Casein kinase II regulation of yeast TFIIIB is mediated by the TATA-binding protein

Ataollah Ghavidel and Michael C. Schultz

Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

The highly conserved protein kinase casein kinase II (CKII) is required for efficient Pol III transcription of the tRNA and 5S rRNA genes in Saccharomyces cerevisiae. Using purified factors from wild-type cells to complement transcription extracts from a conditional lethal mutant of CKII we show that TFIIIB is the CKII-responsive component of the Pol III transcription machinery. Dephosphorylation of TFIIIB eliminated its ability to complement CKII-depleted extract, and a single TFIIIB subunit, the TATA-binding protein (TBP), is a preferred substrate of CKII in vitro. Recombinant TBP purified from Escherichia coli is phosphorylated efficiently by CKII and, in the presence of a limiting amount of CKII, is able to substantially rescue transcription in CKII-deficient extract. Our results establish that TBP is a key component of the pathway linking CKII activity and Pol III transcription and suggest that TBP is the target of a CKII-mediated regulatory mechanism that can modulate Pol III transcription.

[Key Words: Transcription; RNA polymerase III; TFIIIB; TBP; casein kinase II]

Received July 10, 1997; revised version accepted September 5, 1997.

The genes transcribed by RNA polymerase III (Pol III), like many transcribed by RNA Pol II, are strictly regulated according to cell cycle position, growth rate, and developmental phase (Hoeffler et al. 1988; Tower and Sollner-Webb 1988; Wolfe and Brown 1988; White et al. 1989, 1995a; Hartl et al. 1993; Reynolds 1993; Gottesfeld et al. 1994; Sethy et al. 1995). Pol III transcription also responds to a number of pathological conditions. For example, oncogenic transformation is generally associated with increased Pol III transcription (discussion in White et al. 1990, 1996). The regulation of class III gene expression operates principally at the level of the transcription initiation machinery, which includes three fundamental protein components, RNA Pol III, TFIIIB, and TFIIIC (for review, see Geiduschek and Kassavetis 1992; Willis 1993). TFIIIB is a complex of the highly conserved TATA-binding protein (TBP) with at least two other proteins generally referred to as TBP-associated factors or TAFs. There are two Pol III-specific TAFs in Saccharomyces cerevisiae (TFIIIB70/Brf1p and TFIIIB90/Tfc5p; gene identification summarized in Kumar et al. 1997), and one of these, Brf1p, is conserved in human (Wang and Roeder 1995). TFIIIC, composed of six subunits in yeast, is the principal sequence-specific DNA-binding component of the Pol III machinery (for review, see Geiduschek and Kassavetis 1992; Willis 1993).

TFIIIB and TFIIIC are the primary targets of the regulatory mechanisms that affect Pol III transcription. For example, TFIIIB is the target of the mechanism that represses Pol III transcription in metaphase extracts from Xenopus eggs (Hartl et al. 1993; Gottesfeld et al. 1994; Leresche et al. 1996) and mammalian tissue culture cells (White et al. 1995b). In mammalian cells and yeast both TFIIIB and TFIIIC are limiting in stationary phase (Tower and Sollner-Webb 1988; Sethy et al. 1995) and TFIIIC is limiting early in S phase in mammalian cells (White et al. 1995a). In the case of TFIIIB, the regulatory functions of individual subunits are becoming clear. The TBP subunit of Xenopus TFIIIB may be repressed in metaphase extracts (Leresche et al. 1996), and TBP is limiting for Pol III transcription in Drosophila tissue culture cells (Trivedi et al. 1996). In mammalian cells the metaphase silencing of Pol III transcription results from repression of one or more of the Pol III TAFs (White et al. 1995b), and a decline in Brf1p expression partly accounts for decreased TFIIIB activity in the stationary phase of yeast (Sethy et al. 1995). Considering these results, there has been intense interest in identifying the signaling pathways that impinge on the Pol III transcription machinery. A substantial body of evidence suggests that these signaling pathways involve protein phosphorylation events. As a result, protein kinases and phosphatases involved in the regulation of Pol III transcription are actively being sought.

One highly conserved S/T/Y protein kinase implicated in the regulation of Pol III transcription is casein kinase II (CKII; for review, see Pinnata and Schatz 1990; Tuazon and...
Regulation of TATA-binding protein by CKII

Traugh 1991; Litchfield and Lüscher 1993; Allende and Allende 1995). The CKII holoenzyme, a heterotetramer of two catalytic α subunits (αα‘) and two noncatalytic β subunits (ββ‘), is generally implicated in transcriptional regulation by the observation that RNA synthesis is severely inhibited when a temperature-sensitive mutant of yeast CKIIα‘ is shifted to the restrictive temperature (Hanna et al. 1995). We further reported that efficient Pol III transcription of the yeast 5S rRNA and tRNA genes requires CKII, and we have presented evidence suggesting that CKII activity is required for efficient initiation (Hockman and Schultz 1996). Taking advantage of the ability to prepare a CKII- and Pol III-deficient extract from the yeast temperature-sensitive mutant of CKII, we have pursued a biochemical approach aimed at identifying the component of the Pol III transcription machinery that responds to CKII inactivation. We show that the transcription factor TFIIIB is specifically defective in extracts depleted of CKII and that TBP, the central transcription factor in the nucleus, is the target of CKII among the subunits of TFIIIB.

