The Protein Kinase CK2 Phosphorylates SNAP190 to Negatively Regulate SNAPC DNA Binding and Human U6 Transcription by RNA Polymerase III*

Received for publication, March 15, 2007, and in revised form, July 31, 2007 Published, JBC Papers in Press, August 1, 2007, DOI 10.1074/jbc.M702269200

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Human U6 small nuclear RNA gene transcription by RNA polymerase III requires the general transcription factor SNAPC, which binds to human small nuclear RNA core promoter elements and nucleates pre-initiation complex assembly with the Brf2-TFIIIB complex. Multiple components in this pathway are phosphorylated by the protein kinase CK2, including the Bdp1 subunit of the Brf2-TFIIIB complex, and RNA polymerase III, with negative and positive outcomes for U6 transcription, respectively. However, a role for CK2 phosphorylation of SNAPC in U6 transcription has not been defined. In this report, we investigated the role of CK2 in modulating the transcriptional properties of SNAPC and demonstrate that within SNAPC, CK2 phosphorylates the N-terminal half of the SNAP190 subunit at two regions (amino acids 20–63 and 514–545) that each contain multiple CK2 consensus sites. SNAP190 phosphorylation by CK2 inhibits both SNAPC DNA binding and U6 transcription activity. Mutational analyses of SNAP190 support a model wherein CK2 phosphorylation triggers an allosteric inhibition of the SNAP190 Myb DNA binding domain.

In humans, polymerase specificity for snRNA gene transcription depends upon the architecture of the core promoter region. snRNA genes containing solely a proximal sequence element (PSE) are transcribed by RNA polymerase II, whereas those containing juxtaposed PSE and TATA box elements are transcribed by RNA polymerase III (1). The human small nuclear RNA-activating protein complex (SNAPC) is a general transcription factor that coordinates human snRNA gene transcription for both polymerases (2) through its ability to bind to the PSE (3), while serving as a target for numerous regulatory factors that influence snRNA gene transcription. SNAPC, also known as PTF (4), consists of five subunits called SNAP190 (PTFα), SNAP50 (PTFβ), SNAP45 (PTFδ), SNAP43 (PTFγ), and SNAP19 (2, 5–10). Within this complex, PSE-specific binding is mediated by two of these factors SNAP190, which contains a Myb DNA binding domain (5), and SNAP50, which contains an unorthodox, but evolutionarily conserved zinc finger DNA binding domain (11). Neither SNAP190 nor SNAP50 can bind DNA alone, but instead are coordinated by SNAP43, which interacts with both factors to facilitate formation of a complex that is capable of DNA binding (12, 13).

In addition to its role in PSE recognition, SNAP190 plays a pivotal role in snRNA gene transcription by interacting with the TATA-box-binding protein (TBP), stimulating TBP promoter recruitment to TATA-box containing snRNA genes (14). Two regions within SNAP190 have been defined as playing a role in TBP recruitment. One TBP recruitment region (TRR1) is located toward the N terminus (15) and is adjacent to the region involved in SNAPC assembly with SNAP19 and SNAP43 (16). A second TBP recruitment region (TRR2) is located within the Myb DNA binding domain and was proposed to interact with the TBP DNA binding domain (13); these interactions are likely stabilized by adjacent binding of both factors at the PSE and TATA box, respectively. SNAP190 is also a direct target of the transcriptional activator protein Oct-1 (17, 18), which binds to an octamer sequence in the distal sequence element of human snRNA genes.

As a central player in snRNA gene transcription, SNAP190 is also targeted for regulation through post-translational modification. For example, SNAP190 is phosphorylated both in vivo and in vitro, and in part, phosphorylation is mediated by the protein kinase CK2 that associates with and phosphorylates the N-terminal half of SNAP190 predominately at serine residues (19). CK2 associates with other components of the RNA polymerase III transcriptional machinery, including the Brf1-TFIIIB complex (20), as well as with RNA polymerase III itself (21). The functional consequences of CK2 association with these components of the RNA polymerase III transcriptional machinery are complex. CK2 phosphorylation of RNA polymerase III stimulates U6 snRNA gene transcription (21); however, during mitosis CK2 can instead down-regulate U6 transcription by phosphorylating Bdp1 (22), a component shared by both Brf1-TFIIIB and Brf2-TFIIIB complexes (23). Phosphorylation of Bdp1 disrupts its promoter association (22) and may ensure that RNA polymerase III recruitment or promoter stabilization by Bdp1 does not occur at inappropriate points in the cell cycle.
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A role for SNAP_C phosphorylation in snRNA gene transcription by RNA polymerase III has not been described (21, 22). However, CK2 does inhibit DNA binding by a partial SNAP_C, assembled from components expressed in Escherichia coli (19). Interestingly, the inhibitory effect of CK2 was suppressed by cooperative promoter binding by the partial SNAP_C and TBP on DNA containing a juxtaposed PSE and TATA box, but not on TATA-less probes, suggesting that CK2 phosphorylation of SNAP_C was likely to be detrimental for RNA polymerase II on TATA-less probes, implying that CK2 phosphorylation of SNAP_C on DNA containing a juxtaposed PSE and TATA box, but not

