

Cooperation between Small Nuclear RNA-activating Protein Complex (SNAP_C) and TATA-box-binding Protein Antagonizes Protein Kinase CK2 Inhibition of DNA Binding by SNAP_C*

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Protein kinase CK2 regulates RNA polymerase III transcription of human U6 small nuclear RNA (snRNA) genes both negatively and positively depending upon whether the general transcription machinery or RNA polymerase III is preferentially phosphorylated. Human U1 snRNA genes share similar promoter architectures as that of U6 genes but are transcribed by RNA polymerase II. Herein, we report that CK2 inhibits U1 snRNA gene transcription by RNA polymerase II. Decreased levels of endogenous CK2 correlates with increased U1 expression, whereas CK2 associates with U1 gene promoters, indicating that it plays a direct role in U1 gene regulation. CK2 phosphorylates the general transcription factor small nuclear RNA-activating protein complex (SNAP_C) that is required for both RNA polymerase II and III transcription, and SNAP_C phosphorylation inhibits binding to snRNA gene promoters. However, restricted promoter access by phosphorylated SNAP_C can be overcome by cooperative interactions with TATA-box-binding protein at a U6 promoter but not at a U1 promoter. Thus, CK2 may have the capacity to differentially regulate U1 and U6 transcription even though SNAP_C is universally utilized for human snRNA gene transcription.

Protein kinase CK2 is an important regulator of cellular growth (1–4), and abnormal CK2 activity may contribute to tumor progression (5). CK2 is a tetrameric enzyme composed of two catalytic subunits, α and α' , and two copies of the regulatory β subunit (6). One role for CK2 is to function as a regulatory protein that controls gene transcription. For example, general RNA synthesis in yeast is impaired when a temperature-sensitive mutant of the CK2 α' subunit is shifted to a restrictive temperature (7). This decline in total RNA synthesis also suggests that expression of highly transcribed genes encoding ribosomal (r), transfer (t), and small nuclear RNA (snRNA)¹ is sensitive to levels of functional CK2.

In yeast CK2 is important for active RNA polymerase III

transcription (8) and yet, paradoxically, CK2 has been proposed to be the terminal effector in a DNA damage response pathway that represses RNA polymerase III transcription (9). In humans, CK2 exhibits differential effects on gene transcription during the cell cycle. During mitosis, CK2 inhibits RNA polymerase III transcription, whereas at other stages CK2 can stimulate transcription (10). The nature of the regulation is dictated by CK2 target selection. One key target for CK2 is the general transcription factor TFIIB (11–13). There are at least two versions of human TFIIB that function for transcription of distinct classes of genes (14). The Brf1·TFIIB complex functions for 5 S rRNA and tRNA transcription and is composed of the TATA-box-binding protein (TBP) and the TBP-associated factors, Bdp1 and Brf1. The Brf2·TFIIB complex functions for U6 snRNA transcription and is composed of TBP plus Bdp1 but Brf2 instead of Brf1. Brf1·TFIIB phosphorylation during M phase results in the selective release of Bdp1 from tRNA promoters (15). Hernandez and co-workers (10) further demonstrated that Bdp1 is the critical CK2 target within Brf2·TFIIB for mitotic repression of U6 transcription. Because Bdp1 is a shared component of both TFIIB complexes, CK2 may target this factor to repress global RNA polymerase III transcription. However, CK2 inhibitors also interfere with Brf1·TFIIB binding to the TFIIB complex (12), which itself recognizes intragenic promoter elements of 5 S rRNA and tRNA genes, suggesting that CK2 also has a stimulatory role in RNA polymerase III transcription through enhanced preinitiation complex assembly. Consistent with this positive role, CK2 can also activate RNA polymerase III transcription in human cells (12) and in this process may additionally phosphorylate RNA polymerase III itself (13). Together, these data point to an important but complex role for CK2 control of RNA polymerase III transcription.

Human U6 snRNA genes are interesting because they are transcribed by RNA polymerase III and yet their promoters are similar to other snRNA genes, such as U1 and U2, which are transcribed by RNA polymerase II (16–18). Consequently, the mechanisms regulating human snRNA gene transcription by RNA polymerases II and III may also be shared. Nonetheless, the RNA polymerase II-transcribed genes do not use TFIIB and, thus, rely on other factors for regulatory intervention. Regardless of polymerase specificity, all human snRNA genes contain a distal sequence element encompassing an octamer element that is recognized by Oct-1. Additional sites for the Sp1 (19) and STAF (20) transcriptional activator proteins are adjacently located to the distal sequence element at some snRNA genes (21). Oct-1 activates snRNA transcription by direct protein contacts (22–24) with the basal transcription factor called the snRNA-activating protein complex (SNAP_C) (25), which is also referred to as the proximal sequence element transcription

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¹ The abbreviations used are: snRNA, small nuclear RNA; TBP, TATA-box-binding protein; SNAP_C, small nuclear RNA-activating protein complex; PTF, proximal sequence element transcription factor; PSE, proximal sequence element; RNAi, RNA interference; RT, reverse transcription; GST, glutathione S-transferase; DRB, D-ribofuranosylbenzimidazole; HA, hemagglutinin.

factor (PTF) (26). SNAP_C binds to the proximal sequence element (PSE) common to the core promoters of human snRNA genes and functions for both RNA polymerase II and III transcription (18, 25, 27–31). SNAP_C contains at least five proteins called SNAP190 (PTF α), SNAP50 (PTF β), SNAP45 (PTF δ), SNAP43 (PTF γ), and SNAP19 (27–33). The largest subunit SNAP190 plays a centrally important role in human snRNA gene transcription first by serving as the scaffold for SNAP_C assembly though interactions with most other members of SNAP_C (34, 35). Once the complex is assembled, SNAP190 further recognizes the PSE through its Myb DNA binding domain (30) and is also the direct target for Oct-1 (23, 36). In an unexpected twist, SNAP190 can make DNA contacts within the U1 distal sequence element and stimulate the binding of Oct-1 to this enhancer, suggesting that in some contexts coordinated binding of the activator and general transcription machinery is important for transcriptional activation (24).

Human U6 snRNA genes, but not U1 genes, also contain a TATA box that is located adjacently to the PSE, and this promoter arrangement dictates that transcription occurs by RNA polymerase III (37). The TATA box is recognized by the TBP component of the Brf2-TFIIB complex (35, 38–41). SNAP_C, through its SNAP190 subunit, stimulates TBP binding to the U6 TATA box as an early critical step in RNA polymerase III transcription (35, 38).