Results

Transcription in CKII-deficient extract is rescued by TFIIIB

Two isogenic strains, designated CKA2 and cka2ts (temperature-sensitive), were used in this study. These strains differ only in the sequence of the CKA2 gene (encoding CKIIα‘), which is wild type in CKA2 but carries mutations that confer a temperature-sensitive-lethal phenotype in strain cka2ts (Hanna et al. 1995). Whether the starting material is lysed cells (Hockman and Schultz 1996) or isolated nuclei (not shown), CKII activity in transcription extracts from strain cka2ts is ∼20-fold lower than wild type. To identify the CKII-responsive component of the Pol III transcription machinery, active transcription factors purified from wild-type extract were added to cka2ts extract with low CKII activity and impaired specific transcription. Initially we prepared two complementing fractions from wild-type cells, one enriched in Pol III/TFIIIC (fraction P/C) and another enriched in TFIIIB (Fig. 1A). Both wild-type and cka2ts extracts were responsive to TFIIIB, although the magnitude of the response differed significantly between the extracts. TFIIIB slightly stimulated transcription in wild-type extract (Fig. 1B, lanes 1-4), in agreement with previous reports (see Sethy et al. 1995). In contrast, TFIIIB strongly stimulated transcription in cka2ts extract; the purified factor was able to restore activity in the mutant to that of wild type supplemented with TFIIIB (Fig. 1B, lanes 5-8). Based on this result we propose that the defect in cka2ts extract results primarily from inactivation of TFIIIB.

This conclusion is supported by complementation experiments using the P/C (Pol III/TFIIIC) fraction. Titration of fraction P/C into wild-type extract was either without effect (not shown) or very slightly stimulated transcription (Fig. 1C, lanes 1-4). The P/C fraction slightly stimulated cka2ts extract, but it did not restore transcription to the wild-type level (Fig. 1C, lanes 5-8). This result suggests that neither RNA Pol III nor TFIIIC is significantly affected by the loss of CKII activity.

The Pol III transcription machinery can be partially purified by DEAE chromatography (Riggs and Nomura 1990). This procedure provides a 300 mM KCl cut from DEAE, referred to as fraction D300, in which the difference in transcription capacity between wild-type and cka2ts extracts is accentuated (Fig. 2A). In view of the results in Figure 1, we reasoned that a cka2ts-D300 fraction that is severely impaired compared to the wild type fraction (prepared in parallel) would be highly sensitive to the addition of TFIIIB. TFIIIB massively stimulates transcription in cka2ts-D300 (Fig. 2B). To show that this stimulatory activity corresponds to TFIIIB we supplemented cka2ts-D300 with aliquots of fractions from the

Figure 1. Pol III transcription in CKII-deficient whole cell extract is rescued by TFIIIB. (A) (Top) The P/C and TFIIIB fractions reconstitute Pol III transcription. tRNA transcription (20 ng template/reaction) was assayed using 4 µl of P/C, either added to 10-µl aliquots of fractions from the hydroxyapatite column used to purify TFIIIB (lanes 1-7), or on its own (lane 8). (Bottom) The peak of TFIIIB activity obtained by hydroxyapatite chromatography corresponds to the peak of TBP. Immunoblot of TFIIIB fractions (12-µl aliquots) using antiserum raised against recombinant TBP. (B) TFIIIB (0, 0.4, 1, and 2.5 µl) slightly stimulates 5S rRNA transcription in CKA2 extract (lanes 1-4) and fully restores transcription in cka2ts extract (lanes 5-8). (C) The P/C fraction (0, 0.8, 2 and 5 µl) does not significantly stimulate 5S rRNA transcription in CKA2 or cka2ts extract. The reactions in B and C used 60 µg of extract and plasmid pY5S at 400 ng/reaction.
TFIIIB does not contain an activity that can restore the enzymatic function of temperature-sensitive CKII. Although the fraction that stimulates cka2ts extract precisely coelutes with TFIIIB, our results do not rule out the possibility that the TFIIIB fraction contains a factor that restores CKII activity in cka2ts extract (or the D300 fraction). This factor could be CKII itself or a CKII activator (for review, see Tuazon and Traugh 1991; Allende and Allende 1995). We tested for CKII in the TFIIIB fraction using the peptide phosphorylation assay. CKII activity was measured in 3.2 µl of TFIIIB, an amount that fully rescues transcription in cka2ts-D300 (Fig. 2B). These measurements are compared to the CKII activity in 15 µg of wild-type D300 fraction (see Fig. 2A). As shown, TFIIIB contains ~5% of the CKII activity measured in the CKA2-D300 fraction (Fig. 4A). The content of CKII in the TFIIIB fraction was also assessed by immunoblotting using an antiserum that specifically recognizes the $\beta'$ subunit of yeast CKII. We could not detect CKII in amounts of TFIIIB similar to those used for the reconstitution of transcription (Fig. 4B), confirming the result obtained by assaying kinase activity. Therefore, CKII is substantially depleted in the fractions used for reconstitution of transcription.

To test whether the TFIIIB fraction contained an activity that could stimulate the temperature-sensitive CKII, we assayed bulk CKII activity in the D300 fractions with and without added TFIIIB (Fig. 4C). Increased CKII activity can be measured when TFIIIB is added to cka2ts-D300; however, the activity remains 10-fold lower than in the equivalent reaction in which TFIIIB is added to CKA2-D300. Furthermore, transcription in cka2ts-D300 is not stimulated when purified CKII is used to raise the bulk CKII activity to the level observed upon addition of TFIIIB (not shown). We conclude that the transcription machinery responds directly to CKII depletion and that TFIIIB is the component of the transcription machinery that is inactivated when CKII is depleted.

