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TATA Binding Protein-Associated CK2 Transduces DNA Damage Signals to the RNA Polymerase III Transcriptional Machinery

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Summary

Here we report that RNA polymerase (pol) III transcription is repressed in response to DNA damage by downregulation of TFIIIB, the core component of the pol III transcriptional machinery. Protein kinase CK2 transduces this stress signal to TFIIIB. CK2 associates with and normally activates the TATA binding protein (TBP) subunit of TFIIIB. The β regulatory subunit of CK2 binds to TBP and is required for high TBP-associated CK2 activity and pol III transcription in unstressed cells. Transcriptional repression induced by DNA damage requires CK2 and coincides with downregulation of TBP-associated CK2 and dissociation of catalytic subunits from TBP-CK2 complexes. Therefore, CK2 is the terminal effector in a signaling pathway that represses pol III transcription when genome integrity is compromised.

Introduction

Protein kinase CK2 (or casein kinase II) is a Ser/Thr/Tyr-directed enzyme expressed in all eukaryotes (Allende and Allende, 1995; Pinna and Meggio, 1997). It typically exists as a heterotetramer of homologous catalytic subunits, α and α′, tightly associated with two regulatory subunits: either two copies of the β protein, or one copy each of β and its close relative β′. A long history of study (CK2 was perhaps the first known protein kinase; Dobrowolska et al., 1999) has pointed to important functions for CK2 in cellular regulation. It is required for the proliferation of yeast, worm, and mammalian cells (Hanna et al., 1995; Pepperkok et al., 1994; Fraser et al., 2000). Bulk CK2 activity has been correlated with proliferation rate, and CK2 is implicated in the responses to genotoxic and other stresses as well as signaling events involving the CD5 cell surface receptor (Bidwai et al., 1995; Toczyski et al., 1997; Raman and Kimberly, 1998; Teitz et al., 1990; Fritz and Kaina, 1999; Sayed et al., 2000). Misexpression of CK2 is also causally implicated in transformation in various transgenic and tissue culture models (Seldin and Leder, 1995; Kelliher et al., 1996; Landesman-Bollag et al., 1998; Orlandini et al., 1998). Despite this progress, it is evident that in general the signaling functions of CK2 remain poorly defined (Glover, 1998; Guerra and Issinger, 1999).

One important signaling role of CK2 may be its regulation of RNA polymerase (pol) III transcription. Studies in yeast have shown that CK2 modulates tRNA and 5S rRNA synthesis by regulating pol III initiation (Hockman and Schultz, 1996). The core pol III initiation machinery consists of three protein complexes (White, 1998): (1) RNA pol III, (2) TFIIIC, a sequence-specific DNA binding protein, and (3) TFIIIB. Yeast TFIIIB contains the TATA binding protein (TBP), Brf1p, and Tfc5p, all of which are conserved in mammals (White, 1998; McCulloch et al., 2000; Schramm et al., 2000). The target of CK2 among the components of the pol III transcriptional machinery is the TBP subunit of TFIIIB (Ghavidel and Schultz, 1997). The available evidence suggests that CK2 phosphorylation of TBP activates TFIIIB, although the step in initiation stimulated by CK2 is not known. The physiological significance of the possible regulation of pol III transcription by CK2 also remains to be explored.

In the present study we addressed the regulation of CK2 in the context of its function in pol III transcription. Surprisingly, CK2 is physically associated with its target, TFIIIB, and transduces signals from DNA damage sensors to its binding partner in TFIIIB, the TATA binding protein. The physiological outcome of these events, the repression of pol III transcription, may promote viability during checkpoint arrest and/or limit interference by the transcriptional machinery of the process of DNA repair.

Results

Active CK2 Is Associated with TFIIIB

TBP stably interacts with numerous proteins involved in transcription, and CK2 regulatory subunits tightly bind to some of its substrates (Lee and Young, 1998; Guerra and Issinger, 1999). We therefore tested if the β regulatory subunit of CK2 (Ckb1p) is associated with TFIIIB by monitoring the chromatographic behavior of TBP, Brf1p, and CK2β during TFIIIB purification. Chromosomal *ckb1Δ* strains expressing GST or GST-tagged CK2β in a plasmid vector were used for this experiment. As expected from the diverse functions attributed to CK2 and its known interactions with multiple proteins, the bulk of CK2β and enzyme activity fractionate away from TFIIIB. Nonetheless, on the third resin used in TFIIIB purification (hydroxylapatite), GST-β precisely copurifies with TBP and Brf1p (Figure 1A), TFIIIB activity (Figure 1B), and casein kinase activity (Figure 1C); GST does not cofractionate with TFIIIB (Figure 1A, lane 13). Quantitative immunoblotting revealed a molar ratio of 1:0.4:0.4 for TBP, Brf1p, and GST-β in hydroxylapatite fraction 5 (not shown). The 1:1 stoichiometry of Brf1p and GST-β suggests that the β subunit of CK2 is present in a substantial fraction of TFIIIB molecules.

Consistent with the presence of GST-β in TFIIIB, the casein kinase in TFIIIB obtained at the fourth chromatographic step of purification (Cibacron Blue) was found to have the biochemical properties CK2. TFIIIB was isolated from strain CKA2 (*cka1Δ CKA2*), with wild-type α′ (Cka2p), and strain cka2^{ts} (*cka1Δ cka2-8^{ts}*), in which CK2 activity is defective because of mutations in α′ (Hanna et al., 1995). Its protein profile (silver staining not shown) and content of TBP and Brf1p (Figure 1D) is not different between wild-type and cka2^{ts}. TFIIIB from cka2^{ts} cells,

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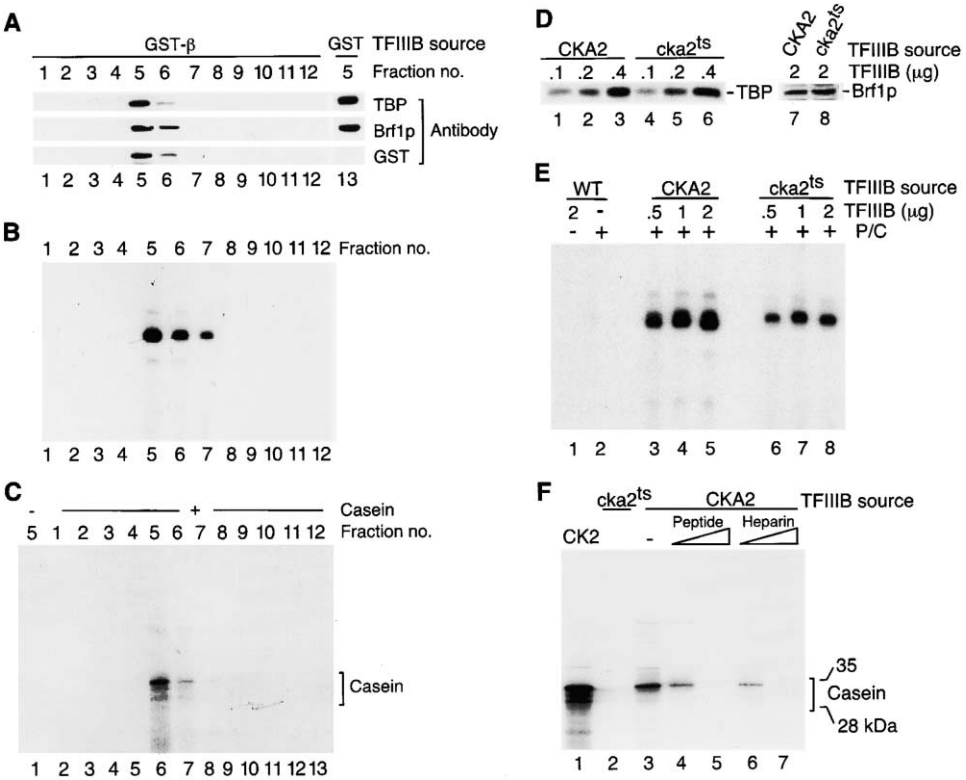