TBP is also required for human snRNA gene transcription by RNA polymerase II (25), but how TBP is recruited to these TATA-less promoters is unclear. Nonetheless, it is likely that SNAP_C contributes to TBP activity at these genes. SNAP_C and TBP co-purify extensively during the biochemical fractionation of SNAP_C (27), and those fractions enriched for SNAP_C and TBP can reconstitute U1 snRNA transcription *in vitro* from extracts that have been depleted of endogenous TBP (25). Thus, SNAP_C plays a pivotal role in snRNA gene transcription by providing core promoter recognition, serving as a target for transcription activation by Oct-1, and coordinating TBP activity and preinitiation complex assembly for both RNA polymerases II and III. Additional RNA polymerase II general transcription factors are also required for U1 transcription including TFIIA, TFIIB, TFIIE, and TFIIF (42). As in RNA polymerase III transcription, CK2 also has a complex role in regulating RNA polymerase II transcription. CK2 phosphorylation of TFIIA and TFIIE stimulates preinitiation complex assembly at the adenovirus major late promoter, whereas TFIIF phosphorylation can stimulate RNA polymerase II elongation. In contrast, CK2 phosphorylation of RNA polymerase II inhibits transcription, potentially by impairing elongation (43).

The striking parallel between RNA polymerase II and III transcription of human snRNA genes prompted an investigation into the role of phosphorylation in U1 transcription. In this study we report that CK2 inhibits overall U1 snRNA gene transcription by RNA polymerase II and can phosphorylate SNAP_C to inhibit its DNA binding. Interestingly, cooperative interactions of SNAP_C with TBP at U6 but not at U1 promoter DNA can overcome the repressive effects of CK2. Together, these data suggest that CK2 may differentially affect preinitiation complex assembly for RNA polymerase II and III transcription of human snRNA genes depending upon the promoter architecture.

MATERIALS AND METHODS

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation assays from HeLa cells were performed using the anti-CK2 α (Ab245), anti-CK2 β (Ab278) antibodies (44) as well as anti-SNAP43 (CS48) and anti-TBP antibodies described previously (45). Enrichment of genomic sequences in the immunoprecipitation reactions was measured by PCR as previously described (45).

RNA interference (RNAi)—CK2 α and CK2 α' cDNA were generated

with a T7 promoter at both ends by reverse transcription (RT)-PCR using total RNA from HeLa cells as a template. The primers for CK2 α are CK2 α forward, 5'-GCGTAATACGACTCACTATAGGAAATAATGAAAAGTTGTTG-3', and CK2 α reverse, 5'-GCGTAATACGACTCACTATAGGCTCTTGACAGTAAAGCCGTGAC-3'. The primers for CK2 α' are CK2 α' forward, 5'-GCGTAATACGACTCACTATAGGCAACAATGAGAGAGTGGTTG-3', and CK2 α' reverse, 5'-GCGTAATACGACTCACTATAGGTCTGTTGATGGTC GTATCGC-3'. LacZ cDNA with a T7 promoter at both ends was generated by PCR using pPelican-lacZ as a template. The primers used are lacZ forward, 5'-TTAATACGACTCACTATAGGAGACGATAACCACCACGTCATCG-3', and lacZ reverse, 5'-TTAATACGACTCACTATAGGAGAGCGGTTACCCAACTTAATCGCC-3'. Resultant cDNAs were subjected to *in vitro* transcription with T7 polymerase to produce double-stranded RNA. After DNase I treatment, double-stranded RNA was incubated with recombinant Dicer, and resultant Dicer-generated small interfering RNA were purified according to the manufacturer's instructions (Invitrogen). Approximately 250 ng of Dicer-generated small interfering RNA for lacZ, CK2 α , or CK2 α' plus 250 ng of CK2 α' Dicer-generated small interfering RNA were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen). Cells were harvested 30 h later, and total RNA was extracted using TRIzol (Invitrogen). RT-PCR was carried out using Titan One Tube RT-PCR System (Roche Applied Science). The primers used to amplify U1 primary transcript are U1prim forward, 5'-ACTTGCTGCTTCACCACGAA-3', and U1prim reverse, 5'-ACAGCCTCATACGCTCACT-3'. The primers used to amplify the total U1 snRNA population are U1 forward, 5'-ATACTTACCTGGCAGGGGAG-3', and U1 reverse, 5'-CAGGGGAAAGCGCGAACGCA-3'. RT-PCR products were separated by 3% Tris borate EDTA-agarose electrophoresis, stained with ethidium bromide, and visualized with Kodak imaging software.

In Vitro Transcription Assays—*In vitro* transcription of human U1 and U6 snRNA genes were performed as described previously (37) with the following modifications. The HeLa cell nuclear extracts were preincubated with Dignam buffer D either with or without recombinant CK2 and kinase inhibitors for 60 min at 30 °C before initiating transcription by the addition of transcription buffers, nucleotide triphosphates, and DNA templates. The amounts of recombinant CK2 and kinase inhibitors used are indicated in the legend to Fig. 2. Transcripts were separated by denaturing PAGE and visualized by Phosphor-Imager analysis (Amersham Biosciences).

Expression and Purification of Recombinant Proteins—GST-SNAP190-(1–719) was expressed in *Escherichia coli* BL21 (DE3) using the vector pSBet-GST-SNAP190-(1–719) and was purified for *in vitro* kinase assays by affinity chromatography using glutathione-Sepharose beads (Amersham Biosciences). Recombinant mini-SNAP_C containing SNAP190-(1–719), SNAP43, and SNAP50 was co-expressed in *E. coli* using the vector combination pSBet-GST-SNAP190-(1–719) and pET21-His-SNAP43/HA-SNAP50. Recombinant mini-SNAP_C was affinity-purified using glutathione-agarose beads followed by digestion with thrombin to release the complex from the GST tag and dialysis against Dignam buffer D containing 80 mM KCl.

Immunoprecipitation and in Vitro Kinase Assays—For the experiment presented in Fig. 3A, 180 μ l of HeLa cell nuclear extract (~10 mg/ml) was incubated with 20 μ l of rabbit anti-SNAP43 (CS48 (27)), anti-SNAP190 (CS398, CS402 (30)), anti-CK2 α (Ab245 (44)), or preimmune antibodies covalently coupled to protein-G agarose beads. Recovered proteins were analyzed by Western blot using a mouse monoclonal antibody against CK2 α (Transduction Laboratories). For Fig. 3B, 40 μ l of HeLa cell nuclear extract was used for each immunoprecipitation. After extensive washing with HEMGT-150 buffer (20 mM Hepes, pH 7.9, 0.1 mM EDTA, 5 mM MgCl₂, 10% glycerol, 0.5% Tween 20, 150 mM KCl), the beads were suspended in 40 μ l of HEMGT-150 buffer containing 2 μ l of [γ -³²P]ATP (6000 Ci/mmol, 150 mCi/ml), and the samples were incubated at 30 °C for 15 min. The beads were then washed extensively in HEMGT-150 buffer, and proteins were separated by 12.5% SDS-PAGE. Radiolabeled proteins were visualized by autoradiography. For Fig. 3C, 100 μ l of HeLa cell nuclear extracts were used for immunoprecipitation. After kinase reactions, proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Radiolabeled proteins were detected first by autoradiography. Subsequently, Western blot analyses were performed using anti-SNAP190 (CS402) antibodies.

