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Regulation of human small nuclear RNA gene transcription by the oncogneic protein kinase CK2

presented by

Liping Gu

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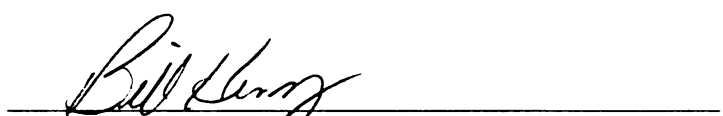
Doctoral degree in Department of Biochemistry and Molecular Biology



Major Professor's Signature

03/15/05

Date

REGULATION OF HUMAN SMALL NUCLEAR RNA GENE TRANSCRIPTION BY THE ONCOGENIC PROTEIN KINASE CK2

By

Liping Gu

A DISSERTATION

Submitted to

Michigan State University

for the Degree of

DOCTORAL OF PHILOSOPHY

Department of Biochemistry and Molecular Biology

2005

ABSTRACT

HUMAN SMALL NUCLEAR RNA GENE TRANSCRIPTION REGULATED BY THE ONCOGENIC PROTEIN KINASE CK2

By

Liping Gu

Protein kinase CK2 participates in many cellular processes including cell cycle, cell differentiation, stress response, and apoptosis. One important role of CK2 is to transduce extracellular signals into the nucleus to control gene expression. CK2 appears to globally regulate protein-coding gene transcription by RNA polymerase II and non-coding gene transcription by RNA polymerases I and III. Human small nuclear RNA genes are transcribed by either RNA polymerase II or RNA polymerase III depending upon their promoter architectures. CK2 regulates RNA polymerase III-specific U6 snRNA gene transcription, but whether CK2 plays a role in regulating the closely related polymerase II-specific U1 snRNA gene transcription has been uncertain.

Herein, I report that CK2 inhibits U1 snRNA gene transcription by RNA polymerase II. CK2 associates with U1 gene promoters, and U1 snRNA expression negatively correlates with endogenous CK2 levels. Thus, CK2 plays a direct role in U1 gene regulation in living cells. CK2 phosphorylates the general transcription factor snRNA activating protein complex ($SNAP_C$) that is required for human snRNA gene transcription. $SNAP_C$ phosphorylation reduces its DNA binding affinity as supported by the observations that CK2 phosphorylation of a recombinant partial $SNAP_C$ can inhibit its

PSE-specific binding before and after DNA association has occurred. Importantly, CK2 phosphorylation of mini-SNAPc restricts promoter recognition by $SNAP_C$ on a U1 promoter and permits higher order complex formation with TBP on a U6 promoter, indicating that the cooperation between SNAPc and TBP can counteract CK2 inhibition of DNA binding by $SNAP_C$ for RNA polymerase III-specific preinitiation complex assembly. Therefore, CK2 may differentially regulate snRNA gene transcription by selectively influencing pre-initiation complex assembly for RNA polymerase II and polymerase III transcription of human snRNA genes depending upon the promoter architectures.

Further investigation showed that SNAP190, the largest subunit of $SNAP_C$, is phosphorylated *in vivo* and can be phosphorylated *in vitro*. CK2 phosphorylates the N-terminal half of SNAP190 at two regions that contain multiple CK2 consensus sites (amino acid 20-63 and 514-545) as determined by mass spectrometric analysis. The region within SNAP190 that is required for CK2 inhibition is contained within amino acids 506-719 encompassing a serine rich region with multiple CK2 consensus sites. The downregulation of $SNAP_C$ DNA binding activity by CK2 phosphorylation may contribute to reduced U1 snRNA gene transcription by RNA polymerase II.

To my family

ACKNOWLEDGEMENTS

I gratefully acknowledge my mentor R. William Henry for his encouragement, support, patience, and understanding. It was a great fortune for me to meet Bill and to join his laboratory when I arrived in Michigan several years ago. Thank you very much for mentoring me during these years in the Ph. D. program and teaching me how to become a scientist.