Figure 1. Association of Active CK2 with TFIIIB

(A) Copurification of CK2 with TFIIIB. Fractions from the hydroxylapatite step of TFIIIB purification were monitored for TFIIIB and GST-tagged CK2β by immunoblotting using the indicated antibodies; for a particular antibody the same volume of each fraction was analyzed. Expression of GST-β (lanes 1–12) and GST (lane 13) in cells grown in CM-Ura medium at 25°C was induced with 0.6 mM $CuSO_4$ for 1 hr prior to harvesting.

(B) Reconstitution of $tRNA^{Tyr}$ transcription using hydroxylapatite fractions from cells expressing GST-β. Reactions contained 2.4 μg of a pol III/TFIIIC fraction (P/C) from wild-type cells and 2 μl of each hydroxylapatite fraction in panel (A). ^{32}P-UTP-labeled $tRNA^{Tyr}$ gene products were resolved in a denaturing polyacrylamide gel and detected by autoradiography.

(C) CK2 activity was assayed in hydroxylapatite fractions (0.5 μl aliquots) from cells expressing GST-β. Assays were performed with 40 μg/ml casein as the exogenous substrate; [γ-^{32}P]GTP was used as the phosphate donor in all in vitro kinase reactions since CK2 is the only known GTP-dependent protein kinase in yeast.

(D) TBP and Brf1p in Cibacron Blue TFIIIB from CKA2 and $cka2^{ts}$ cells were detected by immunoblotting. Cells were grown in rich medium at 25°C.

(E) Low transcriptional activity of TFIIIB (Cibacron Blue fraction) from CK2-deficient cells. Reactions as in (B).

(F) CK2 activity is associated with Cibacron Blue TFIIIB from CKA2 but not $cka2^{ts}$ cells (casein substrate as in (C)).

however, has lower transcriptional activity than the wild-type factor (Figure 1E, lanes 3–8). A GTP-dependent enzyme that phosphorylates both endogenous substrates (not shown) and added casein was readily detected in TFIIIB from *CKA1 CKA2* (not shown) and *cka1*Δ *CKA2* cells (Figure 1F, lane 3). Kinase activity was inhibited by a peptide used to assay conventional CK2 and heparin, a classical CK2 inhibitor (Figure 1F, lanes 4–7); excess unlabeled GTP was also inhibitory in reactions using labeled ATP (not shown). Since CK2 is the only protein kinase known to have these properties, TFIIIB phosphotransferase activity is likely due to CK2. This idea was supported by the observation that TFIIIB-associated kinase activity is repressed in TFIIIB from $cka2^{ts}$ cells (Figure 1F, lane 2). Based upon the above results, we propose that a subpopulation of active CK2 molecules is specifically associated with chromatographically purified TFIIIB.

Further evidence for association of CK2 with TFIIIB was sought by probing TBP immune complexes for the presence of CK2 subunits. In this instance, we used yeast strains expressing GST, or the respective endogenous proteins and GST-tagged versions of α′, β, and β′ (Ckb2p). Immunoblotting of lysates revealed similar expression levels of the tagged CK2 subunits and higher GST expression (Figure 2A, lanes 1–4). As expected, Brf1p was present in TBP complexes isolated from these lysates (data not shown). GST-β, GST-β′, and GST-α′ were also readily detectable in TBP immune complexes, while GST alone was not (Figure 2A, lanes 5–10). A TBP-associated, GTP-dependent kinase that could phosphorylate exogenous casein (Figure 2B, lanes 4, 5) and recombinant TBP (Figure 2C) was also detectable in TBP immune complexes from wild-type cells. Kinase activity was not present in $cka2^{ts}$ immune complexes, and was highly sensitive to competitor peptide substrate and heparin (Figure 2B, lanes 6–11). Collectively, the data demonstrate that enzymatically active CK2 is associated with TBP in vivo as a component of TFIIIB.

CK2 regulatory subunits directly interact with some substrates and in part dictate the enzyme's substrate specificity in vitro. We therefore examined the interac-

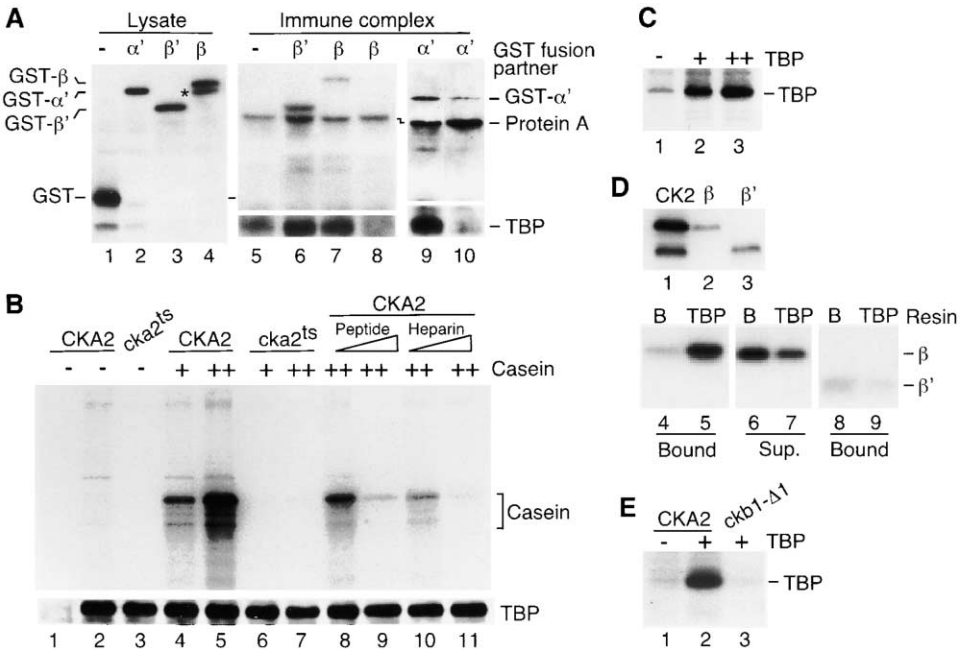

Figure 2. CK2 Subunits and Activity Are Associated with TBP In Vivo

(A) CK2 subunits coimmunoprecipitate with TBP. Lysates (4 μg) and TBP immune complexes from cells expressing the indicated GST fusion constructs, or GST alone (lanes 1, 5), were analyzed by immunoblotting using anti-GST or anti-TBP antibody. Preimmune serum was used for immunoprecipitation in lanes 8 and 10, where the smear in the vicinity of TBP is IgG light chain. A detectable amount of GST-α′ is nonspecifically recovered in lane 10 owing to interaction of α′ with Protein A-Sepharose. The protein in GST-β lysate labeled with an asterisk may be a breakdown product of GST-β.

