for 2 h at 4°C, washed successively with buffers AM-1000/0.1% NP-40, AM-700/0.1%NP-40 and AM-300/0.1%NP-40, and finally resuspended in AM-100. SL1 was either eluted with the epitope peptide (Eberhard *et al.*, 1993), or used attached to magnetic beads for transcription and Western blot analysis (Heix *et al.*, 1997). To measure eSL1 and eΔNSL1 activity, 3 mg of whole-cell extract proteins were incubated with 20 μl of protein A–Sepharose loaded with monoclonal anti-HA antibodies 3F10 (Boehringer Mannheim). After washing, bead-bound SL1 was assayed in the reconstituted transcription system.

In vitro transcription assays

$pHrP_2$, a plasmid containing 5′-terminal human rDNA sequences from –411 to +387 with respect to the transcription start site, was linearized with *Eco*RI and used as template in the cell-free transcription system. 25 µl standard transcription reactions contained 25 µg of extract proteins, 80 ng of linear template DNA, 12 mM Tris–HCl (pH 7.9), 0.1 mM EDTA, 0.5 mM DTE, 5 mM $MgCl_2$, 80 mM KCl, 10 mM creatine phosphate, 12% glycerol, 0.66 mM each of ATP, CTP, GTP, 0.01 mM UTP and 1.5 µCi of [α-^{32}P]UTP. After incubation for 60 min at 30°C, transcripts were isolated and analyzed on 4.5% polyacrylamide gels. SL1 activity was monitored in a reconstituted transcription system containing 50 ng of linearized template DNA ($pHrP_2$/*Eco*RI), 4 µl of a human pol-I-containing fraction (H-400), 2.5 µl of mouse TIF-IA/TIF-IC (poly-L-Lysin–Agarose fraction), and 5 ng of recombinant hUBF immunopurified as a FLAG-tagged fusion protein from baculovirus-infected Sf9 cells (Beckmann *et al.*, 1995; Voit *et al.*, 1997). When SL1 was phosphorylated by incubation with mitotic extract in the presence of ATP, 0.5 µM okadaic acid was added to inhibit cellular phosphatases.

If crude extracts or purified factors were assayed for their capability to rescue transcriptional repression, extracts from asynchronous or mitotic cells were pre-incubated for 30 min at 30°C with 0.2 mM ATPγS in a volume of 12.5 µl to thiophosphorylate, and thus inactivate, the target protein(s) by the mitotic kinase. Then, 2.5 mM DMAP and 0.4 mM AMP-PNP and GMP-PNP were added and transcriptions were started by adding template DNA, nucleotides and the respective protein fractions.

Purification and assay of cdc2/cyclin B kinase

Cdc2/cyclin B was purified either from starfish oocytes as described (Labbé *et al.*, 1991), from mitotic HeLa cells or from baculovirus-infected insect cells. Briefly, 60 µl of extract from mitotic HeLa cells were incubated with 1.5 µg of immobilized α-cyclin B antibodies (Santa Cruz, GNS1) in buffer AM-300 (300 mM KCl, 20 mM Tris–HCl pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, 1 mM PMSF, 1 mM DTE) containing 0.5% NP-40 and 1 mM ATP. As a control, 1.5 µg of α-tubulin antibodies (Oncogene Science) were used. After incubation for 2 h at 4°C, the beads were washed in buffer AM-500 and AM-300, and finally resuspended in buffer AM-100. Alternatively, cdc2/cyclin B was overexpressed in Sf9 cells and purified as described previously (Krude *et al.*, 1997), except that final purification involved chromatography on a MonoS PC1.6/5 column (SMART system, Pharmacia) according to Wihelm *et al.* (1997). Kinase activity was measured in 10 µl assays containing 20 mM Tris–HCl (pH 7.9), 60 mM KCl, 8 mM $MgCl_2$, 0.04 mM ATP, 1.0 µCi of [γ-^{32}P]ATP (5000 Ci/mmol) and 10 µg of substrate peptide (PKTPKKAKKL) as described previously (Casnellie, 1991).