For Fig. 5, B and C, ~5 μ g of GST-SNAP190-(1–719) was bound to glutathione-agarose beads (10 μ l). Immobilized GST-SNAP190-(1–719) was incubated with 10 μ l of HeLa cell nuclear extract for 30 min at 30 °C. The beads were washed extensively with HEMGT-150. *In vitro* kinase assays were then performed directly on the beads. Kinase reactions were also performed using untreated GST-SNAP190-(1–719) plus

10 units of recombinant CK2 (New England Biolabs). Where indicated, kinase reactions were performed in the presence of 20 nM [γ - 32 P]ATP or [γ - 32 P]GTP with or without D-ribofuranosylbenzimidazole (DRB; Sigma) or 3,3',4',5,7-pentahydroxyflavone (quercetin; Sigma).

Tryptic Phosphopeptide Mapping—To obtain material for thin layer chromatography (TLC) analysis, $\sim 1 \mu\text{g}$ of GST-SNAP190-(1–719) was labeled with [γ - 32 P]ATP by using HeLa cell nuclear extracts or recombinant CK2. Phosphorylated GST-SNAP190-(1–719) was gel purified before digestion with sequencing grade modified trypsin (Promega). The tryptic fragments from each of these reactions were spotted individually or were combined at a 1:1 ratio and spotted onto a cellulose TLC plate. Peptides were separated in the first dimension by electrophoresis in pH 1.9 buffer (formic acid (88% w/v)/glacial acetic acid/distilled H_2O , 25:78:897, v/v/v) and in the second dimension by chromatography in chromatography buffer (*n*-butanol/pyridine/glacial acetic acid/distilled H_2O , 15:10:13:12, v/v/v/v) before detection by PhosphorImager analysis (46).

Phosphoamino Acid Analysis—Endogenous SNAP190 was immunoprecipitated, phosphorylated *in vitro* in the presence of [γ - 32 P]ATP, separated by 7.5% SDS-PAGE, and transferred to nitrocellulose membrane. The ~ 190 -kDa radioactive protein corresponding to SNAP190 was excised and hydrolyzed in 5.7 N HCl for 1 h at 100 °C. Recovered phosphoamino acids were vacuum-dried and dissolved in 10 μl of pH 1.9 buffer containing cold phosphoserine, phosphothreonine, and phosphotyrosine mixture. The mixture was separated by one-dimensional electrophoresis (500 V) on cellulose TLC plates (Eastman Kodak Co.) for 1 h at 0 °C in pH 2.5 buffer (67% pH 3.5 buffer (glacial acetic acid, pyridine, water, 50:5:945, v/v/v, containing 0.5 mM EDTA) and 33% pH 1.9 buffer (glacial acetic acid, 88% formic acid, water, 78:25:897, v/v/v)). Unlabeled amino acid standards were visualized by spraying the cellulose plates with ninhydrin. The ^{32}P -labeled amino acid residues were visualized by autoradiography with a PhosphorImager.

Electrophoretic Mobility Shift Assays—Approximately 100 ng of purified mini-SNAP_c and/or 30 ng of TBP were preincubated alone or with 15 or 150 units CK2 and/or 7 mM ATP for 30 min at 30 °C. Electrophoretic mobility shift assays were then performed using DNA probes containing a wild-type mouse U6 PSE with a wild-type or mutant human U6 TATA box as described previously (38, 47).

RESULTS

CK2 Inhibits U1 snRNA Gene Transcription—The conservation of similar promoter architectures among the human snRNA gene family suggests that these genes could be coordinately regulated. That CK2 regulates human U6 snRNA gene transcription by RNA polymerase III (13) prompted us to examine whether CK2 similarly regulates human snRNA gene transcription by RNA polymerase II. First, chromatin immunoprecipitation experiments were performed to determine whether endogenous CK2 could associate with the promoter regions of both U6 and U1 snRNA genes. As shown in Fig. 1A, both U6 and U1 promoter regions were enriched in immunoprecipitation reactions using anti-CK2 α antibodies (lane 7), whereas U1 but not U6 promoter regions were enriched in the anti-CK2 β immunoprecipitation reactions (lane 8). Similar results were obtained in experiments performed with different antibodies directed against CK2 α and CK2 β (data not shown). Possibly the epitopes recognized by the CK2 β antibodies may be occluded by other transcription factors at the U6 promoter. However, this result stands in contrast with that noted previously (13) wherein a more robust CK2 β association with this U6 promoter was noted and only weak U1 promoter association by any CK2 subunit was detected. The reason for this discrepancy is unclear, but differences in chromatin immunoprecipitation or cell growth conditions could potentially affect promoter recovery by CK2 antibodies. The levels of U1 and U6 promoter recovery in this reaction were less than that those observed in either anti-SNAP43 (lane 6) or anti-TBP (lane 9) reactions but markedly greater than that seen in reactions using IgG (lane 5). No significant enrichment of the glyceraldehyde-3-phosphate dehydrogenase exon 2 or U1 upstream regions was observed in any reactions. Therefore, endogenous CK2 associates with the promoter regions of both U1 and U6 genes and suggests the possibility that CK2 could additionally