(B) A TBP-associated protein kinase with the biochemical properties of CK2. TBP was immunoprecipitated from lysates of the indicated strains and assayed for casein (40, 120 μg/ml) kinase activity. Where indicated, the immunoprecipitated complexes were incubated with CK2 peptide (1, 3 mg/ml) or heparin (0.8, 2 μg/ml) for 5 min prior to the addition of casein and ^{32}P-GTP. Labeled proteins were detected by autoradiography, and TBP recovery was monitored by immunoblotting (lower panel). Preimmune serum was used in lane 1.

(C) Recombinant TBP is a substrate of TBP-associated CK2. TBP (40, 120 μg/ml) was added to TBP immune complex from CKA2 cells. The labeling reaction was performed as in (B).

(D) Direct physical interaction of CK2β with TBP. CK2 was autophosphorylated (lane 1) and β and β′ recovered from a preparative SDS-PAGE gel. The isolated subunits were analyzed by SDS-PAGE (lanes 2, 3) and chromatographed on BSA (B) or TBP affinity resins (lanes 4–9); bound and unbound (supernatant) proteins were detected by autoradiography.

(E) CK2 activity in TBP immune complexes is repressed in cells that do not express CK2β (strain ckb1-Δ1; YAPB6 of Bidwai et al., 1995). TBP immune complexes were assayed for their ability to phosphorylate 120 μg/ml of added recombinant TBP as in (C).

tion of in vitro-labeled β and β′ with TBP. Significant retention of β but not β′ on a TBP-affinity matrix was evident (Figure 2D, lanes 4–9). Furthermore, the activity of TBP-associated CK2 is substantially diminished in CK2β null cells (Figure 2E). These results suggest an important role for β in the regulation of TFIIIB-associated CK2.

CK2 Controls Promoter Recruitment of TFIIIB

The early steps in pol III transcription initiation are specific promoter binding of TFIIIC, recruitment of TFIIIB by DNA-bound TFIIIC, and polymerase recruitment by TFIIIB (White, 1998). We previously observed that wild-type and cka2^{ts} TFIIIC have similar DNA binding properties (Ghavidel and Schultz, 1997), and that pol III/TFIIIC (fraction P/C) from cka2^{ts} cells, in combination with wild-type TFIIIB, fully restores transcription in vitro (unpublished). These results raise the possibility that CK2 phosphorylation of TFIIIB regulates its recruitment to promoters. Accordingly, we examined promoter recruitment of TFIIIB by a gel mobility shift assay (Braun et al., 1989) in which the stable complex of TFIIIB with DNA was detected after heparin stripping TFIIIC from the preinitiation complex (Figure 3A). TFIIIB from wild-type cells readily formed a "heparin-resistant" IIIB-DNA complex (Figure 3A, lane 5; Figure 3B lanes 1–4). TFIIIB purified from cka2^{ts} cells, on the other hand, was defective in generating the heparin-resistant IIIB-DNA complex (Figure 3B, lanes 5–8). Dephosphorylation of TFIIIB greatly diminishes its transcriptional activity in vitro (Ghavidel and Schultz, 1997). Consistent with this observation, TFIIIB treated with phosphatase followed by phosphatase inhibitor (Figure 3C, lanes 2–4) has substantially less complex forming ability than untreated TFIIIB (lane 1) or TFIIIB to which the phosphatase and the inhibitor have been added following a brief coincubation (lanes 5–7). We conclude that CK2 phosphorylation of TFIIIB is required for its efficient recruitment to promoters in vitro.

Repression of Pol III Transcription in Response to DNA Damage

Protein kinase CK2 has been implicated in the physiological responses to a variety of cellular insults (Glover, 1998). As a regulator of TFIIIB, CK2 is therefore in a position to modulate tRNA/5S rRNA synthesis according

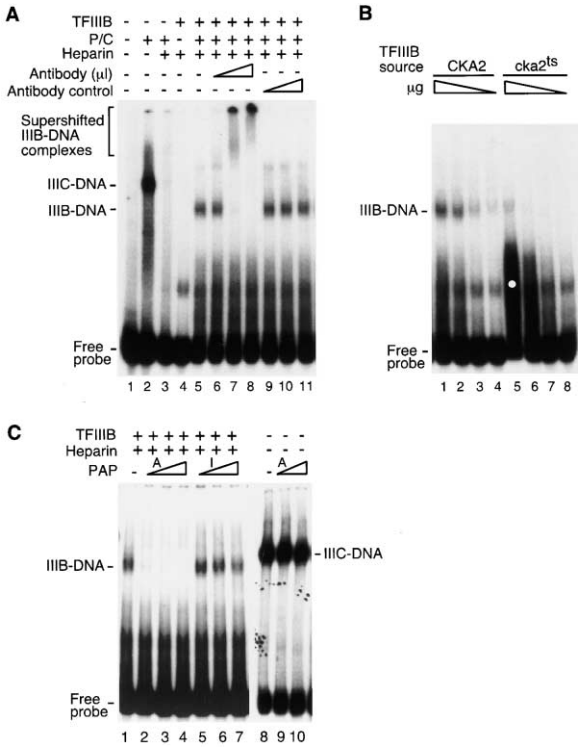

Figure 3. CK2 Controls Promoter Recruitment of TFIIIB

(A) Formation of a stable TFIIIB-DNA complex on a $tRNA^{Tyr}$ promoter. Binding reactions were performed using 0.5 μg pol III/TFIIIC (P/C) and 0.6 μg TFIIIB. Supershifting using anti-TBP antibodies (0.01, 0.04, 0.2 μl) affinity-purified from immune serum and control IgGs from preimmune serum (0.01, 0.04, 0.2 μl) confirmed formation of the IIIB-DNA complex.

(B) TFIIIB from CK2-deficient cells does not form the TFIIIB-DNA complex as efficiently as wild-type TFIIIB. The reactions used 0.6, 0.3, 0.15, and 0.08 μg of TFIIIB in the presence of 150 μg/ml heparin. The broad smear in lane 5 (dot) was not observed in all experiments.

(C) Dephosphorylation of TFIIIB impairs its ability to form a stable complex with promoter DNA. 0.6 μg TFIIIB was pretreated with potato acid phosphatase (PAP, 0.2, 0.4 μg/ml A[ctive]), or coincubated with PAP and phosphatase inhibitor (PAP, I[nactive]). PAP (0.2, 0.4 μg/ml) and its inhibitor do not affect DNA binding by TFIIIC (lanes 8–10). All reactions contained 0.5 μg of fraction P/C.