**EXPERIMENTAL PROCEDURES**

**Recombinant Protein Expression—**In vitro transcription and translation of SNAP_C subunits were performed using rabbit reticulocyte lysates (TnT, Promega), and 1 μg of each pCite2a-derived plasmid encoding full-length or truncated SNAP_C subunits as previously described (16). Translation reactions were performed in the presence of either [35S]methionine or [γ-32P]ATP, and proteins were recovered by anti-SNAP43 immunoprecipitation for visualization by autoradiography. The recombinant partial SNAP_C used for functional studies was assembled by co-expression of GST-SNAP190-(1–719) from the pSBET-SNAP190-(1–719) plasmid along with full-length his-tagged SNAP43 and HA-tagged SNAP50 from the plasmid pET21-HisSNAP43-HASNAP50 in E. coli BL21 DE3. Recombinant complexes were purified by glutathione-Sepharose affinity chromatography, and bound proteins were released from the GST tag by thrombin cleavage. Recovered complexes were then dialyzed against Dignam buffer D-80 (20 mM Hepes, pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.1% Tween 20, 5 mM MgCl₂, 80 mM KCl) prior to in vitro transcription and electrophoretic mobility shift assays (EMSAs), as described below. As indicated in the figure legends, additional complexes containing SNAP190 with further truncations or amino acid substitutions were expressed and purified similarly. Note that full-length SNAP190 contains 1469 amino acids, and unless otherwise noted, SNAP190-(1–719) is hereafter referred to as wild type for the purposes of comparison with these mutant SNAP190-(1–719) derivatives.

**In Vitro Kinase Assays—**For the experiments presented in Figs. 1B and 4, ~3 μg of the indicated GST SNAP190 fusion proteins were bound to glutathione-Sepharose beads (10 μl) and were washed extensively with HEMGT-150 buffer (20 mM HEPES (pH 7.9), 0.5 mM EDTA, 10 mM MgCl₂, 10% glycerol, 0.1% Tween 20, and 150 mM KCl) containing protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium bisulfate, 1 mM benzamidine, 1 μM pepstatin A) and 1 mM dithiothreitol. In vitro kinase assays were performed directly on the beads using 10 units of recombinant CK2 (New England Biolabs) in the presence of 8 nM [γ-32P]ATP (3000 Ci/mmol) for 15 min at room temperature. The beads were then washed with HEMGT-150 buffer to remove unincorporated isotope and CK2. Phosphorylated GST-SNAP190-(1–719) was digested with trypsin, and the resultant fragments were separated by two-dimensional TLC as described (19). Alternatively, phosphorylated GST-SNAP190 proteins were left undigested for analyses by 15% SDS-PAGE. After SDS-PAGE, GST-SNAP190 proteins were visualized by staining with Coomassie Blue prior to autoradiography to detected phosphorylation.

**Phosphopeptide Mapping—**To map SNAP190 phosphorylation sites, ~1 μg of GST-SNAP190-(1–719) immobilized on glutathione-Sepharose beads was treated with 50 μl of HeLa cell nuclear extract in a total volume of 200 μl adjusted with HEMGT-150 buffer. After 1-h incubation at room temperature, the beads were washed extensively with HEMGT-150 buffer and a kinase assay was performed in the presence of 8 nM [γ-32P]ATP and 1 mM unlabeled ATP. After extensive washing with HEMGT-150 buffer, phosphorylated SNAP190-(1–719) was then digested with sequence grade trypsin (Promega) followed either by phosphopeptide purification through a gallium spin column (24) according to the manufacturer’s instructions (Pierce). Purified peptides were directly analyzed or were dephosphorylated with calf intestine alkaline phosphatase (New England Biolabs) prior to analysis by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (PerSeptive Biosystems, Inc., Farmingham, MA) in a positive ion reflector mode using α-cyano-4-hydroxycinnamic acid as a matrix. The purified peptides were also subjected to Ba(OH)₂ treatment for β-elimination (25) prior to liquid chromatography using the Waters CapLC system (Waters Corp., Milford, MA) coupled with quadrupole time of flight tandem mass spectrometric analysis (Q-TOF MS/MS) using the LCQ DECA quadrupole ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) through the Picoview nanoparticle (New Objectives, Cambridge, MA).