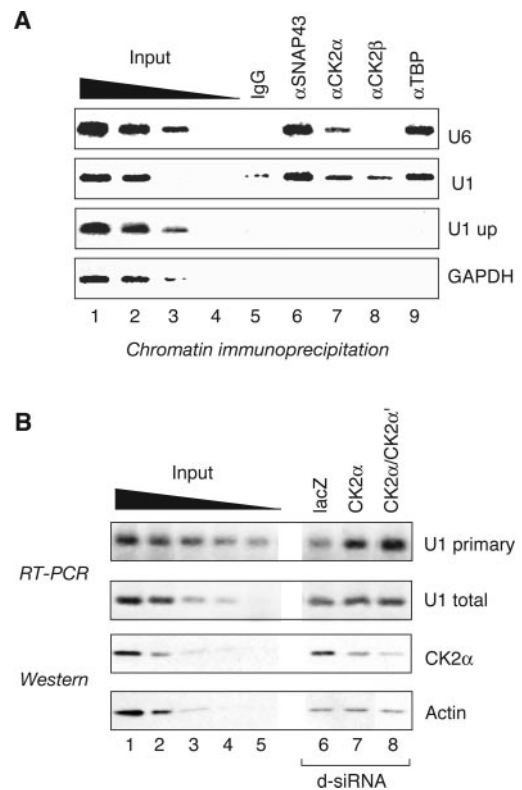


FIG. 1. CK2 inhibits U1 snRNA gene expression. A, endogenous CK2 associates with snRNA gene promoters. Chromatin immunoprecipitation experiments were performed using HeLa cell chromatin and the indicated antibodies. Enrichment of the U6 and U1 promoter regions was detected by PCR and were compared with recovery of the U1 upstream regions and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) exon 2 as negative controls. B, U1 primary transcripts accumulate after CK2 reduction. CK2 levels were reduced by transient transfection of Dicer-generated small interfering RNA (*d-siRNA*) (52) corresponding to CK2 α (lane 7) or CK2 α plus CK2 α' (lane 8). Cells were also treated with LacZ Dicer-generated small interfering RNA as a reference (lane 6) of the U1 primary transcript and total U1 population were monitored by RT-PCR (top). Endogenous CK2 and actin levels were measured by Western analysis (bottom). For reference, lanes 1–5 contain 2-fold decreasing increments of material harvested from untreated cells to serve as a standard curve for each assay.

affect human snRNA gene expression by RNA polymerase II.

To test whether endogenous CK2 influences human U1 gene expression in living cells, CK2 levels were reduced by RNAi, and the effect on U1 snRNA production was monitored by RT-PCR (Fig. 1B). As a negative control, RNAi was also performed using lacZ-specific RNA. Because it was demonstrated that phosphorylation of the carboxyl-terminal domain of RNA polymerase II contributes to 3' processing of human U2 snRNA (48, 49), it was possible that CK2 could also play a role in U1 3' processing. Therefore, in this experiment different primer combinations were used to detect either the primary U1 snRNA transcript that is normally processed rapidly or the total U1 snRNA steady state population. Lanes 1–5 (top panel) show that amplification of the U1 primary transcript and total U1 snRNA levels were directly correlated with the amount of RNA included in the reaction. Interestingly, U1 primary transcript levels were increased in cells treated with CK2 α RNAi (lane 7), and this effect was enhanced by RNAi directed against both CK2 α and CK2 α' (lane 8) as compared with cells treated with LacZ-specific RNAi (lane 6). Steady state U1 snRNA levels remained effectively unchanged by any of these treatments, consistent with their abundant and extremely stable nature. In this experiment endogenous CK2 levels were reduced ~ 2 – 4 -fold relative to the levels observed in the LacZ-RNAi-treated

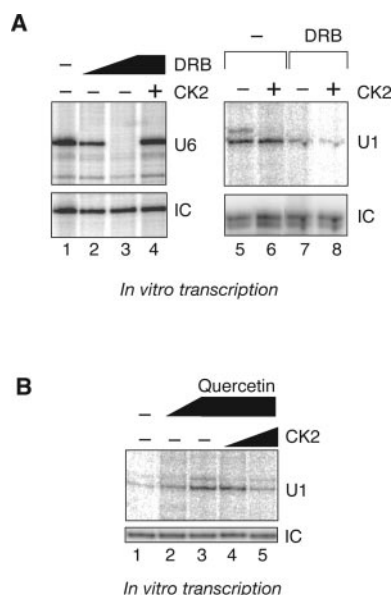


FIG. 2. CK2 represses U1 transcription. *A*, CK2 reverses inhibition of U6, but not U1, transcription by the kinase inhibitor DRB. *In vitro* U6 transcription assays were performed in the absence (lane 1) or presence of DRB (lane 2, 1 μ M DRB; lanes 3 and 4, 7 μ M DRB). The reaction shown in lane 4 also contains 10 units of recombinant CK2. *In vitro* U1 transcription assays were performed using a U1 G-less cassette in the absence (lanes 5 and 7) or presence of 10 units of recombinant CK2 (lanes 6 and 8) either in the absence (lanes 5 and 6) or presence of 7 μ M DRB (lanes 7 and 8). IC, internal control. *B*, *in vitro* U1 transcription is sensitive to the CK2 inhibitor quercetin. Additional U1 transcription assays were performed in the absence (lane 1) or presence of 1 μ M (lanes 2) and 7 μ M quercetin (lanes 3–5), respectively, whereas an additional 10 and 100 units of CK2 were added to reactions shown in lanes 4 and 5, respectively.

cells, whereas actin levels were unaltered by any of the treatments (bottom panel). The increase in U1 primary transcripts with reduced CK2 levels suggests that endogenous CK2 normally stimulates U1 3' processing and/or inhibits U1 transcription.

To determine whether CK2 plays a direct role in U1 snRNA gene transcription, recombinant CK2 and commonly used inhibitors of CK2 were tested for their effect using *in vitro* U1 transcription assays. One characteristic of CK2 is that it can be inhibited by both DRB and quercetin, and therefore, these inhibitors were selected. As a positive control for CK2 activity, *in vitro* U6 transcription was also examined (Fig. 2A). First, U6 transcription was inhibited with increasing amounts of the kinase inhibitor DRB (lanes 2 and 3), and DRB action was reversed by the addition of CK2 (lane 4). Together, these data suggest that CK2 has an overall positive role in U6 transcription consistent with observations previously described (13). No measurable effect on U1 transcription was observed by the addition of recombinant CK2 (compare lanes 5 and 6) perhaps because the HeLa cell extracts used for these experiments contain high levels of CK2 and CK2 activity is not rate-limiting for transcription. U1 transcription was inhibited by DRB (lane 7), but inhibition was not reversed by the addition of CK2 (lane 8), suggesting that in addition to CK2 DRB inhibits a kinase activity that is important for U1 snRNA gene activity. Indeed, the DRB-sensitive elongation factor p-TEFb, which phosphorylates the carboxyl-terminal domain of RNA polymerase II (50), also contributes positively to efficient U1 transcription *in vitro* (data not shown).

Next, the effect of quercetin in U1 transcription was tested. As shown in Fig. 2B and in contrast with DRB, quercetin addition stimulated U1 transcription (lanes 2 and 3). The increased background in reactions containing quercetin indicates

this inhibitor may have nonspecific positive effects on transcription possibly from cryptic promoters on the reporter plasmid. Although quercetin can inhibit a variety of kinases, the increase in U1-specific transcription was reversed by the addition of increasing amounts of recombinant CK2 (lanes 4 and 5), suggesting that CK2 has a direct and overall negative role in controlling U1 transcription. Previously, U2 snRNA gene transcription by RNA polymerase II in nuclear run-on assays was not sensitive to DRB (48, 49), suggesting that CK2 is not involved in the transcription of these genes. The possibility remains that U1 and U2 gene transcription is differentially sensitive to regulation by CK2. Nonetheless, because U1 and U2 genes utilize similar promoter elements and general transcription factors for efficient transcription, a role for CK2 in U2 transcription cannot yet be dismissed.