TFIIIB must be phosphorylated to restore transcription in cka2ts extract

The results presented above demonstrate that TFIIIB responds to changes in CKII activity. This raises the pos-

Figure 2. The transcription defect in CKII-depleted extract is preserved after partial purification of the Pol III transcription machinery and is fully rescued by TFIIIB. (A) tRNA transcription in the 300 m M KCl DEAE fraction from CKA2 and cka2ts extracts. (B) TFIIIB marginally stimulates tRNA transcription in CKA2-D300 (lanes 1-4) and fully restores transcription in cka2ts-D300 (lanes 5-8). The reactions received 0, 2, 5, or 10 µl of hydroxyapatite TFIIIB and 13 µg of D300 fraction. (C) The peak of TFIIIB activity (fraction 36, Fig. 1A) corresponds to the peak of activity capable of rescuing transcription in cka2ts-D300 (lane 5). Transcription was assayed using 13 µg of cka2ts-D300 added to 10-µl aliquots of fractions from the hydroxyapatite column used to purify TFIIIB. The template was at 20 ng/reaction.
sibility that direct CKII phosphorylation of TFIIIB is important for the activity of TFIIIB. We therefore tested whether dephosphorylation of TFIIIB affects its ability to stimulate transcription in cka2ts extract (Fig. 5). TFIIIB was treated with phosphatase in the presence or absence of the phosphatase inhibitor sodium vanadate and added to cka2ts nuclear extract (in which CKII activity is <2.5% of wild type; data not shown). TFIIIB retained its capacity to stimulate cka2ts extract when incubated with phosphatase in the presence of sodium vanadate (cf. lanes 1, 3, 6, 7). In contrast, TFIIIB treated in turn with phosphatase and then sodium vanadate was unable to stimulate cka2ts extract (cf. lanes 1, 3, 4, 5). Because sodium vanadate does not affect transcription on its own (lane 8), we conclude that TFIIIB must be phosphorylated to restore transcription to cka2ts extract.

A single subunit of TFIIIB, the TBP, is phosphorylated by CKII. Four observations suggest that phosphorylation of TFIIIB by CKII can be limiting for transcription in wild-type extracts: (1) TFIIIB is limiting in wild-type extracts (Fig. 1B, lanes 1–4); (2) TFIIIB activity declines when CKII activity declines (Fig. 1B, lanes 5–8); (3) TFIIIB loses transcriptional activity when it is dephosphorylated (Fig. 5); (4) CKII stimulates transcription in some whole cell extracts from CKA2 cells, although our CKII preparations do not contain TFIIIB as assayed by immunoblotting (Fig. 6). These observations suggest that CKII phosphorylation of a TFIIIB subunit is limiting for TFIIIB activity and, therefore, that a component of the wild-type TFIIIB fraction may be a substrate for in vitro phosphorylation by CKII. Likely substrates would include the TFIIIB subunits TBP, Brf1p, and TFc5p, respectively of approximate molecular masses 29, 70, and 90 kD. To examine the CKII phosphorylation of TFIIIB, the Cibachron blue fraction was incubated with CKII in the presence of \[\gamma^{32P}\]GTP as the phosphate donor (Fig. 7A). GTP was chosen because it is an efficient phosphate donor in reactions catalyzed by CKII but not most other protein kinases. The reaction products were analyzed by immunoblotting using a chemiluminescence detection system. The short exposures appropriate for the chemiluminescence assay were insufficient to detect the signal from the \[^{32P}\]labeled proteins. Following immunodetection the membrane was stripped of antibody and then exposed to film to detect the \[^{32P}\] signal.

When incubated alone, yeast CKII autophosphorylates its \(\beta\) and \(\beta'\) subunits (Fig. 7A, lane 1; Bidwai et al. 1994). TFIIIC does not contain significant GTP-dependent protein kinase activity on its own, and when used as a substrate for CKII only minor bands other than the CKII \(\beta\) and \(\beta'\) are labeled (Fig. 7A, lane 2, 3). Although some components of the TFIIIB fraction are labeled when TFIIIB is incubated on its own, these products are not detected (Fig. 7A, lane 4) except in longer exposures of the film (see below). On the other hand, when TFIIIB is used as a substrate for CKII, a single band in addition to the subunits of CKII is heavily labeled (Fig. 7A, lane 5). This band migrates at \(~29\) kD, similar to the molecular mass of yeast TBP (yTBP). The possibility that the labeled 29-kD band is TBP was examined by immunoblot-
ting using a yTBP antiserum (Fig. 7B). As expected, TBP is detected in the TFIIIB (lanes 4, 5) but not the TFIIIC fraction (lanes 2, 3). Furthermore, the protein labeled when TFIIIB is phosphorylated with CKII exactly comigrates with the band that reacts with the TBP antiserum. We also find that the labeled 29-kD band comigrates with recombinant TBP run in parallel and detected by silver staining or immunoblotting (not shown).

We were surprised to observe prominent labeling of only one polypeptide among those present in the TFIIIC and TFIIIB fractions, as our most purified TFIIIC and TFIIIB preparations contain ~30 and 20 polypeptides, respectively, and many of the nine proteins comprising TFIIIC and TFIIIB contain CKII consensus sites (Hockman and Schultz 1996). Nonetheless, only TBP is significantly labeled by CKII. We conclude that the in vitro phosphorylation of TFIIIB-associated TBP by CKII is highly specific.