**SNAP_C Immunodepletion and in Vitro Transcription Assays—**To deplete SNAP_C from HeLa nuclear extracts, 150 μl of extract was incubated with 50 μl of protein G-agarose beads pre-coupled with rabbit pre-immune, anti-SNAP43 (CS48) (2), or anti-SNAP190 (CS402) (5) antibodies for 1 h at room temperature. Extracts were then used for in vitro U6 transcription as described previously (1) with the following modifications. For the reconstitution reactions whose results are depicted in Fig. 6A, the transcription reactions were performed in a total volume of 36 μl containing 1 μg of the DNA template (pU6/Hae/RA.2) with either 8 μl of untreated extract or 14 μl of SNAP_C-depleted extracts. The amounts and constituents of the recombinant mini-SNAP complexes tested are indicated in the figure legend. U6 transcripts were analyzed by RNase T1 protection as described previously (1). For Fig. 6 (B and C), recombinant mini-SNAP_C mS or mΔC was preincubated with Dignam buffer D either with or without recombinant CK2 and ATP for 30 min at room temperature. In vitro transcriptions were

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initiated by the addition of transcription buffers, nucleoside triphosphates, 1 µg of DNA template, and either 8 µl of untreated HeLa nuclear extracts or 14 µl of SNAPC-depleted extract, and reactions were incubated for an additional 60 min at 30 °C.

**Electrophoretic Mobility Shift Assay**—PSE-specific DNA binding by SNAPC was assayed by EMSA as described previously (3) using a DNA probe containing a wild-type mouse U6 PSE with mutant human U6 TATA box (13, 14) with the following modifications. For the experiments presented in Fig. 5A (panel a), ~20 ng of purified mini-SNAP complexes was preincubated alone or with 5 or 50 units of recombinant CK2 and/or 1 mM ATP for 30 min at 30 °C, as indicated. EMSAs were then performed by addition of the radiolabeled DNA probes, and reactions were incubated for an additional 30 min at 30 °C. To determine whether CK2 could affect SNAPC after DNA binding (Fig. 5A, panel b), mini-SNAP complexes were first incubated with DNA probes for 30 min at 30 °C. Subsequently, 5 or 50 units of recombinant CK2 and 1 mM ATP were added, and reactions were incubated for an additional 30 min at 30 °C. Samples were fractionated on a 5% nondenaturing polyacrylamide gel (39:1) in TGE running buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA).

**RESULTS**

Two Regions within SNAP190-(1–719) are phosphorylated by CK2—Previous experiments demonstrated that endogenous CK2 associates with SNAPC and can efficiently phosphorylate the largest SNAPC subunit, SNAP190 (19). To determine whether the presence of a particular SNAPC subunit is required for efficient SNAP190 phosphorylation, the phosphorylation of recombinant SNAPC subunits was examined for proteins that were expressed individually or were co-translated using rabbit reticulocyte lysates. During translation, proteins were labeled with [35S]methionine to serve as markers for subunit expression or with [γ-32P]ATP to detect phosphorylation by kinases resident in the extract. SNAPC proteins were specifically recovered by immunoprecipitation for analysis by SDS-PAGE and autoradiography. In these assays, SNAP190 was efficiently phosphorylated regardless of whether other SNAPC subunits were co-expressed (data not shown). SNAP190 phosphorylation was sensitive to CK2 inhibitors and could be supported by GTP (data not shown), a hallmark of CK2, suggesting that CK2 contributes to SNAP190 phosphorylation in this context, although other kinases may also target SNAP190.

To identify the regions of SNAP190 that are phosphorylated when it is assembled within SNAPC, truncated SNAP190 proteins were co-translated along with SNAP43 and SNAP19, and the various partial complexes were recovered by anti-SNAP43 immunoprecipitation (Fig. 1A). Because preliminary data indicated that SNAP45 and SNAP50 were dispensable for efficient SNAPC assembly, these subunits were omitted from these analyses for simplicity. As with full-length SNAP190-(1–1469) (data not shown), strong SNAP190 phosphorylation was observed with SNAP190 containing amino acids 1–719 (lane 8), but only modest phosphorylation was detected for SNAP190-(1–505) or SNAP190-(1–216) when co-expressed with SNAP43 and SNAP19 (lanes 9 and 10). Therefore, robust SNAP190 phosphorylation requires the N terminus of SNAP190 containing amino acids 1–719. Interestingly, the pattern of SNAP43 phosphorylation in these assays was parallel to that seen with SNAP190; the majority of SNAP43 recovered in these assays was phosphorylated when co-expressed with

![Figure 1. The N-terminal region of SNAP190 is extensively phosphorylated.](image-url)