I would also like to thank the members of my thesis committee, Zachary F. Burton, Kathleen A. Gallo, James H. Geiger, John L. Wang, and R. William Henry for their time and effort in my guidance during these years.

I would like to thank the members of the Henry lab including Craig Hinkley, Heather Hirsch, Zakir Ullah, Gauri Jawdekar, Anastasia Gridasova, Tharakeswari Selvakumar, and Xianzhou Song for sharing their expertise and friendship. I would also like to thank my friends and colleagues in this department and other departments. Special thanks to Rhonda Hussain, Susanne Hoffmann-Benning, Keith Ray, and Walter Esselman and his student Weiqin Chen for technique assistance.

I would like to thank my husband Ruanbao Zhou and my son Kai Ruan for their tremendous support throughout all these years. I would also like to thank my parents and siblings in China for their love and understanding.

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KEY TO SYMBOLS AND ABBREVIATIONS

2, 3-DPG	2, 3-diphosphoglycerate
6-TG	6-thioguanine
APE/Ref-1	apurinic endonuclease
ARC	apoptosis repressor with caspase recruitment domain
ATF1	activating transcription factor-1
bPrP	bovine prion protein
Brf1-TFIIIB	TFIIIB complex composed of TBP, Brf1 and Bdp1
Brf2-TFIIIB	TFIIIB complex composed of TBP, Brf2 and Bdp1
ChIPs	chromatin immunoprecipitations
Chk2	checkpoint kinase 2
CHOP/GADD153	C/EBP homologous protein transcription factor/growth arrest and DNA damage inducible protein
CK2	casein kinase 2 or casein kinase II
CO-IP	co-immunoprecipitation
CoREST	corepressor for the repressor element 1 silencing transcription factor
CPE	core promoter element
CREB	cAMP-dependent response element binding protein
CTCF	CCCTC-binding factor
CTD	carboxy terminal domain of RNA polymerase II largest subunit
C-terminal	carboxy-terminal
Da	Dalton
DEK	proto-oncogene protein transcribed from a gene named *dek*
DHN1	dehydrin protein 1
DPE	downstream promoter element
DRB	5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole
DSB	double-strand breaks

DSE	distal sequence element
d-siRNA	Dicer generated small interfering RNA
dsRNA	double stranded RNA
ecto-CK2	CK2 released from the surface of intact cells
EMSA	electrophoretic mobility shift assay
FACT	facilitating chromatin-mediated transcription
FCP1	TFIIF-associated CTD phosphatase 1
FGF-2	fibroblast growth factor-2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GTFs	the general transcription factors
HDAC	histone deacetylase
HEXIM1	hexamethylene bisacetamide-inducible protein 1
HP1	heterochromatin-associated protein 1
HS1	haematopoietic lineage cell-specific protein 1
HSF1	heat shock factor-1
HTH	helix- turn-helix motif
ICB90	Inverted CCAAT box Binding Protein of 90 kDa
IP	immunoprecipitation
IR	ionizing radiation
IκBα	Inhibitor of kappaB alpha
MALDI-TOF MS	matrix-assisted laser desorption/ionization-time of flight mass spectrometry
MEF2C	myocyte enhancer factor-2C
mini- $SNAP_C$	complex composed of SNAP43, SNAP50, SNAP190 (1-719)
mRNA	messenger RNA
MS	mass spectrometry
mS	mini- $SNAP_C$
mSΔ(N+C)	complex composed of SNAP43, SNAP50, SNAP190 (63-505)
mSΔC	complex composed of SNAP43, SNAP50, SNAP190 (1-505)
mSΔN	complex composed of SNAP43, SNAP50, SNAP190 (63-719)
MTA-2	multithreaded architecture