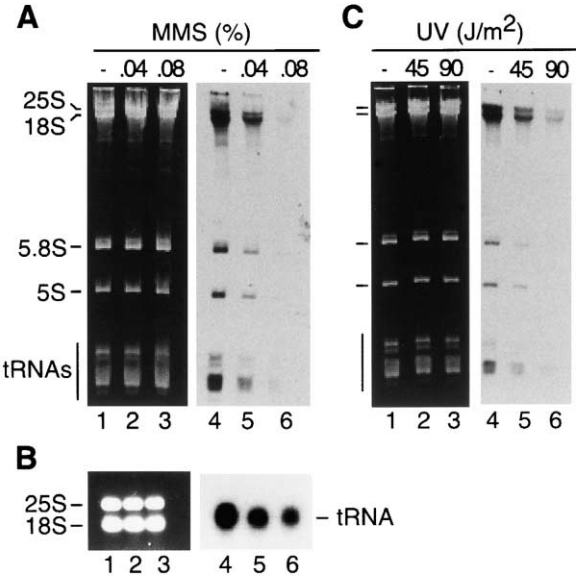

Figure 4. Genotoxic Stress Represses RNA Polymerase III Transcription In Vivo

(A) Analysis of transcription in MMS treated cells by metabolic labeling. ^{3}H-uracil was added to CKA2 cultures after 90 min of growth in the presence of MMS as indicated. After 30 min of labeling total RNA samples were prepared and 10 μg aliquots resolved by denaturing PAGE. The gel was stained with ethidium bromide, photographed (left panel), then processed for fluorography (right panel).

(B) S1 nuclease protection analysis of $tRNA^{Trp}$ transcription in cells treated with MMS. RNA was isolated from CKA2 cells treated for 2 hr with MMS as in (A). Transcripts in 10 μg samples were hybridized to an endlabeled oligonucleotide probe, digested with S1 nuclease, and the products resolved by denaturing PAGE (right panel). Equivalent total RNA recovery was confirmed by electrophoresing 5 μg of each starting sample in a 1% formaldehyde-agarose gel and staining with ethidium bromide to detect the large rRNAs (left panel).

(C) Analysis of transcription in UV irradiated cells by metabolic labeling. CKA2 cells were exposed to the indicated doses of UV irradiation and grown for 1 hr prior to labeling for 30 min with ^{3}H-uracil. Panels as in (A).

to the physiological status of the cell. A survey of stress conditions revealed that methane methylsulfonate (MMS) treatment strongly represses pol III transcription in vivo, as measured by metabolic labeling with ^{3}H-uracil and S1 nuclease protection analysis (Figures 4A and 4B). Because MMS creates DNA lesions (Smith and Grisham, 1983; IARC, 1999), and we used this reagent at concentrations widely employed to study the DNA damage response, the results in Figure 4A suggest that pol III transcription is downregulated in response to genotoxic stress. We therefore monitored the cellular response to UV irradiation at the level of pol III transcription. Substantial repression of tRNA and 5S rRNA synthesis was observed at UV exposures known to induce the DNA damage-responsive genes in yeast (Figure 4C; Aboussekhra et al., 1996). Importantly, the repression is not an indirect effect of cell cycle arrest, since MMS treatment fully downregulates pol III transcription in arrested cdc mutants (G2/M, *cdc15-2*; G1, *cdc28-1*; not shown). Figure 4 reveals that RNA pol I synthesis is also repressed in response to DNA damage, as previously reported (Koch et al., 1976).

TFIIIB Is the Target of a DNA Damage Response Pathway

We reproduced the effect of MMS on pol III transcription in vitro. Extracts from control and MMS-treated cells were used to transcribe exogenous native (undamaged) templates. Transcription directed by the 5S rRNA gene (not shown) and a tRNA gene was inhibited in a dose-dependent manner in crude extract and a chromatographic fraction from MMS-treated cells (Figures 5A and 5B). Damage of the template DNA is therefore unlikely to fully account for the repression of transcription in vivo. Transcriptional downregulation is evidently not due to differential recovery of components of the pol III machinery from MMS-treated cells, since the concentrations of TBP and Brf1p were similar between matched extracts or fractions (Figures 5A and 5B, lower panels). Mixing experiments further suggested that transcrip-

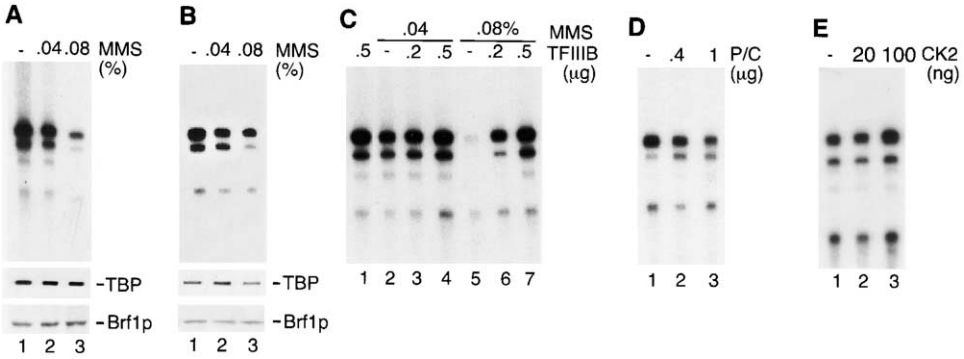

Figure 5. Repression of TFIIIB in Cells with DNA Damage

(A) Pol III transcription is repressed in crude extracts from MMS-treated cells. Runoff transcription directed by a $tRNA^{Tyr}$ gene was assayed in 10 μg whole cell extract from cells treated for 2 hr with MMS (top panel). TBP and Brf1p recovery in an equal amount of each extract was monitored by immunoblotting (lower panel).

(B) MMS-dependent repression of pol III transcription is preserved in a pol III-enriched fraction. Extracts prepared as in (A) were chromatographed in parallel on DEAE-Sepharose to obtain a 0.3 M KCl fraction that contains all components of the pol III transcriptional machinery. Transcription (in 4 μg aliquots) and TBP/Brf1p recovery were assayed as in (A).

(C) TFIIIB fully restores the transcription activity of extract from MMS-treated cells. TFIIIB from control cells was added in the indicated amounts to whole cell extracts from untreated (lane 1) or MMS-treated cells. $tRNA^{Tyr}$ transcription was monitored as in (A).

(D) An active pol III/TFIIIC fraction (P/C) does not restore the transcriptional activity of MMS-treated extract. The P/C fraction used in Figure 1E was titrated into extract from cells treated with 0.08% MMS.

(E) CK2 stimulates pol III transcription in extract from MMS treated cells. CK2 purified from wild-type untreated cells was added to extract from cells treated with 0.08% MMS. The film exposures in (D) and (E) were prolonged to clearly demonstrate the effects of added fractions.

tional repression was not due to a dominant inhibitor in MMS-treated extracts (not shown).