A robust SNAP190 phosphorylation, as a member of SNAPC, requires an extensive N-terminal region. Various truncated SNAP190 proteins, as indicated, were co-translated in vitro along with SNAP19 and SNAP43 using rabbit reticulocyte lysates complemented with either [35S]methionine (lanes 1–5) or [γ-32P]ATP (lanes 6–10). Proteins were recovered by anti-SNAP43 immunoprecipitation and were visualized by autoradiography. Arrows indicate that positions of phosphorylated proteins detected in this assay. Extensive SNAP190 and SNAP43 phosphorylation are observed in reactions containing SNAP190-(1–719) but not in reactions containing other truncated SNAP190 proteins. B, full-length endogenous SNAP190 and recombinant SNAP190-(1–719) can be phosphorylated in similar regions. Endogenous SNAP190 was recovered from HeLa cell nuclear extracts by anti-SNAP43 immunoprecipitation followed by "on-bead" in vitro kinase assays with SNAPC-associated kinases in the presence of [γ-32P]ATP. Phosphorylated SNAP190 was gel-purified and digested with trypsin, and the resultant peptides were separated by thin layer electrophoresis in the first dimension and thin layer chromatography in the second dimension (left panel). The pattern of tryptic peptide separation was compared with recombinant GST-SNAP190-(1–719) that was phosphorylated by recombinant CK2 (right panel). The directions of electrophoresis and chromatography are indicated by arrows.
SNAP190-(1–719) but not with SNAP190-(1–505) or SNAP190-(1–216). Unlike SNAP190, SNAP43 was not efficiently phosphorylated when expressed individually (lane 6) or co-translated with SNAP19 alone (lane 7). The assembly of SNAP190 into a complex with SNAP43 and SNAP19 was not disrupted by truncation of the SNAP190 C-terminal region, because SNAP43 interacts with the N-terminal region of SNAP190 (16). Thus, SNAP190 may also direct kinase activity toward SNAP43, with SNAP19 likely playing an auxiliary role by facilitating the association between SNAP43 and SNAP190 (10).

To determine whether similar regions in endogenous SNAP190 and recombinant SNAP190 can be phosphorylated, endogenous SNAP190 was immunopurified from HeLa nuclear extracts for subsequent in vitro phosphorylation by endogenous SNAPC-associated kinases. Phosphopeptide mapping for full-length SNAP190 was then performed by two-dimensional TLC of tryptic peptides, and the migration of phosphorylated peptides was compared with those obtained from recombinant SNAP190-(1–719) phosphorylated by recombinant CK2. As shown in Fig. 1B, a subset of the tryptic phosphopeptides from full-length SNAP190 migrated similarly to that observed for all of the tryptic peptides derived from recombinant SNAP190-(1–719), although additional phosphopeptides were seen with endogenous SNAP190. Thus, both recombinant SNAP190-(1–719) and endogenous full-length SNAP190 can be phosphorylated in similar regions that harbor sites for CK2 recognition. Interestingly, at higher ATP concentrations, only two strongly phosphorylated tryptic peptides were observed for the recombinant SNAP190 phosphorylated by either recombinant CK2 or the endogenous SNAPC-associated kinases (data not shown and Ref. 19), possibly because higher ATP concentrations favors the complete and uniform phosphorylation of peptides that harbor multiple CK2 sites.

Because the previous studies suggest that SNAP190 is extensively phosphorylated within the N-terminal region of the protein, a more detailed phosphopeptide mapping study was then performed to identify those sites that are targeted for phosphorylation. In the following studies, recombinant GST-SNAP190-(1–719) was immobilized on glutathione-Sepharose beads as bait to recover endogenous kinases from HeLa nuclear extracts prior to SNAP190 phosphorylation during in vitro kinase assays with [γ-32P]ATP. Although CK2 was suspected to be the predominant kinase recovered through SNAP190 association (Fig. 1 and Ref. 19), rather than using recombinant CK2, HeLa extracts were chosen as the kinase source to allow for the possibility that additional SNAP190 phosphorylation by other kinases would also be revealed. Next, phosphorylated GST-SNAP190-(1–719) was gel-purified and digested with trypsin, and phosphopeptides were affinity-purified using a gallium column (24). Purified phosphopeptides were analyzed directly by MALDI-TOF MS or were dephosphorylated by alkaline phosphatase prior to analysis. Theoretically, a peptide that is phosphorylated at a single site should give a mass addition of +80 Da relative to the mass calculated from its native sequence, and the mass of the phosphorylated peptide should be converted back to its calculated value after phosphatase treatment.

As shown in Fig. 2, five peaks with ~80 Da or multiples of 80-Da differences were observed, diagnostic of potential phosphopeptides (top panel). These masses potentially correspond to multiple different phosphorylated peptides, or alternatively, to a single peptide that is multiply phosphorylated. After phosphatase treatment only one peak at m/z 4624.83 was revealed (bottom panel), suggesting that the mass spectral peaks at m/z 4704.53, 4784.38, 4864.42, and 4944.95 Da correspond to the mass of the SNAP190 peptide from amino acids 20–63 (calculated MH’ m/z is 4624.07 Da, observed MH’ m/z is 4624.17 Da) with 1-, 2-, 3-, and 4-phosphate groups. After phosphatase treatment, these peaks shift to the peak at m/z 4624.83 Da. B, the primary sequence of the SNAP190 tryptic peptide containing amino acids from 20–63 with the consensus CK2 sites within this peptide bracketed. Serine residues within the CK2 consensus motif are highlighted with asterisks.
peptide at \( m/z \) 4624.17 is likely to be SNAP190-(20–63) with a calculated MH\(^+\) at \( m/z \) 4624.07. The amino acid sequence of SNAP190-(20–63) is shown in Fig. 2B, and interestingly, this 44-amino acid region within SNAP190 contains five consensus CK2 sites. Together, these data strongly suggest that CK2 can phosphorylate SNAP190 at multiple serine residues within this region.