muTATA	mutant TATA box sequence
NAP-2	nucleosome assembly protein 2
NE	nuclear extract
NFATc	nuclear factor of activated T cells
NFκB	nuclear factor kappa B
N-terminal	amino-terminal
OIR	Oct-1 interact region
PA-PLA1α	phosphatidic acid-preferring phospholipase 1A
PBP	proximal sequence element binding protein
PEST domain	proline-glutamic acid-serine-threonine domain
PIC	Pre-initiation complex
PK60S	protein kinase 60S
PKA	protein kinase A
pol	RNA polymerase
pptase	phosphatase
PSE	proximal sequence element
pSer	phosphoserine
P-TEFb	positive transcription elongation factor-b
PTF	proximal sequence element transcription factor
pThr	phosphothreonine
PTP	phosphotyrosine phosphatase
pTyr	phosphotyrosine
Q-TOF MS/MS	quadrupole time of flight mass spectrometry
quercetin	3, 3', 4', 5, 7-pentahyroxyflavone
RB	the retinoblastoma tumor suppressor protein
RbAp48	the Rb-associated protein
RNAi	RNA interference
RNPs	ribonucleoprotein particles
RNPS1	RNA-binding protein prevalent during S phase
rRNA	ribosomal RNA
RRR	arginine rich region

RT-PCR	reverse transcriptase polymerase chain reaction
SANT	DNA-binding domain in SWI-SNF, ADA, N-CoR and TFIIIB
SBF	the SPH binding factor
SL1/TIF-IB	promoter selectivity factor or transcription initiation factor IB
$SNAP_C$	the snRNA activating protein complex
snRNAs	small nuclear RNAs
snRNPs	small nuclear ribonucleoprotein particles
SOD	superoxide dismutase
SPH	*SphI* postoctamer homology
SRR	serine rich region
SSB	single-strand breaks
SSRP1	structure-specific recognition protein 1
Staf	the selenocysteine tRNA gene transcription activating factor
SV 40	Simian virus 40 large T antigen
TAF	TBP-associated factor
TBB	4, 5, 6, 7-tetrabromobenzotriazole
TBP	TATA binding protein
TGE running buffer	buffer containing 50 mM Tris, 380 mM glycine, 2 mM EDTA
TLC	thin layer chromatography
TLE	thin layer electrophoresis
TLR-3	Toll-like receptor 3
tRNA	transfer RNA
TRR	TBP recruitment region
uPA	urokinase
UPE	upstream promoter element
UV	ultraviolet light
VSMC	vascular smooth muscle cell
WCE	whole cell extract
wtPSE	wild type PSE sequence
wtTATA	wild type TATA box sequence
XRCC1	the X-Ray cross-complementing group 1 protein

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Human small nuclear RNA gene transcription

1.1. The functional significance of snRNAs

The final products of human small nuclear RNA genes are small nuclear RNAs (snRNAs), which play fundamental roles in processing of other RNAs. For example, U1, U2, and U6 snRNAs are essential components of small nuclear ribonucleoprotein particles (snRNPs) that control pre-messenger (m) RNA splicing (Burge et al., 1999). U3 snRNA is involved in ribosomal (r) RNA processing (Fatica, et al., 2002; Grandi, et al., 2002), while H1 snRNA is the catalytic subunit of RNase P responsible for transfer (t) RNA maturation (Bartkiewicz et al., 1989). In addition, snRNA functions in regulating mRNA gene transcription. For example, U1 snRNA associates with TFIIH and stimulates TFIIH-dependent RNA polymerase (pol) II transcriptional initiation (Kwek et al., 2002). 7SK snRNA, on the other hand, associates with a positive transcription elongation factor-b (P-TEFb) (Peng et al., 1998; Wei et al., 1998) and the hexamethylene bisacetamide-inducible protein 1 (HEXIM1) (Ouchida et al., 2003; Michels et al., 2003). HEXIM1 sequesters the 7SK•P-TEFb ribonucleoprotein particle (RNP) and represses P-TEFb kinase and transcriptional activities by inhibiting P-TEFb phosphorylation of the carboxy terminal domain (CTD) of RNA pol II (Nguyen et al., 2001; Yang et al., 2001; Yik et al., 2003; Chen et al., 2004; Yik et al., 2005). Futhermore, snRNA can mediate a preimmune

signal. For example, U1 snRNA, a component of U1-70-kDalton (kDa) RNP, is capable of inducing innate immunity signaling through activation of Toll-like receptor 3 (TLR-3) (Hoffman et al., 2004). Human snRNAs function in diverse cellular processes, the levels of snRNA gene transcription need to be well regulated when cells undergo cell proliferation, differentiation and embryo development (Caceres et al., 1992; Cheng et al., 1997; Glibetic et al., 1992; Lund et al., 1987; Meissner et al., 1995; Nash et al., 1987; Robert et al., 2002).