Endogenous CK2 Targets SNAP190 for Phosphorylation at Multiple Sites—SNAP_C recognizes the core promoters of human snRNA genes and plays an important early role in coordinating transcription of snRNA genes by both RNA polymerases II and III. The findings that CK2 can affect both human U1 and U6 transcription (Fig. 2 and Ref. 13) implicates SNAP_C as a potential target for CK2. First, we examined whether CK2 co-purifies with SNAP_C. As shown in Fig. 3A, endogenous CK2 from HeLa cell nuclear extract was recovered with SNAP_C during immunoprecipitation using anti-SNAP190 (lanes 4 and 5) or anti-SNAP43 (lane 3) antibodies but not while using IgG antibodies (lane 2). These levels of recovered CK2 are significantly less than that observed in reactions using antibodies against CK2 α (lane 7), suggesting that only a minor proportion of SNAP_C is associated with CK2 or that the interaction between SNAP_C and CK2 is not stable. In separate experiments, recombinant CK2 alone did not cross-react with the anti-SNAP43 antibodies (data not shown), suggesting that recovery of CK2 in these assays requires SNAP_C. These results indicate that endogenous CK2 associates with SNAP_C.

Next, to determine whether any subunits of SNAP_C can be phosphorylated by SNAP_C-associated kinase(s), including CK2, the anti-SNAP43 immunoprecipitated proteins were directly assayed for kinase activity by incubation with [γ -³²P]ATP. As shown in Fig. 3B, robust phosphorylation of a 190-kDa protein was observed in the anti-SNAP43 immunoprecipitated samples (lane 1), suggesting that SNAP190 is extensively phosphorylated in this assay. After a longer exposure (as shown in Fig. 3B), proteins of 60 kDa, 43 kDa (labeled SNAP43), and 19 kDa (labeled with an asterisk (*)) in size were additionally observed. The identity of the 60-kDa protein is unknown; however, these results suggest that SNAP43 and SNAP19 were also phosphorylated in these assays but to a much lesser extent than SNAP190.

To confirm the identity of the proteins phosphorylated by the SNAP_C-associated kinase, immunopurified SNAP_C was used for *in vitro* kinase assays followed by Western blot analysis using SNAP_C-specific antibodies (Fig. 3C). A 190-kDa protein is phosphorylated in kinase assays using material recovered by either anti-SNAP43 (lane 2) or anti-SNAP190 (lane 3) immunoprecipitation but not by immunoprecipitation with preimmune antibodies (lane 1). As expected, no phosphorylation was observed when [γ -³²P]ATP was not included in the kinase reaction (lane 4). Lanes 5 and 6 show the results of anti-SNAP190 Western blot analysis for the same reactions shown in lanes 3 and 4. SNAP190 is detected in both reactions regardless of whether [γ -³²P]ATP is added or not. Importantly, SNAP190 as detected by Western blot analysis co-migrated with the 190-kDa protein that is phosphorylated by the SNAP_C-associated kinase, indicating that the 190-kDa phosphoprotein is indeed SNAP190. In similar experiments, SNAP43 co-migrated with

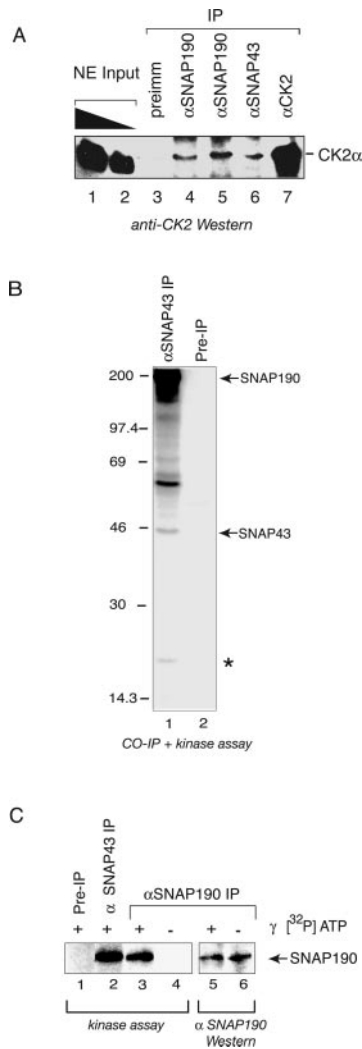


FIG. 3. Endogenous CK2 associates with SNAP_C. *A*, HeLa cell nuclear extracts (*NE*) were immunoprecipitated (*IP*) with preimmune (*lane 3*), anti-SNAP190 (*lane 4*, CS398; *lane 5*, CS402), anti-SNAP43 (*lane 6*, CS48), or anti-CK2 α (*lane 7*, Ab245) antibodies as indicated. Recovered proteins were analyzed by Western blot analysis using antibodies directed against the α subunit of CK2. *Lanes 1* and *2* contain 3 and 1 μ l of nuclear extract, respectively. *B*, multiple subunits of SNAP_C are phosphorylated *in vitro* by a SNAP_C-associated kinase activity. Immunoprecipitation reactions were performed from HeLa cell nuclear extracts using anti-SNAP43 (*lane 1*) or preimmune (*lane 2*) antibodies that were immobilized on protein G-agarose beads. After extensive washing, an *in vitro* kinase assay was performed on the beads in the presence of [γ -³²P]ATP. Proteins were then separated by 15% SDS-PAGE, and radiolabeled proteins were detected by autoradiography. The positions of SNAP190 and SNAP43 are labeled, whereas a protein that migrates similarly to SNAP19 is indicated by an asterisk. *C*, SNAP190 co-migrates with a 190-kDa phosphoprotein. HeLa cell nuclear extracts were immunoprecipitated with preimmune (*lane 1*), anti-SNAP43 (*lane 2*), or anti-SNAP190 antibodies (*lanes 3* and *4*). Recovered samples were subjected to an *in vitro* kinase assay in the presence (*lanes 1–3*) or absence (*lane 4*) of [γ -³²P]ATP. Proteins were separated by 7.5% SDS-PAGE and transferred to nitrocellulose membrane. Radiolabeled proteins were detected by autoradiography for 1 h (*lanes 1–4*). This membrane was then used for Western blot analysis using antibodies directly against SNAP190. *Lanes 5* and *6* are the same as *lanes 3* and *4* but exposed on film for 5 s. The position of SNAP190 is indicated.

the 43-kDa phosphoprotein (data not shown), suggesting that SNAP43 is also phosphorylated by the SNAP_C-associated kinase. A similar experiment performed to determine whether the 19-kDa phosphoprotein observed in the *in vitro* kinase assays is SNAP19 was inconclusive because our anti-SNAP19 antibodies were not sensitive enough to detect SNAP19 in this