In vitro phosphorylation and dephosphorylation of SL1

Anti-TBP antibodies (3G3; Brou *et al.*, 1993) were immobilized on magnetic beads (Dynal) and added to a fraction containing partially purified SL1 (S-700; Schnapp and Grummt, 1996). After incubation for 2 h at 4°C, the beads were washed with buffers AM-1000/0.1% NP-40, AM-700/0.1% NP-40 and AM-300/0.1% NP-40, and bound complexes were eluted in AM-300/0.1% NP-40 with 2 mg/ml of the epitope petide (P81; Brou *et al.*, 1993) in the presence of 0.1 mg/ml insulin. Aliquots of immunopurified SL1 or purified recombinant TAF_Is (Heix *et al.*, 1997) were incubated with bead-bound cdc2/cyclin B in the presence of [γ-^{32}P]ATP, and labeled subunits were analyzed by SDS–PAGE and autoradiography. Histidine (His)-tagged TBP and ΔNTBP were expressed in *E.coli* and purified on a Ni^{2+}-chelate column according to the manufacturer's protocol (Qiagen).

For dephosphorylation, SL1 was bound to magnetic beads via anti-TAF_I95 antibodies, resuspended in buffer AM-100 and incubated for 20 min at 30°C with 8 units of SAP (Amersham Life Science). After addition of 20 mM KF and 1 mM sodium vanadate, the immobilized SL1 was washed with AM-1000/0.1% NP-40, AM-700/0.1% NP-40 and AM-300/0.1% NP-40, and finally resuspended in buffer AM-100.

Metabolic labeling and phosphopeptide analysis of SL1

Asynchronous or M-phase arrested HeLa cells (4×10^6) were cultured for 30 min in phosphate-free DMEM, 10% FCS. Then, 1 mCi/ml [^{32}P]orthophosphate was added and cells were metabolically labeled by culturing for 5 h in the absence or presence of nocodazole. Cells were lysed in a buffer containing 20 mM Tris–HCl (pH 7.9), 1% NP-40, 250 mM KCl, 5 mM $MgCl_2$, 20% glycerol, 1 mM EDTA, 10 mM KH_2PO_4, 20 mM KF, 1 mM sodium orthovanadate, protease inhibitor cocktail (Boehringer Mannheim) and 1 mM DTT. SL1 was immunoprecipitated with anti-TBP antibodies (3G3; Brou *et al.*, 1993), eluted with the epitope peptide and precipitated with anti-$mTAF_I$95 antibodies (Heix *et al.*, 1997). Proteins were separated by SDS–PAGE and visualized by autoradiography.

For expression in insect cells, 3×10^6 Sf9 cells were co-infected with baculoviruses encoding FLAG-tagged human TAF_I110, TAF_I68, TAF_I48 and TBP (Heix *et al.*, 1997) in the absence or presence of baculoviruses encoding human His-tagged cdc2 and cyclin B. Cells were metabolically labeled for 4 h, 42 h post-infection, in the presence of 1 mCi/ml [^{32}P]orthophosphate in phosphate-free Grace's medium (Gibco-BRL) containing 10% dialyzed FCS. The cells were lysed in RIPA buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl, 0.5% sodium deoxycholate, 0.5% NP-40, 0.5% SDS, 10 mM EGTA, 20 mM KF, 1 mM sodium orthovanadate, 10 mM K_2HPO_4, 2 mg/ml of each leupeptin, aprotinin and pepstatin) as described previously (Voit *et al.*, 1995) and radiolabeled TAF_Is were immunoprecipitated using M2-anti-FLAG antibodies covalently attached to agarose beads (Kodak). The region containing TAF_I110 was cut out and processed for tryptic phosphopeptide mapping according to Boyle *et al.* (1991).

UBF–SL1 interaction assay

Recombinant N-terminally FLAG-tagged hUBF was immobilized on M2–agarose beads (Kodak) at 2 µg/µl of packed beads as described previously (Voit *et al.*, 1997). As a control, beads saturated with the FLAG epitope peptide were used. Two microliters of the respective resin were incubated with 30 µl of whole-cell extract (~300 µg protein) in buffer AM-300 for 30 min at room temperature. The beads were washed with AM-300 containing 0.1% NP-40 and subjected to Western blot analysis using affinity-purified α-TAF_I95 antibodies.

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