Although two SNAP190 tryptic peptides were strongly phosphorylated by endogenous CK2, as measured by SDS-PAGE analysis (data not shown), only one phosphorylated peptide was identified by MALDI-TOF MS, possibly because the other phosphorylated peptide was not efficiently ionized. Therefore, the purified tryptic phosphopeptides were also analyzed by \( \beta \)-elimination coupled with Q-TOF MS/MS to identify the second site of phosphorylation within SNAP190. Although unmodified serine residues are not affected by \( \beta \)-elimination, phosphoric acid groups are removed from phosphorylated serine leading to a net 18-Da loss relative to the modified serine residue (26, 27). Using this approach, numerous phosphorylated fragments encompassing the serine-rich region (514–545) of SNAP190 were observed, suggesting that this region of SNAP190 is extensively phosphorylated. Fig. 3 (top panel) shows one example of a spectrum for a MS/MS ion after \( \beta \)-elimination of a peptide with \( m/z \) 627.11 Da, which corresponds to amino acid residues 519–543 (S\(^{530}\)SSTSSSSGSSGSSSSSSSSSE\(^{543}\) + 3H\(^+\)). In this spectrum, a total of 20 y-series product ions (ions from the C-terminal end of the peptide) and 6 b-series (ions from the N-terminal end of the peptide) could be assigned to SNAP190. An enlarged spectrum highlighting the \( y(5)^{+}\), \( y(6)^{+}\), \( y(7)^{+}\), \( y(9)^{+}\), \( y(10)^{+}\), \( y(13)^{+}\), \( y(16)^{+}\), \( y(17)^{+}\), and \( y(18)^{+}\) ions is shown in Fig. 3A (bottom panel). The mass differences between \( y(5)^{+}\) and \( y(6)^{+}\) is 43.5 (87/2) Da, indicating that serine 538 is not phosphorylated, whereas the mass differences between \( y(6)^{+}\) and \( y(7)^{+}\), \( y(9)^{+}\) and \( y(10)^{+}\), \( y(16)^{+}\) and \( y(17)^{+}\), and \( y(17)^{+}\) and \( y(18)^{+}\) are 34.5 ((87–18)/2) Da due to \( \beta \)-elimination of phosphoserine. Within this peptide, 14 different serine residues exhibited an 18-Da loss, suggesting that 14 serines were phosphorylated.

We further analyzed 62 spectra corresponding to peptides encompassing the SNAP190 serine-rich region, and the data are summarized in Fig. 3B. These analyses revealed that each of 20 serine residues within this region was phosphorylated, although the combination of phosphorylation events at different serine residues within this region was complex. Interestingly, this region of SNAP190 harbors only three putative CK2 consensus sites (SXX(D/E)) at Ser\(^{540}\), Ser\(^{541}\), and Ser\(^{542}\) complemented by the downstream acidic residues Glu\(^{543}\), Glu\(^{544}\), and Asp\(^{545}\). It is likely that phosphorylation at these consensus CK2 sites introduces a negative charge to create an additional CK2 recognition site at the nearby serine located at the N-3 position propagating additional phosphorylation throughout this region.

A schematic representation of SNAP190 and the positions of the experimentally determined CK2 sites relative to other functional landmarks within this protein are shown in Fig. 4A. The first CK2 site within amino acids 20–63 overlaps with the TBP recruitment region 1 (34–83) and is adjacent to the leucine zipper region within SNAP190 that is required for complex recruitment region 1 (34–83) and is adjacent to the leucine zipper region within SNAP190 that is required for complex recruitment.
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assembly with SNAP43 and SNAP19 (16). The second CK2 site within amino acids 514–545 is adjacent to the Myb DNA binding domain (amino acids 263–503), which also constitutes a second TBP recruitment region (13). Next, in vitro kinase assays were performed using various truncated GST-SNAP190 molecules that lack the putative CK2 sites to confirm that CK2 predominately phosphorylates these two target regions. As shown in Fig. 4B, GST-SNAP190-(1–719) was effectively phosphorylated by recombinant CK2, whereas phosphorylation of GST-SNAP190-(63–719), which lacks the N-terminal CK2 sites, was modestly reduced. In contrast, phosphorylation of GST-SNAP190-(1–505) was markedly reduced, whereas deletion of both the N-terminal and serine-rich regions resulted in barely detectable phosphorylation. Together, these results suggest that these two regions are targeted by CK2; however, the bulk of phosphorylation occurs within the serine-rich region adjacent to the SNAP190 Myb DNA binding domain.