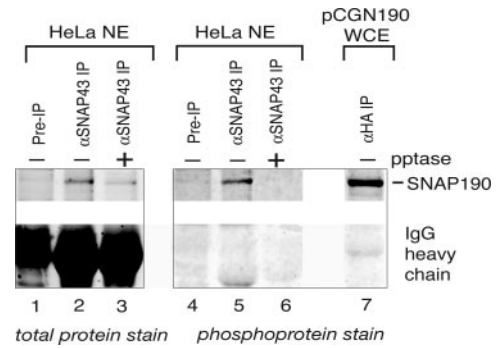


FIG. 4. Endogenous SNAP_C is phosphorylated. HeLa cell nuclear extracts (*NE*) were immunoprecipitated (*IP*) by either preimmune (*lanes 1* and *4*) or anti-SNAP43 antibodies (*lanes 2* and *3* and *lanes 5* and *6*). The protein complex recovered by anti-SNAP43 immunoprecipitation was left untreated (*lanes 2* and *5*) or dephosphorylated by calf intestine alkaline phosphatase (*pptase*, *lanes 3* and *6*). As an additional size marker, HA-SNAP190 was overexpressed in HeLa cells by transient transfection and was immunoprecipitated using an anti-HA antibody (*lane 7*). Proteins were separated by 7.5% SDS-PAGE and were stained first with Pro-Q diamond phosphoprotein dye (*lanes 4–7*) and then with SYPRO Ruby total protein dye (*lanes 1–3*). WCE, whole cell extract.

assay. Taken together, these results demonstrate that a SNAP_C-associated kinase phosphorylates SNAP190 and SNAP43.

To determine whether endogenous SNAP_C is phosphorylated, α SNAP43 immunoprecipitation reactions were performed from HeLa cell nuclear extracts, and recovered proteins were directly analyzed by Pro-Q diamond staining (Molecular Probes), which specifically detects phosphorylated proteins. As shown in Fig. 4, a protein of \sim 190 kDa was detected in the anti-SNAP43-immunoprecipitated sample (*lane 5*) that was not observed in a similarly immunoprecipitated sample treated with phosphatase (*lane 6*), indicating that this protein is phosphorylated. This 190-kDa protein was not detected in the sample recovered by immunoprecipitation with nonspecific antibodies (*lane 4*). Furthermore, the 190-kDa protein co-migrated with HA-tagged SNAP190 recovered by anti-HA immunoprecipitation from transiently transfected HeLa cells (*lane 7*), suggesting that the endogenous 190-kDa phosphoprotein is SNAP190. This same gel was analyzed by Sypro ruby staining to detect the total level of recovered proteins. The 190-kDa protein was detected in both the untreated (*lane 2*) and phosphatase-treated (*lane 3*) samples, although SNAP190 staining was reduced in the latter sample. In all immunoprecipitated samples, significant levels of IgG heavy chain were detected by Sypro ruby staining (*bottom panel*) but not by Pro-Q diamond staining, further demonstrating that phosphorylated proteins are specifically detected in this assay. Therefore, we conclude that endogenous SNAP190 is phosphorylated. Other subunits of SNAP_C were not detected in these assays (not shown) perhaps because they are not phosphorylated *in vivo* or the recovered levels of phosphorylated protein in these assays were below the threshold of detection using the Pro-Q diamond stain.

We had previously observed that recombinant SNAP43 was preferentially phosphorylated when assembled into SNAP_C, and furthermore, efficient SNAP43 phosphorylation required SNAP190-(1–719), suggesting that SNAP190 is responsible for recruiting a kinase activity to the complex (data not shown). In those experiments, SNAP190-(1–719) was also extensively phosphorylated. Therefore, to determine whether CK2 interacts with SNAP190, GST-SNAP190-(1–719) was used to affinity-purify kinase(s) activity from HeLa nuclear extracts, and anti-CK2 Western analysis was performed (Fig. 5A). Significant amounts of CK2 associated with GST-SNAP190-(1–719)