We used factors purified from untreated cells in an attempt to reconstitute transcription in MMS-treated extract. TFIIIB fully restored transcription in MMS-treated extract (Figure 5C). In contrast, a fraction containing pol III and TFIIIC but not TFIIIB was inactive (Figure 5D). Collectively, these results indicate that TFIIIB is specifically downregulated in response to DNA damage. Since a single purified factor fully rescues transcription in MMS-treated extract, we conclude that transcriptional repression in vivo in response to genotoxic stress is not a result of general cytolysis. Consistent with the notion that a CK2-dependent effect on TFIIIB underlies the downregulation of transcription by MMS, CK2 holoenzyme purified from control cells stimulated tRNA transcription in MMS-treated extracts (Figure 5E). CK2, however, even in larger amounts than shown, has only a modest effect compared to TFIIIB. In this assay, the low activity of CK2 may be the result of its apparent inhibition (up to 95%) when added to crude extracts (Zandomeni et al., 1986; our unpublished data); the latter effect is possibly due to self-aggregation which reduces CK2 activity upon its dilution to physiological salt concentrations from high-salt storage buffer (Miyata and Yahara, 1992).

TBP-Associated CK2 Activity Is Downregulated in Response to DNA Damage

Since CK2 has been implicated in the DNA damage response (Bidwai et al., 1995; Toczyski et al., 1997), and TFIIIB is a target of CK2 in cells exposed to MMS, a change in the biochemical properties of TBP-associated CK2 could potentially mediate the repression of TFIIIB in DNA-damaged cells. This possibility was tested by analyzing TBP immune complexes isolated from cells expressing GST-tagged CK2 subunits (strains as in Figure 2). The overall expression level of tagged CK2 subunits was unchanged after UV irradiation (Figure 6A, 'Lysate') and MMS exposure (not shown). While there is no change in GST-β or GST-β′ recovery following UV irradiation (Figure 6A, lanes 3, 4, 8, 9), the recovery of GST-α′ in TBP immune complexes declines to the baseline level represented by nonspecific binding of GST-α′ to Protein A Sepharose beads (Figure 6A, lanes 13–16), and TBP-associated kinase activity is reduced (Figure 6B; a similar result is obtained using MMS, see Figure 6C). The dissociation of the catalytic subunit from TBP complexes following DNA damage, with a concomitant decrease in TBP-associated CK2 activity, is a plausible molecular mechanism for the DNA damage-dependent repression of pol III transcription.

DNA Damage-Induced Transcriptional Repression In Vivo Requires CK2 and a Potential CK2 Phosphoacceptor Site in TBP

To confirm the role of CK2 in genotoxic stress signaling to the pol III transcriptional machinery, we monitored tRNA and 5S rRNA synthesis in wild-type cells and CK2 and TBP mutants exposed to UV irradiation. We reasoned that if pol III downregulation in response to DNA damage involves CK2, then mutations that perturb CK2 function should dampen damage-induced transcriptional repression. As we previously reported, the $cka2^{ts}$ mutant has impaired pol III transcription when grown at the permissive temperature (Hockman and Schultz, 1996). UV irradiation further represses pol III transcription, but the fold of repression is considerably lower than in wild-type cells (Figure 7A lanes 3, 4 compared to 1, 2; Figure 7B). Similar to mutation of the catalytic subunit, disruption of the regulatory CK2β subunit (strain ckb1Δ) results in reduced TBP-associated kinase activ-

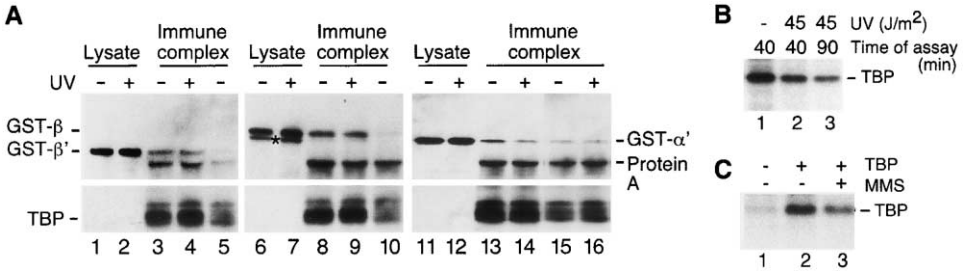

Figure 6. Regulation of TBP-Associated CK2 in Cells Exposed to Genotoxic Stressors

(A) The subunit composition of TBP-associated CK2 in UV irradiated cells expressing GST-β′ (lanes 1–5), GST-β (lanes 6–10), and GST-α′ (lanes 11–16). Lysates were prepared after UV irradiation (60 J/m^2) and 4 μg of each was assayed for expression of GST-CK2 fusion constructs by immunoblotting against GST. Our TBP antibody did not detect TBP in this amount of lysate. Immune complexes were recovered by precipitation using preimmune serum (lanes 5, 10, 15, 16) or anti-TBP antibody (lanes 3, 4, 8, 9, 13, 14) and probed with anti-GST or anti-TBP antibody. The protein in GST-β lysate labeled with an asterisk may be a breakdown product of GST-β.

(B) TBP-associated CK2 activity is downregulated in UV irradiated cells. Kinase activity was measured in TBP immune complexes from untreated and UV-treated cells. TBP immune complexes were prepared 40 and 90 min after irradiation and assayed as in Figure 2C using 120 μg/ml recombinant TBP as substrate.

(C) TBP-associated CK2 activity is downregulated in MMS-treated cells. Kinase activity was measured as above in TBP immune complexes from untreated and 0.08% MMS-treated cells (2 hr).

ity (Figure 2E), impaired pol III transcription (Figure 7A, lane 5), and a reduced fold of pol III repression following UV exposure (Figure 7A lanes 5, 6 compared to lanes 1, 2; Figure 7B). Consistent with the recovery of both catalytic and regulatory subunits in complex with TBP (Figure 6A), these results suggest a requirement for CK2 holoenzyme in mediating pol III repression following DNA damage. Accordingly, mutations in both catalytic and regulatory subunits of CK2 (strain cka2^{ts} ckb1Δ) effectively inhibit repression of pol III transcription following UV irradiation (Figure 7A, lanes 7, 8 compared to lanes 1, 2; Figure 7B) or MMS treatment (not shown). Collectively, these findings demonstrate that properties of CK2, as defined by the *cka2^{ts}* and *ckb1Δ* mutations, are essential for the enzyme's ability to activate transcription under benign conditions and to respond to DNA damage signals.