To extend the results obtained using cellular TFIIIB and purified CKII we tested whether recombinant TBP can be phosphorylated by CKII. Yeast TBP was expressed in Escherichia coli and purified to >95% homogeneity by ion exchange chromatography. Recombinant TBP, whether or not it was pretreated with phosphatase, was phosphorylated by CKII in the presence of $[^{32}P]ATP$ as the phosphate donor (Fig. 7C, lanes 1–5; the same result was obtained with GTP (data not shown)). Consistent with the idea that CKII is responsible for the observed phosphorylation of cellular and recombinant TBP, phosphorylation is completely blocked by heparin, a widely used inhibitor of CKII (not shown). This observation, combined with the results in Figure 7, A and B, demonstrates that in our experiments the only component of the wild-type Pol III transcription machinery that is a good substrate for CKII is the TBP subunit of TFIIIB.

Figure 6. (A) TFIIIB does not substantially copurify with CKII. Analysis of TFIIIB by immunoblotting using a polyclonal antiserum raised against recombinant TBP. (Lane 1) Five microliters of hydroxypatite TFIIIB; (lane 2) 1 µl of purified TBP; (lane 3) 15 µl of purified CKII. (B) CKII can stimulate 5S rRNA transcription in whole cell extract (30 µg) from log phase cells. Plasmid pY'5S was used as the template at 400 ng/reaction.

Figure 4. Rescue of transcription by TFIIIB does not involve restoration of CKII activity. (A) The TFIIIB fraction does not contain a significant amount of CKII activity. Bulk CKII activity is compared in 15 µg of CKA2–D300 extract (see Fig. 2A) and 4 µl of hydroxypatite TFIIIB. CKII activity is expressed as a percentage of activity in 15 µg of CKA2–D300 (arbitrarily assigned the value of 100%). (B) The TFIIIB fraction does not contain a significant amount of the β' subunit of CKII. Analysis of the TFIIIB fraction was performed by immunoblotting with a polyclonal antiserum raised against a carboxy-terminal peptide of yeast β'. (Lane 1) Purified CKII (1 µl); (lane 2) hydroxypatite TFIIIB (15 µl); (lane 3) Cibachron blue TFIIIB (15 µl). (C) TFIIIB does not contain an activity that can restore the enzymatic function of temperature-sensitive CKII. Bulk CKII activity is expressed relative to wild type, which is arbitrarily assigned the value of 100%. The reactions used 6 µg of whole cell extract (WCE), 15 µg of D300, and 4 µl of hydroxypatite TFIIIB.

Figure 5. TFIIIB must be phosphorylated to restore transcription in cka2ts extract. TFIIIB was treated with phosphatase (CIP) in the presence or absence of sodium vanadate and then added to cka2ts nuclear transcription extract. The final composition of each reaction is indicated in the panel above the autoradiograph; the timing of CIP inactivation by sodium vanadate is indicated below the lane numbers. Plasmid pY5S was used as the template at 400 ng/reaction.

Figure 7. TFIIIB does not substantially copurify with CKII. Analysis of TFIIIB by immunoblotting using a polyclonal antiserum raised against recombinant TBP. (Lane 1) Five microliters of hydroxypatite TFIIIB; (lane 2) 1 µl of purified TBP; (lane 3) 15 µl of purified CKII. (B) CKII can stimulate 5S rRNA transcription in whole cell extract (30 µg) from log phase cells. Plasmid pY'5S was used as the template at 400 ng/reaction.
Upon longer exposure of gels such as those in Figure 7 we detect a TFIIIB-associated activity that phosphorylates several proteins in the TFIIIB preparation, including TBP (Fig. 7C, lane 6; cf. with short exposure in A, lane 4). The level of TBP phosphorylation by the TFIIIB-associated kinase, however, is minor compared with that obtained when CKII is added to TFIIIB (lane 9). The molecular identity of the TFIIIB-associated kinase is not clear at the present time. It is unlikely to be a conventional form of CKII, as it is relatively insensitive to heparin (not shown) and is not associated with a significant amount of the β' subunit (Fig. 4B).

Our results suggest that CKII phosphorylation of TBP increases the activity of TFIIIB. These results could be explained if the stability of TBP is affected by CKII. For example, the observation that transcription is severely impaired in cka2ts–D300 (Fig. 2A) could reflect loss of TBP during chromatography. To test whether changes in the amount of TBP could explain our results, we assessed the relative concentration of TBP in whole cell extract and various fractions from CKA2 and cka2ts cells by immunoblotting. We observe, in two independent sets of samples, that the concentration of TBP is virtually identical in CKA2 and cka2ts whole cell extracts and fractions from DEAE (Fig. 7D). We conclude that our results cannot be explained by an effect of CKII on the stability or chromatographic properties of TBP.

TBP and a limiting amount of CKII rescues Pol III transcription in CKII-deficient extract

We next tested whether the activity of TBP as a Pol III transcription factor is influenced by CKII. This was done by supplementing nuclear transcription extract with recombinant TBP and purified yeast CKII, either alone or in combination (Fig. 8A). We observe significant stimulation of transcription in cka2ts extract by TBP alone (lanes 1–3). [Large amounts of TBP inhibit transcription (lane 4), perhaps by nonspecific occlusion of the template or by squelching.] The observed stimulatory effect of TBP on its own suggests that a TBP-dependent step in transcription is impaired as a consequence of CKII depletion. The effect of adding TBP and CKII together supports this idea. By comparing lanes 1–3 with 5–7 in Figure 8A, it is clear that the amount of TBP required to maximally stimulate cka2ts extract is significantly low-