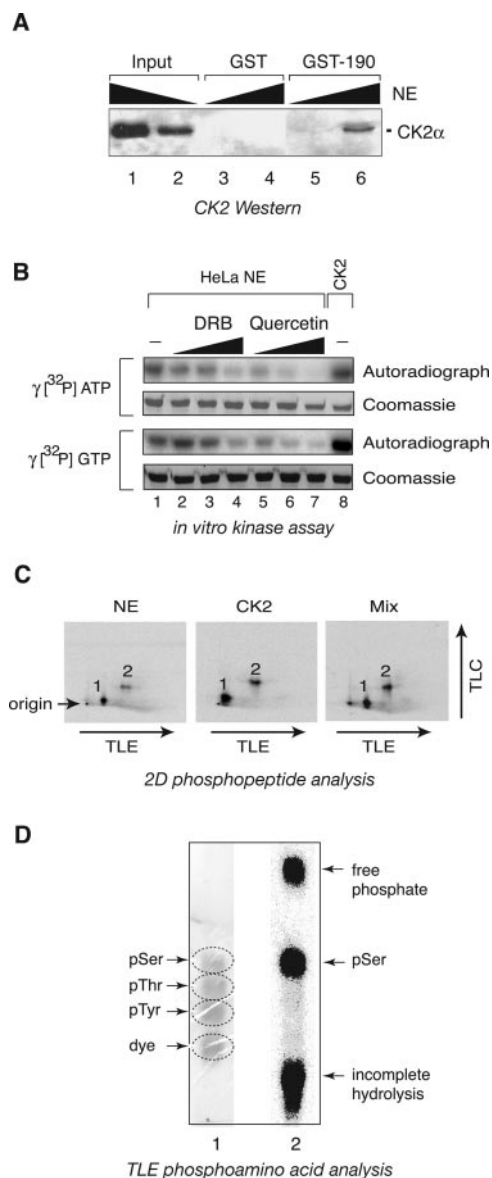


FIG. 5. Endogenous CK2 phosphorylates the amino-terminal region of SNAP190. **A**, endogenous CK2 can interact with SNAP190-(1-719). Approximately 3 μ g of recombinant GST (lanes 3 and 4) or GST-SNAP190-(1-719) (lanes 5 and 6) were bound to glutathione-agarose beads and incubated with 60 or 180 μ l of HeLa cell nuclear extract (NE). Associated proteins were separated by 12.5% SDS-PAGE for anti-CK2 α Western analysis. Lanes 1 and 2 contain 3 and 1 μ l of nuclear extract, respectively. **B**, the SNAP190-associated kinase activity exhibits properties of CK2. Recombinant GST-SNAP190-(1-719) bound to glutathione-agarose beads was pretreated with HeLa cell nuclear extracts, washed, and then incubated with either [γ - 32 P]ATP (top panel) or [γ - 32 P]GTP (bottom panel) in the presence of 1, 7, and 50 μ M DRB (lanes 2, 3, and 4, respectively) or 1, 7, and 50 μ M quercetin (lanes 5, 6, and 7, respectively). GST-SNAP190-(1-719) in lane 8 was treated with recombinant CK2 (10 units). The amounts of GST-SNAP190-(1-719) used in each reaction were visualized by Coomassie Blue staining before autoradiography. **C**, recombinant CK2 and the SNAP190-associated kinase from HeLa cell nuclear extracts phosphorylate the same regions within SNAP190-(1-719). Analysis of tryptic fragments obtained from radiolabeled GST-SNAP190-(1-719) phosphorylated by the SNAP190 associated kinase (left panel) or by recombinant CK2 (middle panel) was performed by two-dimensional thin layer electrophoresis in the first dimension followed by thin layer chromatography in the second dimension. The tryptic peptides obtained from each of these samples were mixed at 1:1 ratio for analysis (right panel). The major phosphopeptides are indicated. The directions of electrophoresis and chromatography are indicated by arrows. **D**, CK2 phosphorylates SNAP190 at serine residues. Radioactively labeled endogenous SNAP190 was acid-hydrolyzed and dissolved in pH 1.9 buffer containing cold phosphoserine (pSer), phosphothreonine (pThr), and phospho-

relative to that observed in reactions containing GST (compare lanes 5 and 6 to lanes 3 and 4), indicating that CK2 can interact with SNAP190.

To investigate whether CK2 is the predominant kinase that associates with GST-SNAP190-(1-719) in these assays, the effect of kinase inhibitors on GST-SNAP190-(1-719) phosphorylation by the HeLa cell-derived kinase was investigated. As shown in Fig. 5B (top panel), both DRB (lanes 2-4) and quercetin (lanes 5-7) were effective in limiting the extent of GST-SNAP190-(1-719) phosphorylation as compared with the untreated sample (lane 1). A second hallmark of CK2 is that it is capable of utilizing GTP as a phosphoryl group donor. Indeed, robust phosphorylation of GST-SNAP190-(1-719) was observed when [γ - 32 P]GTP was included in the reaction (bottom panel, lane 1), and this GTP-based phosphorylation was inhibited by DRB (lanes 2-4) and quercetin (lanes 5-7), consistent with the idea that endogenous CK2 can associate with and phosphorylate SNAP190.

To further examine the extent of SNAP190 phosphorylation by endogenous CK2, GST-SNAP190-(1-719) was phosphorylated either by recombinant CK2 or by endogenous kinase(s) present in the HeLa cell extract that are capable of associating with SNAP190. Subsequently, phosphorylated GST-SNAP190-(1-719) was digested with trypsin, and the radiolabeled peptides were compared by two-dimensional TLC (Fig. 5C). This analysis revealed two major SNAP190 tryptic peptides that were phosphorylated by the kinase recruited from HeLa cell extracts (left panel) and by recombinant CK2 (middle panel). The tryptic peptides from both these kinase reactions were then mixed and analyzed as before (right panel). As had been observed for the individual analysis of the tryptic peptides, only two predominate spots were observed, suggesting that recombinant CK2 and the kinase activity from HeLa cells phosphorylate SNAP190 within the same regions, thus providing additional evidence confirming that the HeLa cell-derived kinase is CK2. Endogenous SNAP190 that was phosphorylated by HeLa cell-derived CK2 was also hydrolyzed for phosphoamino acid analysis (Fig. 5D), which revealed that phosphorylation occurs predominately on serine residues in this assay. Given the above observations, we conclude that the major SNAP190-associated kinase in HeLa nuclear extracts is CK2.

CK2 Restricts SNAP_C Promoter Recognition—An examination of the amino acid sequence of SNAP190-(1-719) revealed a total of 13 CK2 consensus motifs containing serines, most of which are clustered around regions involved in cooperative promoter recognition by SNAP_C in the presence of TBP (35, 38). Other CK2 sites are contained within the Myb DNA binding domain of SNAP190, in particular, the Rh and Ra Myb repeats (30). Because CK2 had previously been shown to inhibit DNA binding by other Myb-domain proteins (51), these observations immediately suggested the possibility that CK2 could potentially inhibit both DNA binding and TBP recruitment by SNAP_C.

To test whether CK2 could affect DNA binding by SNAP_C, electrophoretic mobility shift assays were performed with DNA probes resembling either a U6 (wild-type PSE and TATA) or U1 promoter (wild-type PSE with mutant TATA). The recombinant SNAP_C used in these reactions is a partial complex containing full-length SNAP43 and SNAP50 along with SNAP190-(1-719), hereafter referred to as mini-SNAP_C, and this complex

tyrosine (pTyr). The mixture was separated by one-dimensional thin layer electrophoresis (TLE), and phosphoamino acids were visualized with ninhydrin (lane 1). Subsequently, radiolabeled phosphoamino acids were detected by PhosphorImager analysis (lane 2). Identical results were obtained with full-length HA-SNAP190 and GST-SNAP190-(1-719) (not shown).

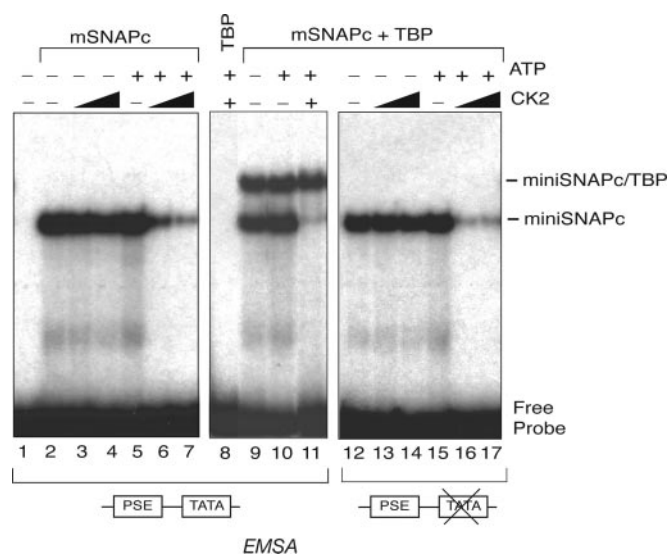


FIG. 6. TBP and SNAP_C cooperate at U6 but not at U1 promoter probes to overcome CK2 inhibition of DNA binding by SNAP_C. An electrophoretic mobility shift assay was performed using U6-like (wild-type PSE, wild-type TATA) or U1-like (wild-type PSE, mutant TATA) probes in the absence (lane 1) or presence of recombinant mini-SNAP_C (lanes 2–7 and 9–17). Reactions shown in lanes 8–17 also contained recombinant full-length human TBP. As indicated, reactions were performed in the absence or presence of increasing amounts of recombinant CK2 (15 or 150 units) and ATP (7 mM). The reaction in lane 11 contained 150 units of CK2. The positions of the miniSNAP_C-DNA and miniSNAP_C-TBP-DNA complexes are indicated. EMSA, electrophoretic mobility shift assay.

is competent for both U1 and U6 promoter binding. As shown in Fig. 6, robust U6 promoter binding was observed by recombinant mini-SNAP_C in the absence (lane 2) or presence of increasing amounts of recombinant CK2 (lanes 3 and 4) or ATP (lane 5) alone. However, PSE recognition by mini-SNAP_C was dramatically inhibited by the addition of ATP along with recombinant CK2 (lanes 6 and 7), suggesting that SNAP_C phosphorylation by CK2 inhibits its ability to recognize the PSE.