One interesting feature of the serine-rich region is that it is flanked by an acidic region toward the C terminus (543–548). We hypothesized that these acidic amino acids are important components of CK2 recognition motifs. Indeed, alanine substitution of the three most adjacent acidic residues (Glu543, Glu544, and Asp545) strongly reduced GST-SNAP190-(1–719) phosphorylation, further suggesting that the initial serine phosphorylation adjacent to these acidic residues nucleates extensive CK2 phosphorylation throughout the serine-rich region (Fig. 4C). In contrast, the conversion of the three most adjacent serine residues (Ser540, Ser541, and Ser542) into aspartic acid residues did not substantively affect SNAP190 phosphorylation. The serine-rich region is also flanked by a positively charged arginine-rich region toward the N terminus (amino acids 506–513). Previous

FIGURE 4. The SNAP190 serine-rich region is a major target for CK2 phosphorylation. A, a schematic representation of SNAP190 and the position of the CK2 sites (red) relative to other functional regions are shown (top panel). Myb domain, Myb DNA binding domain; TRR1, TBP-interacting region 1; TRR2, TBP-interacting region 2; OIR, Oct-1 interacting region; 43/19, SNAP43 and SNAP19 interacting region; and 45, SNAP45 interacting region (modified from Ref. 16). The wavy vertical line around position 719 indicates the C terminus of the truncated SNAP190 protein used for these studies. Sequences of the SNAP190 amino acids 501–513 and amino acids 514–545 are also given. The locations of amino acid substitutions introduced within the two CK2 targeted regions are shown. B, the serine-rich region of SNAP190-(1–719) is extensively phosphorylated by CK2. GST-SNAP190 fusion proteins that lack the CK2 phosphorylation sites were expressed in E. coli and were purified using glutathione-Sepharose beads for in vitro kinase assays with recombinant CK2. A schematic representation of the various GST-SNAP190 fusion proteins tested is shown on the left. Phosphorylation signals were detected by autoradiography (lane 1), and the amount of GST-SNAP190 proteins used in each reaction was detected by staining with Coomassie Blue (lane 2). Deletion of the serine-rich region strongly diminishes SNAP190-(1–719) phosphorylation by recombinant CK2. C, the CK2 recognition motifs within the serine-rich region are critical for SNAP190 phosphorylation. GST-SNAP190-(1–719) fusion proteins harboring mutations in the putative CK2 recognition sequences were subjected to in vitro kinase assays with recombinant CK2. Phosphorylation signals were detected by autoradiography (top) and total protein was measured by Coomassie staining (bottom). The corresponding locations of the indicated amino acid substitutions are shown in A.
studies have shown that stretches of positively charged residues can stimulate CK2 activity (28), and consistently, glutamine substitution of six arginine residues (Arg506 to Arg511) reduced GST-SNAP190-(1–719) phosphorylation to levels similar to that seen with alanine substitution of the acidic residues. These results argue that both the positive and negative charged regions flanking the serine-rich region are important for SNAP190 phosphorylation by CK2. In contrast, alanine substitution of five serine residues for those CK2 motifs within the N-terminal cluster (amino acids 28–59) only modestly affected phosphorylation and is consistent with a similarly modest effect when this region is deleted (Fig. 4B). These results indicate that the serine-rich region is the favored substrate for phosphorylation by recombinant CK2.

SNAP190 Phosphorylation by CK2 Inhibits DNA Binding by SNAP190—Because the most extensively phosphorylated region of SNAP190 is adjacent to its DNA binding domain, we next considered the hypothesis that CK2 phosphorylates the serine-rich region to inhibit DNA binding by SNAP190. To test this idea, the effect of CK2 was compared for DNA binding by a partial recombinant “mini-SNAP190” containing either SNAP190-(1–719) (called the mS complex) or SNAP190-(1–505), which lacks the serine-rich region (called the mSAC complex). As shown in Fig. 5A, both the mS and mSAC complexes bound well to a PSE-containing probe in the absence of CK2 (lanes 2 and 8). In each case, addition of CK2 alone did not affect DNA binding in the absence of added ATP (lanes 3, 4, 9, and 10) nor did ATP alone in the absence of CK2 (lanes 5 and 11). However, DNA binding was abolished when both CK2 and ATP were preincubated with the mS complex (lanes 6 and 7). In contrast, DNA binding by mSAC complex was unaffected by CK2 and ATP (lanes 12 and 13). Thus, CK2 phosphorylation of the SNAP190 serine-rich region inhibits DNA binding by SNAP190.

To determine whether CK2 could also affect SNAP190 after it had bound to DNA, mini-SNAP190 containing SNAP190 with or without the serine-rich region was pre-bound to DNA for 30 min followed by treatment of the DNA binding reactions with CK2 and ATP. Again, CK2 inhibited DNA binding by the mS complex (lanes 16 and 17), but not the mSAC complex (lanes 19 and 20). Because SNAP190 has a slow off-rate (~50% of the SNAP190-DNA complex in EMSA remains by 1 h (29)), the nearly complete loss of the SNAP190-DNA complex by 30 min after CK2 treatment indicates that CK2 can likely remove SNAP190 from DNA. The contribution of the positively and negatively charged regions flanking the serine-rich region to the CK2 inhibition of DNA binding was also investigated. As shown in Fig. 5B, CK2 treatment of the mS complex reduced DNA binding relative to the untreated complex (compare lanes 3 and 4 to lane 2). Substitution of amino acids within the arginine-rich region (lane 5) or negatively charged region (lane 8) did not substantively affect DNA binding in the absence of CK2 treatment, however, both mutant complexes became unresponsive to CK2 inhibition (lanes 6, 7, 9, and 10). These results indicate that both sets of charged regions flanking the serine-rich region are important for CK2-mediated inhibition of DNA binding.