1.2 Human snRNA gene transcription

Human snRNA genes are transcribed by either RNA pol II or RNA pol III. The polymerase specificity depends upon their promoter architectures (for a review, see Hernandez, 2001).

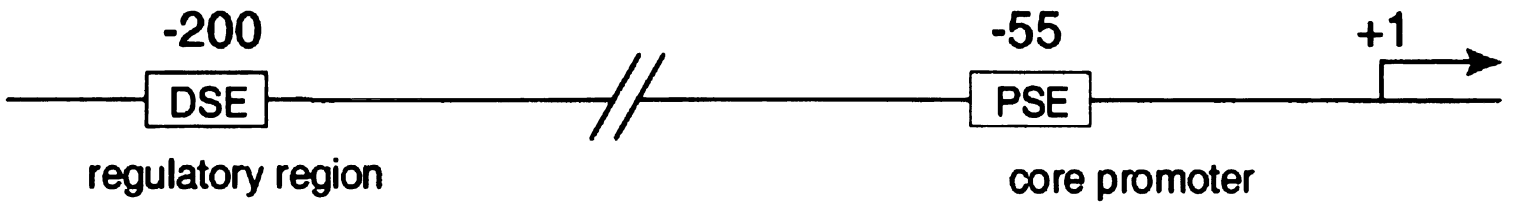
1.2.1 The structures of human snRNA gene promoters

U1 and U2 snRNA gene promoters are representatives of pol II-specific snRNA promoters, whereas U6 and 7SK snRNA gene promoters are representatives of pol III-specific snRNA promoters. As illustrated in Figure I-1, two distinct and conserved cis-acting regulatory regions that are important for snRNA gene transcription are present in both human RNA pol II and pol III snRNA gene promoters. A proximal sequence element (PSE) is located in the core promoter region centered near position -55 relative to the transcription initiation start site and a distal sequence element (DSE) is located in the regulatory region near position –220, and serves as a transcriptional enhancer. The PSE is

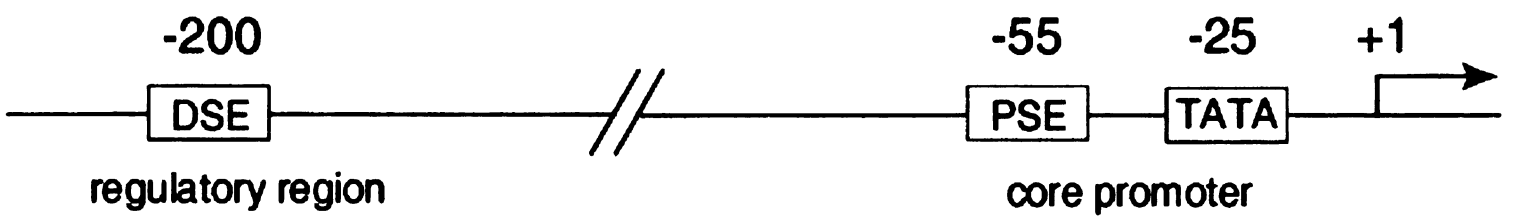
Figure I-1. Schematic representation of cis-elements of human snRNA promoters. The cis-elements on RNA pol II transcribed snRNA gene promoters, such as the U1 snRNA promoter, include a proximal sequence element (PSE) centered near position -55 relative to the transcription initiation start site and a distal sequence element (DSE) near position -200 at the regulatory region. RNA pol III-transcribed snRNA genes, represented by the U6 snRNA promoter, contain an additional TATA-box near position -25 adjacent to the PSE at the core promoter.