Figure 7. The TBP subunit of TFIIIB is preferentially phosphorylated by CKII. (A) Autoradiograph showing the labeled products resulting from in vitro phosphorylation of TFIIIB (10 µl) and affinity-purified TFIIIC (10 µl) with purified CKII (5 µl). The positions of the prestained molecular weight markers and the β subunits of CKII are shown on the left and right, respectively. (B) Immunoblotting analysis of the products in A using antiserum against TBP. As judged by the position of the markers, the immunoreactive band in lanes 4 and 5 exactly comigrates with the labeled 29-kD band in A, lane 5. (C) Recombinant TBP is phosphorylated by CKII in vitro. Reactions contained CKII on its own (lane 1; 0.5 µl), TBP on its own (0.1 µl; lane 2), CKII plus increasing amounts of recombinant TBP (0.1, 0.2, 0.5 µl; lanes 3–5), TFIIIB on its own (Cibachron blue fraction, 12 µl; lane 6), and CKII plus increasing amounts of TFIIIB (4, 8, 12 µl; lanes 7–9). Kinase/s in TFIIIB phosphorylate a number of proteins (white dots) that are not detected in shorter exposures (A, lane 4). (D) TBP is not depleted in whole cell extract or the DEAE fractions of cka2ts cells. Western blot analysis of whole cell extract (WCE; 42 µg per lane), the DEAE flowthrough (FT; 22 µg/lane), and 300 mM KCl fractions (300; 28 µg/lane) using TBP antiserum. Recombinant TBP (0.5 µl, lane 9) is the marker.

Figure 8. TBP and a limiting amount of CKII rescues Pol III transcription in CKII-deficient extract. (A) Nuclear extract (16 µg) from cka2ts cells was supplemented with TBP alone (lanes 1–4) or increasing amounts of TBP in the presence of CKII (0.5 µl, lanes 6–8). The amount of CKII used in this experiment only slightly stimulated transcription on its own (cf. lanes 1 and 4). (B) The D300 fraction (11 µg) from cka2ts cells was supplemented with buffer (lane 1), TBP (2 µl, lane 2), CKII (2.5 µl, lane 3), and TBP plus CKII (lane 4). Plasmid pY5S was used as the template at 400 ng/reaction.
phorylation of TBP is required for efficient Pol III transcription in vitro. Consistent with this proposal, we note that yTBP contains three motifs that match the CKII consensus S/Txx acidic [the Y specificity motif is not present in yTBP (Wilson et al. 1997)]. Because the amino-terminal 60 residues of yTBP are dispensable for function in vivo (Reddy and Hahn 1991), potential CKII sites in this region are unlikely to play a significant role in Pol III transcription. The remaining 180 residues of TBP contain potential CKII sites at S128 and S183. Modelling studies using the VADAR program (Wishart et al. 1994) indicate that S183, which is buried in the crystal structure of TBP (Chasman et al. 1993), is highly unlikely to be solvent exposed. This residue therefore is unlikely to be available for phosphorylation by CKII. S128, on the other hand, is solvent exposed, highly conserved (Kim et al. 1993), and the CKII consensus sequence at S128 encompasses a small surface of TBP that is likely to be solvent exposed. This residue therefore might be inhibitory. The level of activation/inhibition may vary according to the site of phosphorylation, with some inhibitory sites possibly being dominant over sites required for maximal activity. The latter scenario has been described in another context; the p34cdc2 component of the p34cdc2–cyclin B complex is regulated by dominant inhibitory and stimulatory phosphorylation events (for review, see King et al. 1994). These considerations suggest the working hypothesis that TFIIIB is regulated by positive and negative phosphorylation events and that CKII is involved in the positive phosphorylation event.

**Discussion**

We have identified TFIIIB as the component of the Pol III transcription machinery that responds to the inhibition of CKII activity. We show further that TFIIIB must be phosphorylated to restore transcription in a CKII-depleted extract and that a combination of TBP and a limiting amount of CKII rescues transcription in a CKII-depleted extract to a higher level than the sum of TBP and CKII added individually. We have therefore identified an effector (CKII) and target (TFIIIB/TBP) that may be components of a mechanism for regulating Pol III transcription in yeast.

**Regulation of TFIIIB by phosphorylation**

Previous studies using extracts from Xenopus eggs suggest that the phosphorylation status of TFIIIB is important for its activity (Hartl et al. 1993; Gottesfeld et al. 1994; Leresche et al. 1996). For example, the inactive form of TFIIIB from metaphase extracts can be inactivated by phosphatase treatment. Conversely, the active inter-form of TFIIIB from metaphase extracts can be activated by CKII and TBP together. Based on these results and the evidence presented in Figures 1–7 we propose that direct CKII phosphorylation of TBP is required for efficient Pol III transcription in yeast.