SNAP190 Phosphorylation by CK2 Inhibits U6 Transcription—A key function of SNAP190 is to promote preinitiation complex assembly, and thus the inhibition of SNAP190-DNA binding by CK2 suggests that snRNA gene transcription could be regulated via this mechanism. To investigate this possibility, we first tested whether the CK2-targeted regions were required for U6 transcription. As shown in Fig. 6A, removal of endogenous SNAP190 from HeLa extracts debilitated U6 transcription (lane 2) relative to that supported by the untreated extract (lane 1), and addition of the mS complex restored robust U6 transcription (lanes 3–5). Thus, transcrip-
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A critical early event in the transcription of human snRNA genes involves promoter recognition by the transcription factor SNAP<sub>C</sub>. To initiate RNA polymerase III transcription of U6 and related snRNA genes, SNAP<sub>C</sub> and TBP cooperate for DNA transcription supported by cell extracts depleted of endogenous SNAP<sub>C</sub> is dependent upon the recombinant complex. As observed for the SNAP190-(1–719)-containing complex, mutant mini-SNAP<sub>C</sub> harboring SNAP190 with deletions of the N-terminal CK2 sites (lanes 6–8), the serine-rich region (lanes 9–11), or both regions (lanes 12–14) continued to support robust levels of U6 transcription at roughly equivalent levels. Thus, none of the CK2 sites is essential for U6 transcription, and thus, may instead play a regulatory role governing RNA polymerase III transcription. Therefore, whether phosphorylation of the CK2 sites within SNAP190-(1–719) could contribute to U6 regulation was investigated (Fig. 6B). As expected, U6 transcription was reconstituted by the untreated mS complex (compare lane 2 to lane 1); however, the same complex that was subjected to phosphorylation with 100 units of recombinant CK2 for 30 min was unable to reconstitute U6 transcription (lane 3). Although less pronounced, CK2 addition simultaneously with the mS complex also reduced U6 transcription (lane 4). Together, these observations indicate that CK2 phosphorylation of SNAP<sub>C</sub> may likely influence U6 transcription.

To determine whether the serine-rich region of SNAP190 is important for CK2 inhibition of U6 transcription, in vitro transcription assays were reconstituted with the unmodified mS and mS ΔC complexes, or with the same complexes that were phosphorylated with recombinant CK2 (Fig. 6C). In these assays, both the unmodified mS and mS ΔC complexes supported U6 transcription, although the efficacy for reconstituted transcription by each complex was different as compared with that previously described for the simple reconstitution experiments shown in Fig. 6A. The pattern of transcriptional activity is reminiscent of that observed for the DNA binding studies presented in Fig. 5A, and therefore, we surmise that these complexes are not equally stable during the preincubation period in this assay. Nonetheless, CK2 phosphorylation of the mC complex significantly decreased U6 transcription, whereas the mS ΔC complex was recalcitrant to CK2. Addition of equivalent amounts of recombinant CK2 (20 units) to transcription reactions without preincubation with mini-SNAP<sub>C</sub> had no reproducible effect on transcription for reactions reconstituted with either the SNAP190-(1–719) or SNAP190-(1–505)-containing complexes. Thus, the inhibition of transcription observed when the mS complex is pretreated with CK2 indicates that CK2 is acting predominately through SNAP<sub>C</sub> to inhibit transcription in this experimental system. We conclude that CK2 phosphorylation of the SNAP190 serine-rich region inhibits U6 transcription by RNA polymerase III.

DISCUSSION

A critical early event in the transcription of human snRNA genes involves promoter recognition by the transcription factor SNAP<sub>C</sub>. To initiate RNA polymerase III transcription of U6 and related snRNA genes, SNAP<sub>C</sub> and TBP cooperate for DNA transcription supported by cell extracts depleted of endogenous SNAP<sub>C</sub> is dependent upon the recombinant complex. As observed for the SNAP190-(1–719)-containing complex, mutant mini-SNAP<sub>C</sub> harboring SNAP190 with deletions of the N-terminal CK2 sites (lanes 6–8), the serine-rich region (lanes 9–11), or both regions (lanes 12–14) continued to support robust levels of U6 transcription at roughly equivalent levels. Thus, none of the CK2 sites is essential for U6 transcription, and thus, may instead play a regulatory role governing RNA polymerase III transcription. Therefore, whether phosphorylation of the CK2 sites within SNAP190-(1–719) could contribute to U6 regulation was investigated (Fig. 6B). As expected, U6 transcription was reconstituted by the untreated mS complex (compare lane 2 to lane 1); however, the same complex that was subjected to phosphorylation with 100 units of recombinant CK2 for 30 min was unable to reconstitute U6 transcription (lane 3). Although less pronounced, CK2 addition simultaneously with the mS complex also reduced U6 transcription (lane 4). Together, these observations indicate that CK2 phosphorylation of SNAP<sub>C</sub> may likely influence U6 transcription.