* Images in this dissertation are presented in color.

U1 snRNA gene promoter (pol II)



U6 snRNA gene promoter (pol III)



sufficient to nucleate the assembly of a RNA pol II transcription initiation complex and to direct basal RNA pol II transcription *in vitro*. The basal RNA pol III snRNA gene promoters additionally contain a TATA box located around position -25 and at a fixed distance downstream of the PSE. Together the PSE and TATA box direct basal RNA pol III transcription (Hernandez and Lucito 1988; Mattaj et al. 1988; Kunkel and Pederson 1989; Lobo and Hernandez 1989).

1.2.2 Trans-acting factors required for snRNA gene transcription

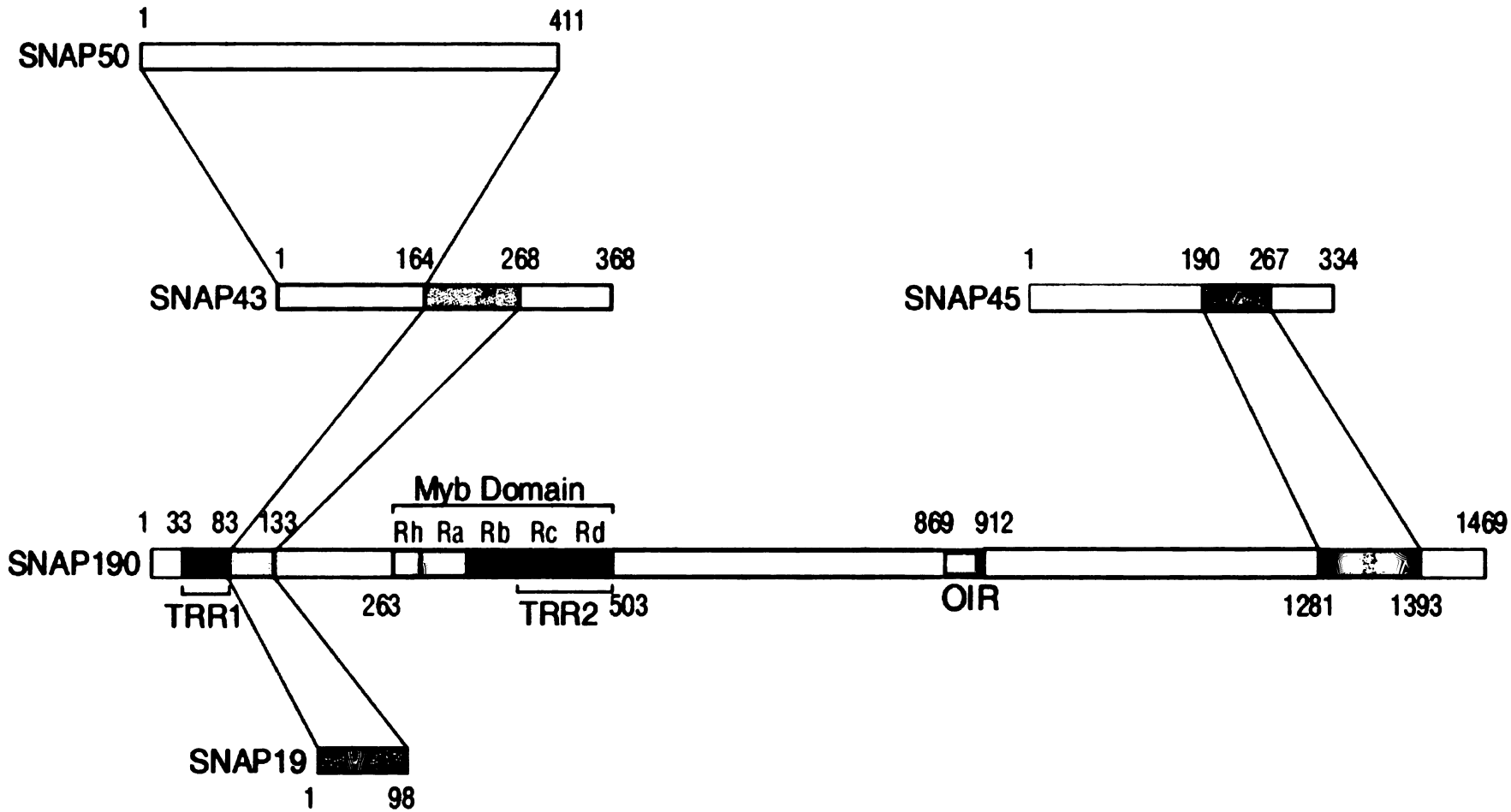
1.2.2.1 The DSE binding factors

All human snRNA genes contain a DSE encompassing an octamer sequence ATGCAAAT that is recognized by the Oct-1 or Oct-2 POU domain (Murphy et al, 1992). In addition, many snRNA gene DSEs contain a GC-box (GGGGCGGGGA) and an *SphI* postoctamer homology (SPH) motif that are recognized by the transcription factor Sp1 (Ares et al., 1987) and a zinc finger containing transcription factor referred to as the SPH binding factor (SBF) (Roebuck et al, 1990; Zamrod and Stumph, 1990) or as the selenocysteine tRNA gene transcription activating factor (Staf) (Schuster et al., 1995; Schaub et al., 1997; Myslinski et al., 1998; Rincon et al., 1998), respectively. Oct-1 binds to the octamer sequence of the DSE and activates snRNA transcription by direct protein-protein contacts with the basal transcription factor called the snRNA activating protein complex ($SNAP_C$) (Henry et al., 1995; Mittal et al., 1996; Henry et al., 1998b; Ford et al., 1998; Mittal et al., 1999; Hovde et al., 2002).

1.2.2.2 The PSE binding factor