A conserved CK2 consensus site in yeast TBP, Ser-128, is likely important for the regulation of pol III transcription by CK2 (Ghavidel and Schultz, 1997; Ghavidel et al., 1999). If phosphorylation of Ser128 plays a role in transcriptional regulation in cells with DNA damage, then its substitution by a nonphosphorylatable residue should at least partly block the class III gene expression response to DNA damage. To explore this possibility, we constructed a strain in which Ser128 was changed to Ala (strain S128A). S128A cells grow slower than wild-type at 22°C, and this difference is accentuated at 37°C (Figure 7C). S128A and wild-type cells, however, have the same viability at 22°C and 37°C (not shown) and express TBP at a similar level (Figure 7C). Polymerase III transcription is impaired in S128A cells at the permissive temperature, and it is further repressed after UV irradiation (Figure 7D, lanes 3, 4; Figure 7E). Importantly, however, the fold of repression of pol III transcription in response to DNA damage is dampened in S128A mutant compared to wild-type cells (Figure 7D, lanes 3, 4 compared to 1, 2; Figure 7E). Like the S128A mutant, and in agreement with a previous report, the P65S mutant of TBP is defective in pol III transcription (Schultz et al., 1992), yet unlike S128A, it maintains a fold of repression that is similar to the wild-type cells (Figure 7D, lanes 5, 6 compared to lanes 1, 2; Figure 7E). Therefore, the dampening effect of the S128A mutation on DNA damage-dependent repression of transcription is not a generic property of functionally compromised TBP molecules. In summary, downregulation of pol III transcription is impaired by elimination of a single solvent-exposed CK2 consensus site on a surface of TBP that is involved in multiple protein-protein interactions (Nikolov and Burley, 1994) and is required for efficient pol III transcription under normal conditions (Cormack and Struhl, 1993). These findings support a model in which TBP is a critical target of the CK2-dependent DNA damage response in yeast.

Our data reveal that in addition to pol III, synthesis of pol I transcribed rRNAs also declines following DNA damage (Figure 7A, quantitation of 5.8S rRNA not shown). Significantly, mutation of either the catalytic or regulatory subunits of CK2 is associated with impaired synthesis of both classes of gene products in vivo (Figure 7A, lanes 1, 3, 5, 7). Furthermore, CK2 mutations reduce the fold of repression of pol I transcription after DNA damage (Figure 7A, lanes 1–8; quantitation not shown) by the same amount that is observed for pol III genes. We conclude that CK2 is required for optimal tRNA and rRNA synthesis under benign conditions, and also mediates coordinated repression of class I and III gene expression in response to genotoxic stress. More generally, our results suggest that CK2-mediated coupling of pol I and pol III transcription could play an important role in the global regulation of protein synthesis.

Discussion

Our data provide insights into the DNA damage response and the role of CK2 in intracellular signaling. We establish that coordinate downregulation of rRNA and tRNA synthesis is a hallmark of the DNA damage response in yeast. Additionally, we characterize a signaling function of CK2 that makes a major contribution to

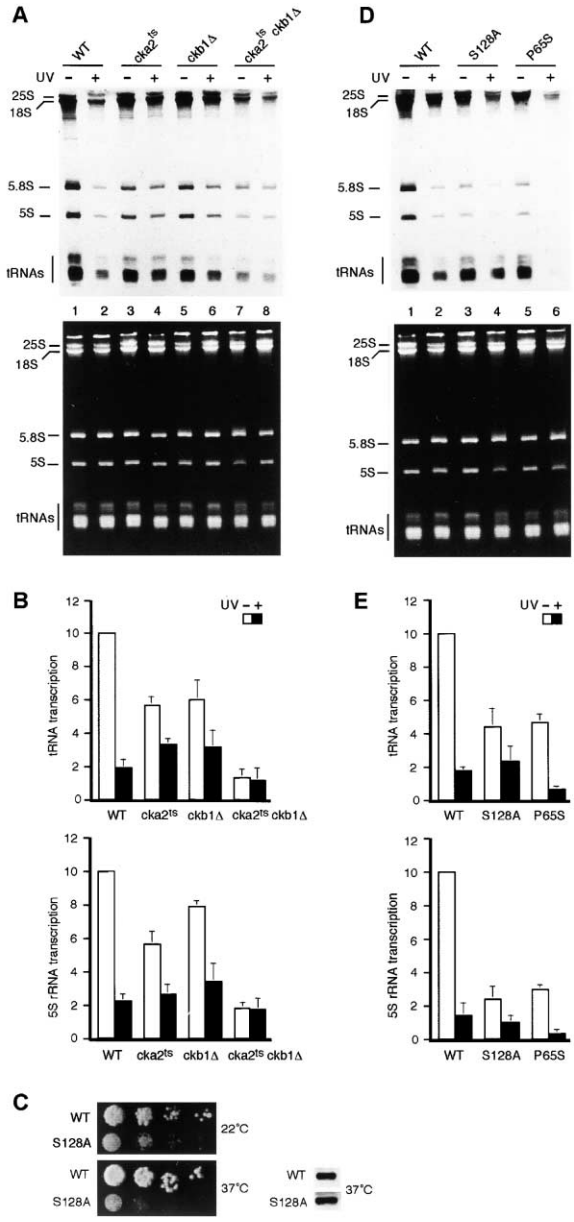

Figure 7. Full Transcriptional Repression in Response to DNA Damage Requires Active CK2 and a Potential CK2 Site in TBP

(A) Transcriptional response of CK2 mutants to DNA damage. A temperature-sensitive strain of CK2 (cka2^{ts}), a CK2β null mutant (ckb1Δ), the cka2^{ts} ckb1Δ double mutant, and isogenic wild-type (WT) cells grown at 27°C were UV irradiated (60 J/m^2) and grown for an additional 1 hr prior to labeling for 30 min with ^{3}H-uracil. Panels as in Figure 4A.

(B) Quantitation of the pol III transcription of the tRNA and 5S rRNA genes in untreated (open bars) and UV treated (filled bars) CK2 mutants. The graphs plot transcription in arbitrary units with wild-type normalized to 10, and show the means and the standard deviations of four independent experiments.

(C) 10-fold serial dilutions of strains harboring wild-type or the S128A allele of TBP were grown for 3 days at the indicated temperature. The right panel shows TBP expression in 30 μg of whole cell lysate prepared from wild-type and S128A yeast strains grown at 37°C.

(D) Transcription in untreated and UV irradiated wild-type (WT) and TBP mutant cells (S128A and P65S) grown at 27°C. Metabolic labeling was performed as in panel (A).

(E) Quantitation of DNA damage effects on pol III transcription in TBP mutants.

the repression of pol III transcription in response to DNA damage. Consistent with this finding, CK2 has previously been implicated in the DNA damage response. Thus, *ckb2* null cells have a subtle UV sensitivity phenotype (Bidwai et al., 1995), similar to that conferred by the *rad5-535* allele (Fan et al., 1996), and mutations in *ckb1* and *ckb2* also impair adaptation of cells to the G2/M double strand break checkpoint (Toczyski et al., 1997).

Why Repress Pol III Transcription in Response to DNA Damage?

CK2-mediated pol III repression could serve three functions in cells with DNA damage. Toczyski et al. (1997) suggested that CK2 is important in the adaptation response to genotoxic stress because it plays a critical role in enhancing survival during checkpoint arrest. By extension, one function of CK2-mediated pol III (and pol I) repression in cells subjected to genotoxic stress could be to divert cellular resources from the energetically costly process of transcription to DNA repair and other processes required to maintain viability while the damage is repaired.