The molecular target of CKII phosphorylation

Our evidence indicates that the regulation of TFIIIB by CKII occurs through direct CKII phosphorylation of the TBP subunit of TFIIIB. Specifically, the observation that CKII stimulates transcription in wild-type transcription extract correlates with the finding that only one polypeptide in our wild-type TFIIIB fraction, TBP, is efficiently phosphorylated by CKII in vitro. Furthermore, a limiting amount of CKII increases the stimulation of transcription in cka2ts extract by a fixed amount of TBP. Based on these results we propose that direct phosphorylation of TBP by CKII is required for efficient Pol III transcription in vitro. Our work potentially relates to the mounting evidence that TFIIIB is a key target of regulatory mechanisms that
govern the output of Pol III genes. In mammalian cells the response of the Pol III transcription machinery to a wide variety of internal and external cues is governed by mechanisms that regulate TFIIIB (see introductory section). In Xenopus the cell cycle regulation of Pol III transcription operates at the level of TFIIIB, perhaps through differential phosphorylation of its 92-kD and TBP subunits (Leresche et al. 1996). The Drosophila Pol III transcription machinery is sensitive to the cellular concentration of TBP, and in yeast the silencing of transcription in stationary phase is mainly due to an effect on TFIIIB/Brf1p (Sethy et al. 1995; Trivedi et al. 1996). In summary, TFIIIB is the target of a spectrum of regulatory effects that can act on distinct subunits of TFIIIB. The protein kinases involved in these diverse regulatory mechanisms remain to be fully characterized. Our results raise the possibility that CKII is one such kinase. Thus, the activity of CKII toward TBP may be regulated and therefore contribute directly to the regulation of Pol III transcription in vivo. On the other hand, CKII phosphorylation of TBP might be constitutive and ensure that the maximal level of Pol III transcription is achieved when the demand for Pol III products is at its peak. These hypotheses will be amenable to critical experimental analysis in yeast.

Materials and methods

Buffers

Buffers are described as follows: (YDBI) 50 mM KCl, 5 mM EGTA, 0.05 mM EDTA, 2.5 mM DTT, 0.4 mM PMSF, and 20% glycerol. (Buffer B) 1 mM EDTA, 1 mM DTT, 0.4 mM PMSF, and 20% glycerol. (Buffer L) 1 mM EDTA, 5 mM MgCl₂, 2.5 mM DTT, 0.2 mM PMSE, 10% glycerol, and 0.05% N-P-40. Buffers YDBI, B and L also contained 20 mM HEPES-KOH (pH 7.9) and 0.3 µg/ml of leupeptin. (Buffer K) 0.5 mM EDTA, 0.4 mM PMSF, 0.6 µg/ml of leupeptin, 0.6 µg/ml of pepstatin, and 20% glycerol. (Buffer T) 20 mM Tris-HCl (pH 8), 1 mM EDTA, 1 mM DTT, 1 mM PMSE, 2 mM benzamidine-HCl, and 10% glycerol. The concentration of KCl (mM) in buffers B, K, L, and T is designated by the number after the letter abbreviation.

Strains and extract preparation

Extracts were prepared from S. cerevisiae strains CKA2, cka2Δ, and the protease-deficient BJ5626 (Jones 1991) grown to an OD at 600 nm of 1–2 (see Hockman and Schultz 1996). CKA2 and cka2Δ were grown at room temperature, and BJ5626 at 30°C. Whole cell extracts were prepared after breaking the cells under liquid nitrogen, using a motorized mortar grinder for large-scale extracts (Schultz et al. 1997). Nuclear extracts were prepared in parallel from strains CKA2 and cka2Δ according to Dunn and Wobbe (1995).

Purification and assay of transcription factors from yeast

Large-scale whole cell extracts were fractionated according to Kassavetis et al. (1989), with modifications that take into account the presence of an inhibitor that is concentrated on Bio-Rex 70. The transcriptionally active 92-kD ammonium sulfate cut (2.8 grams of protein) of whole cell extract (7 grams) was dialyzed to YDBI and applied to 140 ml of Bio-Rex 70 (Bio-Rad). This column was step-eluted with 100, 250, and 500 mM KCl in YDBI. The 500 mM KCl fraction contained all components of the Pol III transcription machinery, although it was transcriptionally inert because of an inhibitor that is separated from Pol III/TFIIIC on DEAE and from TFIIIB on hydroxyapatite. The Bio-Rex 500 mM KCl fraction (160 mg) was dialyzed to B100 and applied to a 40-ml DEAE-Sepharose Fast Flow (Pharmacia) column equilibrated in B100; fractions were collected from the 100, 300, and 600 mM KCl washes. TFIIIB (108 mg), eluted in the flowthrough, was dialyzed to buffer K containing 25 mM KPO₄ (buffer K25). Fifty milligrams of this fraction was loaded onto a 20-ml Bio-Gel hydroxyapatite (Bio-Rad) column equilibrated in buffer K25. The column was washed with buffer K50 and developed with a 75-mM gradient (50-300 mM KPO₄) that eluted TFIIIB at ~200 mM KPO₄. TFIIIB fractions (~3 mg) were dialyzed against buffer B200 and applied to a 3-ml Cibacron blue-Sepharose column (Fluka). Following washing with buffer B200, TFIIIB was eluted with B1000. Pol III and TFIIIC (20 mg) eluted together in the 300 mM cut from DEAE. Ten milligrams of this fraction (P/C) was dialyzed to buffer L1000, precleared with 0.6 mg of pSemp3, and applied to a 1.3-ml TFIIIC affinity column [synthetic box B+29-mer coupled in 10 repeat fragments (average) to Sepharose CL2B (Kassavetis et al. 1989)]. The column was washed with buffer L200, and TFIIIC was eluted with L1000. Bulk Pol III activity was measured according to Schultz and Hall (1976). TFIIIB was monitored during chromatography by immunoblotting with a polyclonal antiserum against TBP (1:10,000 dilution). TFIIIB was also monitored by its ability to complement fraction P/C, which is transcriptionally inactive and devoid of TBP but enriched in bulk Pol III activity and TFIIIC DNA-binding activity. TFIIIC was monitored by its DNA-binding activity according to the method of Kassavetis et al. (1989). The Pol III machinery was obtained in a single fraction (D300) by applying whole cell extract in YDBI to a DEAE-Sepharose Fast Flow column (Riggs and Nomura 1990). The column was washed with 90 mM KCl/YDBI, and the transcription machinery was then eluted with 300 mM KCl/YDBI.