In addition to direct promoter recognition, SNAP_C functions to recruit TBP to the TATA box that is adjacent to the PSE within human U6 snRNA genes (47). Therefore, the effect of mini-SNAP_C phosphorylation on its ability to cooperatively bind with TBP on the U6 promoter was tested. In these assays full-length human TBP alone does not bind well to the U6-specific probe either in the presence or absence of CK2 and ATP (lane 8, and data not shown, respectively). As expected, formation of a super-shifted complex is observed with mini-SNAP_C and TBP in reactions performed without CK2 and ATP (lane 9). The addition of only ATP to similar reactions did not affect cooperative DNA binding by mini-SNAP_C and TBP (lane 10), but interestingly, cooperative DNA binding by mini-SNAP_C and TBP was maintained in reactions containing ATP and CK2 under conditions where DNA binding by mini-SNAP_C alone was impaired (lane 11). Reduced formation of the mini-SNAP_C-DNA complex in this reaction indicates that most of the mini-SNAP_C is likely phosphorylated. Therefore, CK2 restricts promoter recognition by mini-SNAP_C alone while permitting higher order complex formation with TBP on the U6-specific probe.

Next, whether TBP could overcome CK2-mediated inhibition of DNA binding by SNAP_C on U1-specific probes was tested. On these TATA-less probes, only PSE binding by mini-SNAP_C was observed in the absence (lane 12) or presence of CK2 (lanes 13 and 14) or ATP (lane 15) individually. TBP also did not rescue PSE recognition by mini-SNAP_C on the U1-related probe in reactions containing CK2 and ATP (lanes 16 and 17). There-

fore, TBP requires direct promoter recognition via the TATA box to overcome CK2-imposed restricted promoter recognition by mini-SNAP_C. Together, these results suggest the intriguing possibility that CK2 inhibits DNA binding by SNAP_C while permitting robust promoter recognition by SNAP_C and TBP on DNA probes containing an appropriate arrangement of promoter elements.

DISCUSSION

Human snRNA genes are transcribed by two different RNA polymerases depending upon the core promoter structure of these genes. Within the core promoter, the PSE is common to all human snRNA genes regardless of polymerase specificity, and consequently, the basal transcription factor SNAP_C that binds this element is used for transcription by RNA polymerases II and III (27–31). Our data demonstrate that CK2 inhibits RNA polymerase II transcription of human U1 snRNA genes *in vitro* and *in vivo*. This observation is consistent with chromatin immunoprecipitation experiments demonstrating that CK2 is present at endogenous human U1 and U6 snRNA gene promoters in HeLa cells (Fig. 1A and Ref. 13), which directly links this kinase to the regulation of snRNA gene transcription in the cell.

The data presented herein further support a role for SNAP_C as a target for CK2 regulation. In other experiments examining the role of CK2 for human U6 transcription, SNAP_C phosphorylation had minimal impact (10), raising the possibility that SNAP_C is not a target for CK2 regulation. As noted by the authors of that study, the recombinant SNAP_C used was expressed in insect cells, and it could have been already phosphorylated and, thus, refractive to further effects of CK2. In that system CK2 stimulated transcription by phosphorylating RNA polymerase III but also inhibited transcription by phosphorylating the Bdp1 subunit of TFIIIB (10). To determine whether CK2 affects SNAP_C function we examined the effect of phosphorylation on recombinant SNAP_C expressed in *E. coli*. Our studies indicate that CK2 does impair SNAP_C binding to the PSE within the U6 promoter but adjacent TBP binding to the TATA-box can rescue SNAP_C recruitment. Thus, CK2 may play an important role in ensuring that SNAP_C is not engaged in nonproductive preinitiation complex formation at inappropriate sites in the genome by restricting DNA binding and requiring multiple factors for promoter recognition. In contrast, TBP did not rescue SNAP_C binding to DNA containing the U1-like arrangement of promoter elements, suggesting that SNAP_C phosphorylation by CK2 could be important for the repressive effects of CK2 observed in U1 transcription. Whether CK2 can disable SNAP_C already bound to DNA is not known, but if so, this would suggest that CK2 could act after preinitiation complex assembly. It will be important to determine the cellular context for CK2 action on SNAP_C.

In our assays we observed that two subunits of SNAP_C, SNAP190 and SNAP43, were phosphorylated by CK2. Both SNAP43 and SNAP190 interact with TBP and are candidates for regulatory intervention by CK2 to influence TBP recruitment at snRNA gene promoters. However, we favor the idea that SNAP190 plays a dominant role in this process. First, SNAP190 is a better substrate for CK2. Second, SNAP190 contains an unusual Myb DNA binding domain consisting of four and a half Myb repeats (30), and CK2 was shown previously to inhibit DNA binding by the c-Myb nuclear oncoprotein (51). That phosphorylated SNAP_C can bind DNA cooperatively with TBP in reactions wherein SNAP_C is unable to bind DNA alone (Fig. 6) argues that most SNAP_C in these reactions is phosphorylated and argues against the idea that CK2 inhibits SNAP_C though phosphorylation of a residue that is critical for DNA interaction. Instead, we speculate that phosphorylation

induces a conformational change in SNAP190, rendering it unable to recognize the PSE. Interestingly, a number of CK2 sites within SNAP190 are located adjacently to the TBP-recruiting region (TRR)-1 and TRR-2 that are involved in TBP recruitment to the U6 promoter (35, 38). TRR-2 coincides with the SNAP190 Rc and Rd Myb repeats, and interaction with TBP may unveil the SNAP190 Myb DNA binding domain to allow promoter recognition. It is not known that these same regions are important for TBP recruitment to U1 promoters, but as we demonstrate, TBP does not overcome CK2-mediated inhibition of DNA binding by SNAP_C to U1-like arrangement of promoter elements. This notion further suggests that CK2 may have the capacity to differentially regulate U1 and U6 transcription even though SNAP_C is universally used for snRNA gene transcription. As with human U6 transcription, it remains possible that CK2 phosphorylates different factors during the cell cycle to enact either positive or negative outcomes on U1 transcription.

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