Two possible functions are more proximally related to the DNA damage events. In yeast, DNA repair is inhibited by pol III transcription, perhaps because elongating polymerase obstructs the repair machinery (Aboussekhra and Thoma, 1998). It follows that CK2-signaled repression of pol III transcription might be important in the DNA damage response because this repression ensures adequate access of the repair machinery to damaged DNA. Finally, we do not rule out a role for tRNA/rRNA downregulation in establishment of DNA damage checkpoints. Translational capacity will decrease in vivo when these RNAs are depleted in response to genotoxic stress, perhaps to levels at which key cell cycle regulators are not synthesized in sufficient amounts to promote cell cycle progression (Neufeld and Edgar, 1998; Polymenis and Schmidt, 1999). In this respect, it is noteworthy that CK2 has been implicated in the regulation of translation and G1/S and G2/M progression (Hanna et al., 1995). Besides its downregulation functioning to repress pol III transcription in response to DNA damage, we presume that reactivation of CK2 after damage repair is necessary for restoring the cell's full capacity for translation.

TBP-Associated CK2 as the Effector Kinase in a Genotoxic Stress Signaling Pathway

The fundamental logic of the regulation of TBP-associated CK2 is distinctive among stress-response kinases. Whereas most stress signaling kinases are normally off in the absence of the appropriate stimulus, TBP-associated CK2 is most active in the absence of signaling from upstream components of the DNA damage pathway, and its regulation in response to the appropriate stress signal occurs by a negative mechanism. This system of signaling ensures constitutive activation of TFIIIB under benign conditions (low genotoxic stress).

The repression of TBP-associated CK2 activity in response to DNA damage is likely due to dissociation of α/α′ from the TBP-CK2 complex. This mechanism of regulation has a precedent in mammalian cells, where

dissociation of CK2α from protein phosphatase 2A (PP2A) underlies the inactivation of PP2A-associated CK2 activity in response to platelet-derived growth factor (Hériche et al., 1997). Our data further support the hypothesis that the ability of TBP-associated CK2 to maximally stimulate pol III transcription is modulated by CK2β, whose presence in the complex is likely determined in part by its direct physical interaction with TBP. The crystal structure of human CK2β reveals a set of surface grooves and an acidic domain (domain I) that may serve as docking sites for interacting proteins (Chantalat et al., 1999). Sequence comparison indicates that these structural elements will be conserved in yeast CK2β, with the acidic domain an attractive candidate for interaction with highly basic areas on the convex face of TBP that have already been characterized as the binding sites for other proteins (Nikolov and Burley, 1994). How exactly the interaction of TBP with CK2β regulates TBP-associated CK2 has yet to be determined. Nonetheless, our results, and the observation that CK2α activity toward yeast TBP is robustly stimulated by recombinant CK2β (Maldonado and Allende, 1999), are consistent with the proposal that CK2β plays an important and direct role in the regulation of pol III transcription by CK2. Interestingly, support for the hypothesis that the regulatory subunits of CK2 directly modulate CK2 activity in vivo has waned over recent years (Pinna and Meggio, 1997). Clearly, the present results revitalize this hypothesis.

Our results illuminate a DNA damage signaling pathway that impinges directly on TFIIIB. Effects on pol II transcription are unlikely to contribute to TFIIIB regulation by this pathway, since MMS treatment of yeast does not affect the abundance of mRNAs for any subunit of CK2 or TFIIIB (Jelinsky and Samson, 1999) or cause a change in TBP or Brf1p expression levels (Figure 5). The possibility that effects on pol II transcription can otherwise influence the DNA damage-dependent repression of class III gene transcription, however, is not excluded. For example, transcripts of the Rpc19p subunit of pol III are repressed in cells exposed to MMS (Jelinsky and Samson, 1999). While this effect does not account for the repression of transcription reported here (pol III activity is unaffected in MMS-treated cells), it could after prolonged exposure underlie depletion of pol III to a level that might become limiting for transcription in vivo.

The pol III transcriptional machinery, CK2, and many DNA damage checkpoint genes are highly conserved in eukaryotes (Allende and Allende, 1995; White, 1998; Zhou and Elledge, 2000). CK2 has also been implicated in the DNA damage response in mammals: its β subunit suppresses the UV sensitivity phenotype of xeroderma pigmentosum cells (Teitz et al., 1990), and inhibition of CK2 increases the sensitivity of CHO cells to MMS cytotoxicity (Fritz and Kaina, 1999). These observations raise the intriguing possibility that CK2 plays a role in DNA damage signaling to the pol III transcription machinery in all eukaryotes.

Experimental Procedures

In Vitro Pol III Transcription

Growth media were prepared according to Ausubel et al. (1995). Purification of transcription factors, CK2, and recombinant yeast TBP according to published procedures has been described (Ghavidel and Schultz, 1997). The hydroxylapatite and Cibacron Blue fractions, representing the third and fourth chromatographic steps of TFIIIB purification respectively, (Kassavetis et al., 1989) were used as noted in the text. Preparation and assay of transcription in S100 extracts and reconstitution of transcription using purified factors were performed as outlined in Hockman and Schultz (1996) and Ghavidel and Schultz (1997).

In Vivo Transcription

Total RNA was prepared according to Schmitt et al. (1990) from cells grown to early log (A_{600} 0.2− 0.3). Newly synthesized $tRNA^{Tyr}$ was measured by S1 protection analysis (Hockman and Schultz, 1996). Transcription was also monitored by in vivo labeling of 5 ml cultures with [5,6^{-3}H]-uracil (38.5 Ci/mmol, NEN, added to 15 μCi/ml). After 30 min, total RNA was isolated and resolved by electrophoresis in an 8% acrylamide gel containing 7 M urea. The gel was stained with ethidium bromide to assess steady state tRNA and rRNA levels, then processed for fluorography (EN^{3}HANCE, NEN). Quantitation of images of stained gels and fluorographs, acquired with a flatbed scanner, was performed using the Image Gauge program (v. 3.0, Fuji). Transcription signals were normalized against RNA recovery as determined by analysis of stained gels.

Electrophoretic Mobility Shift Assay

Assays were performed essentially according to Braun et al. (1989) using 0.5 ng of an endlabeled EcoRI/HinDIII fragment that contains the entire coding region of the *SUP4* $tRNA^{Tyr}$ gene (from pTZ1, kindly provided by Peter Geiduschek). To examine the effect of phosphorylation on DNA binding, TFIIIB was incubated with Potato Acid Phosphatase (PAP, Sigma) for 1 hr at 30°C, followed by addition of 30 mM β-glycerophosphate to inhibit the phosphatase. As a control, PAP was briefly incubated with inhibitor before adding to TFIIIB. The entire mixture was then added to preformed IIIC-DNA complex (we note that addition of PAP to P/C before DNA abolishes IIIC-DNA formation). After a further 30 min incubation at room temperature, heparin was added (150 μg/ml final concentration) to strip TFIIIC from the DNA. The final composition of all reactions (12 μl) was: 20 mM HEPES [pH 7.9], 120 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 5% glycerol, 0.4 mM DTT, 0.3 mg/ml BSA, and 6 μg/ml of pGEM3 as nonspecific competitor.