Expression and purification of recombinant TBP

Yeast TBP was expressed in E.coli strain BL21(DE3) and purified according to a protocol developed by Steve Hahn (pers. comm.; see also Reddy and Hahn 1991). Briefly, cells containing plasmid pSh228 were induced with 0.4 mM IPTG for 2 hr. The soluble fraction resulting from sonication of cells in buffer T50 was applied to DEAE-Sepharose Fast Flow, and the flowthrough collected. This material was applied to M mono S (Pharmacia) and eluted with a 50–500 mM KCl gradient (in buffer T). TBP was recovered in the 250–300 mM KCl fractions.

Purification of CKII and assay by peptide phosphorylation

CKII was purified according to Bidwai et al. (1994) from cells broken under liquid nitrogen using a mortar grinder (Schultz et al. 1997). The heparin–agarose fraction of CKII (85% pure) was used for most experiments. Peptide phosphorylation was performed as described (Hockman and Schultz 1996), except that a TCA precipitation step was introduced to remove polyproteins from the reaction mix prior to spotting onto P81 paper. This step reduces the variability between replicate assays. Specifically, 25 µl reactions are stopped by adding TCA to 5%, incubated on ice for 10 min, and then spun for 5 min in a microcentrifuge. Twenty microliters of the supernatant is then spotted onto P81 paper and processed for scintillation counting.
Preparation and use of CKII \(\beta^8\) antiserum

A peptide (DLTKSGFKT-3') corresponding to the 10 carboxy-terminal residues of CKII \(\beta^8\) (amino acids 219–228; Reed et al. 1994) was coupled to keyhole limpet hemocyanin (KLH) at the peptide’s amino terminus and used to immunize New Zealand white rabbits. After collecting naive serum, the rabbits were injected with 1 mg of KLH-conjugated peptide. The injection was repeated 2, 5, 9, and 13 weeks after the first immunization; serum was screened by ELISA (Harlow and Lane 1988) using the target peptide coupled to BSA and peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch Labs). The reaction product was detected using a peroxidase substrate serum was screened by ELISA (Harlow and Lane 1988) using the target peptide coupled to BSA and peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch Labs). The reaction product was detected using a peroxidase substrate (. 20% horse serum in TBST (100 mM Tris-HCl at pH 7.5, 150 mM NaCl, 5 mM MgCl\(_2\), 0.1% Tween 20). The immunoreactive species were detected using horseradish peroxidase-conjugated anti-rabbit antibody and the ECL chemiluminescence detection system (Amersham).

In vitro kinase and phosphatase reactions

Purified CKII was incubated with potential substrates in 20 µl final volume containing 15 mM HEPES–KOH (pH 7.9), 130 mM KCl, 10 mM MgCl\(_2\), 2.5 mM EGTA, 0.2 mM EDTA, and 1 µl \([\gamma-32P]GTP\) or \([\gamma-32P]ATP\) (NEN; 3000 Ci/mmol). The reactions were performed for 15 min at 30°C, stopped with SDS-PAGE loading buffer, and the products resolved as described under immunoblotting. Hydroxyapatite TFIIIB was dephosphorylated by incubation for 20 min at room temperature with the calf alkaline intestinal phosphatase (Sigma) in a buffer containing 20 mM Tris-HCl (pH 8.5), 50 mM NaCl, 5 mM MgCl\(_2\), 0.1 mM ZnCl\(_2\), and 1 mM DTT. Sodium vanadate (Sigma) was used at 0.5 mM.

In vitro transcription reactions

Multiple round transcription reactions were performed according to Hockman and Schultz (1996) using pY5S for 5S rRNA transcription (Schultz et al. 1992) and pTZ1, which contains the SUP4 trnAT\(^{Ty}\) gene, for tRNA transcription (Kassavetis et al. 1989). Details are noted in the legends to Figs. 1–3, 5, 6, and 8. The final buffer composition of all reactions in an experiment was identical. Reconstitution of transcription in ck\(a^2\) extract with CKII and TBP was performed without preincubation of CKII and TBP, as CKII phosphorylation of TBP prior to mixing with cell extract usually dampens rather than enhances the stimulation of transcription (not shown). We speculate that in a kinase reaction containing only purified TBP and CKII, phosphorylation of TBP occurs at some sites that both are not available when TBP is assembled into TFIIIB and must be unmodified for TBP to participate in transcription.

Acknowledgments

We are grateful to Darren Hockman for expert technical assistance, especially in development of the CKII assay (according to helpful suggestions from Colin Rasmussen) and the preparation of antisera. Ron Reeder kindly provided the TBP antiserum, and plasmids pTZ1 and pLNG56 were supplied by Peter Guideshek. pSH228 was from Steve Hahn, who also advised on the purification of recombinant TBP. Max Cummings and Mike Ellison are acknowledged for the molecular modeling, and we thank Claiborne Glover III for helpful discussions and Charlotte Spencer for comments on the manuscript. Peptides were prepared by the Alberta Peptide Institute (Edmonton), and oligonucleotides were synthesized by the DNA Core Facility, Biochemistry Department, University of Alberta. This work was supported by a establishment grant from the Alberta Heritage Foundation for Medical Research and by operating grants from the Canadian National Cancer Institute and Medical Research Council (MRC). M.C.S. is a scholar of the MRC.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 USC section 1734 solely to indicate this fact.