To determine whether the serine-rich region of SNAP190 is important for CK2 inhibition of U6 transcription, in vitro transcription assays were reconstituted with the unmodified mS and mS ΔC complexes, or with the same complexes that were phosphorylated with recombinant CK2 (Fig. 6C). In these assays, both the unmodified mS and mS ΔC complexes supported U6 transcription, although the efficacy for reconstituted transcription by each complex was different as compared with that previously described for the simple reconstitution experiments shown in Fig. 6A. The pattern of transcriptional activity is reminiscent of that observed for the DNA binding studies presented in Fig. 5A, and therefore, we surmise that these complexes are not equally stable during the preincubation period in this assay. Nonetheless, CK2 phosphorylation of the mS complex significantly decreased U6 transcription, whereas the mS ΔC complex was recalcitrant to CK2. Addition of equivalent amounts of recombinant CK2 (20 units) to transcription reactions without preincubation with mini-SNAP<sub>C</sub> had no reproducible effect on transcription for reactions reconstituted with either the SNAP190-(1–719) or SNAP190-(1–505)-containing complexes. Thus, the inhibition of transcription observed when the mS complex is pretreated with CK2 indicates that CK2 is acting predominately through SNAP<sub>C</sub> to inhibit transcription in this experimental system. We conclude that CK2 phosphorylation of the SNAP190 serine-rich region inhibits U6 transcription by RNA polymerase III.
CK2 Phosphorylates SNAP190 to Regulate DNA Binding

FIGURE 7. Model for CK2 inhibition of DNA binding by SNAPC. Extensive phosphorylation of SNAP190 within the serine-rich region is predicted to increase the overall negative charge and facilitate its association with the positively charged arginine rich region located adjacently to the SNAP190 Myb DNA binding domain.

binding to the PSE and TATA-box, respectively, as a prelude to preinitiation complex assembly with additional components of the Brf2-TFIIIB complex, leading to RNA polymerase III recruitment (13, 14). It was demonstrated previously that multiple players in this process, including Bdp1 and RNA polymerase III, are targets for regulatory intervention by the protein kinase CK2 (21, 22). The current study extends this cadre of CK2 targets within the U6 transcriptional machinery to demonstrate that DNA binding and, consequently, the transcriptional function of SNAPC, can be inhibited by SNAP190 phosphorylation by CK2.

Within SNAPC, the largest subunit SNAP190 functions as a direct target for regulatory factors (17), for interactions with other SNAPC components (16), and for TBP interaction (13, 15), while directly contacting DNA through its Myb DNA binding domain (5). This network indicates that SNAP190 is an attractive target for regulatory intervention. Interestingly, two sites of CK2 phosphorylation were identified within SNAP190, including an N-terminally located region (amino acids 20–63) that functions in TBP recruitment (15), and a second serine-rich region (amino acids 514–545) that is adjacent to the Myb DNA binding domain. Of these, the inhibitory effects of CK2 on DNA binding by SNAPC are mediated predominately through extensive phosphorylation of the serine-rich region. We propose an allosteric regulatory model (see Fig. 7) wherein the initial CK2 phosphorylation at the consensus CK2 motifs within SNAP190 can mitigate TBP ability for stimulated DNA binding by phosphorylated SNAPC. Alternatively, promoter association by SNAPC remained intact during repression, but additional downstream functions of promoter-bound SNAPC were additionally inhibited by CK2. The regulated inhibition of SNAPC activity, including promoter recognition, could be important during several cellular processes where down-regulation of RNA polymerase III transcription is critical. For example, in Saccharomyces cerevisiae, CK2 has been proposed to target TFIIIB, interfering with preinitiation complex assembly, to limit RNA polymerase III transcription after DNA damage (38), and CK2 may likewise contribute to U6 regulation in these contexts by targeting SNAPC either alone or in conjunction with Br2-TFIIIB. However, in humans, U6 transcription after DNA damage is also regulated by Maf1 (39) and p53 (40), and whether additional augmentation of these processes by CK2 is required remains unknown. A more likely possibility is that CK2 plays an important role governing SNAPC activity in a cell cycle-dependent manner as one contributor to repression of U6 transcription during mitosis. Indeed, U6 promoter binding of endogenous SNAPC is diminished at M-phase during the same cell cycle window that CK2 phosphorylates the Bdp1 component of Br2-TFIIIB (22). Although U6 promoter association by SNAPC was not diminished to the same extent as that of Bdp1 (22), the current studies suggest that inhibition of multiple targets within the RNA polymerase III machinery could reinforce a strict shut-off of these powerful promoters.

Acknowledgments—We thank Keith Ray and the Michigan State University (MSU) proteomics facility for their assistance with the Q-TOF MS analyses, and the MSU mass spectrometric facility for the MALDI-TOF MS analyses. We additionally acknowledge the many helpful comments from Grace Chen and Allison Gjidoda during their critical readings of the manuscript.

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