Immunoprecipitation and Immune Complex Kinase Assay

Strains expressing GST or GST fusion constructs in plasmid pYEX 4T-1 have been described (Martzen et al., 1999; from Research Genetics, AB), and we determined that GST-β and GST-α′ complement the growth defects of ckb1Δ and $cka2^{ts}$ cells respectively. All steps were performed at 4°C except as indicated. Typically, 100 μl of packed early log cells (grown in CM-Ura at room temperature) was resuspended in 1.5 vol of buffer N-150 (30 mM HEPES [pH 7.9], 150 mM KCl, 2 mM EDTA, 2 mM EGTA, 5% glycerol, 0.01% NP-40) and lysed by 20 s agitation with glass beads in a bead-beater (Biospec, OK). The lysate was incubated with DNase I (0.1 mg/ml) and RNase A (50 μg/ml) for 10 min and microcentrifuged (30 min). 10 μl of packed Protein A-Sepharose beads (Sigma) was added to the supernatant for 30 min in a preclearing step, and the beads then removed. To immunoprecipitate TBP-containing complexes, rabbit polyclonal antiserum raised against recombinant yTBP was then added to 120 μg of supernatant in a volume that gave a final 1:100 (v/v) dilution of the serum. After mixing for 1 hr, 10 μl Protein A-Sepharose beads was added and the reaction incubated for an additional 30 min. The beads were then spun down and extensively washed with buffer N-150 containing 0.1% NP-40. Immune complex kinase reactions were performed at room temperature for 15 min. The final reactions (10 μl) contained: 25 mM Tris [pH 8], 12 mM $MgCl_2$, 80 mM KCl, 1 mM DTT, and 1 μl [γ-^{32}P]GTP (6000 Ci/mmol, NEN). The entire reactions were boiled in SDS sample buffer, and the products resolved by SDS-PAGE. After electroblotting onto nitrocellulose, the phosphorylated proteins were detected by autoradiography. The membrane was subsequently probed by immunoblotting (Ghavidel and Schultz, 1997) to verify equal loading of TBP or to detect GST-tagged proteins.

Antibodies and Recombinant Proteins

New Zealand white rabbits were immunized and boosted with near homogeneous recombinant yeast TBP prepared as described (Ghavidel and Schultz, 1997). For supershifts, anti-TBP IgGs were purified by ammonium sulfate precipitation and TBP-Affi-Gel 10 (BioRad) affinity chromatography. Goat anti-GST antibody was from AP Biotech. Anti-Brf1 antibody from Ian Willis was used as described (Sethy et al., 1995). Purified recombinant TBP, Brf1 (from Steve Hahn and I. Willis), and GST-β′ were used as standards for quantitative immunoblotting in which chemiluminescence (ECL, AP Biotech) was directly quantitated using a FluorChem digital imaging system and the AlphaEaseFC program (Alpha Innotech Corp.). For expression of yeast CK2β′ in *E. coli*, the *CKB2* gene was cloned between the BamHI and EcoRI sites of pGEX-4T-1; the resultant aminoterminally tagged GST fusion protein was batch purified using glutathione sepharose 4B (AP Biotech) according to the manufacturers' recommendations.

In Vitro Assay of TBP Binding to Purified CK2 Subunits

40 μg (400 μl) of recombinant TBP was microconcentrated to 60 μl in an Ultrafree-MC Centrifugal Filter (Millipore) and incubated with 40 μl of CNBr-activated, preswelled Sepharose 4B beads (AP Biotech). After overnight incubation at 4°C, the beads were quenched overnight with 1 M ethanolamine [pH 8], followed by several washes in buffer X-150 (20 mM Tris [pH 8], 150 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 0.01% NP-40). Coupling efficiency was monitored by immnuoblotting against TBP. As a control, 40 μg of BSA (New England Biolabs) was similarly cross-linked to beads. The individual regulatory subunits of CK2 were obtained by gel purification (Cho et al., 1999). Briefly, 120 μg of highly enriched CK2 (heparin-agarose fraction; Ghavidel and Schultz, 1997) was autophosphorylated, resolved by preparative SDS-PAGE, and the ^{32}P labeled regulatory subunits detected by wet gel autoradiography. Proteins were eluted from gel slices, denatured in 6 M guanidine-HCl, renatured at room temperature, and acetone precipitated. Approximately half of each recovered protein sample was diluted in 20 μl of buffer (X-150 plus 50 μg/ml BSA) and incubated for 90 min at 4°C with 5 μl of Sepharose beads cross linked to TBP or BSA. The beads were then spun down, washed several times in buffer X-150, boiled in SDS-PAGE sample buffer, and electrophoresed.

Construction of Yeast Mutants

The *CKB1* gene encoding CK2β was disrupted in isogenic CKA2 and cka2^{ts} strains (YDH6 and YDH8, described in Hanna et al., 1995) to construct ckb1Δ and cka2^{ts} ckb1Δ, respectively. An integrating fragment was obtained by PCR using oligonucleotides 5′-CCGAATCTGTAAATTGGTGAATTGGTATTTAGAAGCGACCATTAGCTAAAAGAGAGAGAAAACTCGTACGCTGCAGGTCGAC and 5′-GGTAAAGTACATATGTATATGGTAGGGCAAAGAGGAAAATAAATCAAGGGTTAAATCGATGAATTCGAGCTCG (underlined residues are complimentary to the region immediately flanking the CKB2 open reading frame, and the residues at the 3′ ends are the sequence complimentary to the regions flanking the *kan*^{r} gene). Using these oligonucleotides, the *kan*^{r} gene was PCR amplified from the *kan* MX4 module (Wach et al., 1994) and transformed into yeast cells which were subsequently selected on plates containing 200 μg/ml geneticin (G418). The selected transformants were initially assayed by PCR and subsequently probed by Southern blotting, using the endlabeled Sal1-Sca1 fragment of the *kan*^{r} gene, to confirm proper integration. TBP Ser128 was changed to Ala in plasmid pSH225 using the oligonucleotide 5′-GCTTTAATTTTTGCGTCAGGGAAAATGGTTGTTACCGGTGCAAAAGCTGAGGATGACTC (changed residues underlined) and the Transformer Site-directed Mutagenesis Kit (Clontech, CA). Plasmid-borne wild-type TBP in an *spt15*Δ background was replaced by plasmid shuffling with pSH225 containing wild-type or Ser128Ala TBP (details in Schultz et al., 1992; reagents kindly provided by S. Hahn).

Induction of DNA Damage and Assessment of Viability after Genotoxic Stress

30 ml cultures were grown to an A_{600} of 0.2 in CM medium and irradiated with a 254 nm germicidal UV lamp at the dose rate of 1 J/m^2/s while stirring in a 15 ml Petri dish (Siede et al., 1993). Following UV or MMS treatment, cells were plated and viability was measured by counting the number of colonies after 3 days at room temperature. Under these conditions, viability was 52% and 18% after 45 and 90 J/m^2 of UV irradiation, and 70% and 22% after 2 hr of treatment with 0.04% and 0.08% MMS respectively.

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