References

Allende, J.E. and C.C. Allende. 1995. Protein kinase CK2: An enzyme with multiple substrates and a puzzling regulation. FASEB J. 9: 313–322.

Bidwai, A.P., D.E. Hanna, and C.V.C. Glover. 1992. Purification and characterization of casein kinase II (CKII) from \(\Delta cka1 \Delta cka2\) Saccharomyces cerevisiae rescued by Drosophila CKII subunits. The free catalytic subunit of casein kinase II is not toxic in vivo. J. Biol. Chem. 267: 18790–18796.

Bidwai, A.P., J.C. Reed, and C.V.C. Glover. 1994. Casein kinase II of Saccharomyces cerevisiae contains two distinct regulatory subunits, \(\beta^8\) and \(\beta^8\). Arch. Biochem. Biophys. 309: 348–355.

Chasman, D.I., K.M. Flaherty, P.A. Sharp, and R.D. Kornberg. 1993. Crystal structure of yeast TATA-binding protein and model for interaction with DNA. Proc. Natl. Acad. Sci. 90: 8174–8178.

Cormack, B.P. and K. Struhl. 1993. Regional codon randomization: defining a TATA-binding protein surface required for RNA polymerase III transcription. Science 262: 244–248.

Dunn, B. and C.R. Wobbe. 1995. Preparation of protein extracts from yeast. In Current protocols in molecular biology (ed. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl), pp. 13.13.1–13.13.9. Greene/Wiley, New York, NY.

Gelduschek, E.P. and G.A. Kassavetis. 1992. RNA polymerase III transcription complexes. In Transcriptional regulation (ed. S.L. McKnight and K. Yamamoto), pp. 247–280. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Gottesfeld, J.M., V.J. Wolf, T. Dang, D.J. Forbes, and P. Hartl. 1994. Mitotic repression of RNA polymerase III transcription in vitro mediated by phosphorylation of a TFIIIB component. Science 263: 81–84.

Hanna, D.E., A. Rethinaswamy, and C.V.C. Glover. 1995. Casein kinase II is required for cell cycle progression during G1 and G2/M in Saccharomyces cerevisiae. J. Biol. Chem. 270: 25905–25914.

Harlow, E. and D. Lane, eds. 1988. Antibodies. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Hartl, P., J. Gottesfeld, and D.J. Forbes, 1993. Mitotic repression of transcription in vitro. J. Cell Biol. 120: 613–624.

Hockman, D.J. and M.C. Schultz. 1996. Casein kinase II is required for efficient transcription by RNA polymerase III.
Regulation of TATA-binding protein by CKII

and TATA-lacking RNA polymerase III promoters in Droso-
phila cells. Mol. Cell. Biol. 16: 6909–6916.

Tuazon, P.T. and J.A. Traugh. 1991. Casein kinase I and II—
Multipotent serine protein kinases: Structure, function,
and regulation. Adv. Second Messenger Phosphoprotein Res.
23: 123–164.

Wang, Z. and R.G. Roeder. 1995. Structure and function of a
human transcription factor TFIIIB subunit that is evolution-
arily conserved and contains both TFIIIB- and high-mobility-
group protein 2-related domains. Proc. Natl. Acad. Sci.
92: 7026–7030.

White, R.J., D. Stott, and P.W. Rigby. 1989. Regulation of RNA
polymerase III transcription in response to F9 embryonal car-
icoma stem cell differentiation. Cell 59: 1081–1092.

White, R.J., D. Stott, and P.W. Rigby. 1990. Regulation of RNA
polymerase III transcription in response to Simian virus 40
transformation. EMBO J. 9: 3713–3721.

White, R.J., T.M. Gottlieb, C.S. Downes, and S.P. Jackson.
1995a. Mitotic regulation of a TATA-binding-protein-con-
taining complex. Mol. Cell. Biol. 15: 1983–1992.

White, R.J., T.M. Gottlieb, C.S. Downes, and S.P. Jackson.
1995b. Cell cycle regulation of RNA polymerase III transcrip-
tion. Mol. Cell. Biol. 15: 6653–6662.

White, R.J., D. Trouche, K. Martin, S.P. Jackson, and T. Louzari-
des. 1996. Repression of RNA polymerase III transcription by
the retinoblastoma protein. Nature 383: 88–90.

Willis, I.M. 1993. RNA polymerase III. Genes, factors and tran-
scriptional specificity. Eur. J. Biochem. 212: 1–11.

Wilson, L.K., N. Dhillon, J.Thorner, and G.S. Martin. 1997.
Casein kinase II catalyzes tyrosine phosphorylation of the
nucleolar immunophilin Fpr3. J. Biol. Chem. 272: 12961–12967.

Wishart, D.S., L. Willard, F.M. Richards, and B.D. Sykes. 1994.
VADAR: A comprehensive program for protein structure
evaluation. Version 2.1. University of Alberta, Edmonton,
Canada.

Wolffe, A.P. and D.D. Brown. 1988. Developmental regulation
of two 5S ribosomal RNA genes. Science 241: 1626–1632.
Casein kinase II regulation of yeast TFIIIB is mediated by the TATA-binding protein

Ataollah Ghavidel and Michael C. Schultz

Genes Dev. 1997, 11:
Access the most recent version at doi:10.1101/gad.11.21.2780

References
This article cites 39 articles, 21 of which can be accessed free at:
http://genesdev.cshlp.org/content/11/21/2780.full.html#ref-list-1

License

Email Alerting Service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.