The PSE binding factor is a multi-subunit complex called $SNAP_C$ (Sadowski et al. 1993), also referred to as the proximal sequence element transcription factor (PTF) (Murphy et al., 1992) or the proximal sequence element binding protein (PBP) (Waldschmidt et al., 1991; Meissner et al., 1995). $SNAP_C$ consists of at least five subunits SNAP190, SNAP50, SNAP45, SNAP43, and SNAP19, which are named according to their apparent molecular masses. The cDNAs encoding the SNAP19 (Henry et al. 1998b), SNAP43 (Henry et. al, 1995) or PTF γ (Yoon and Roeder, 1996), SNAP45 (Sadowski et al., 1996) or PTF δ (Yoon and Roeder, 1996), SNAP50 (Henry et. al, 1996) or PTF β (Bai et al., 1996), and SNAP190 (Wong et al., 1998) subunits have been isolated. The protein-protein contacts within $SNAP_C$ have been mapped by reconstitution of partial complexes and coimmunoprecipitations of various *in vitro* translated full-length or truncated $SNAP_C$ subunits (Henry et al., 1996; Henry et al., 1998a; Henry et al., 1998b; Wong et al., 1998; Mittal et al., 1999; Ma and Hernandez, 2001; Gu and Henry, unpublished data). As summarized in Figure I-2, SNAP190 interacts with SNAP45, SNAP43 and SNAP19, while SNAP50 joins the complex by interacting with SNAP43. Further analysis revealed that SNAP190 (Yoon et al. 1995) and SNAP50 (Henry et al. 1996; Bai et al., 1996) are in close contact with the DNA when $SNAP_C$ is bound to the PSE. SNAP190 possesses a Myb DNA binding domain consisting of four complete repeats (RaRbRcRd) and a half repeat (Rh) (Wong et al. 1998), and a baculovirus-assembled recombinant $SNAP_C$ with an RhRaRb deletion within SNAP190 is capable of binding specifically to the PSE and directing snRNA gene transcription (Wong et al.

Figure I-2. SNAP190 acts as a scaffold protein at snRNA promoters. Domains within SNAP190 that interact with other $SNAP_C$ subunits and other trans-regulators at snRNA promoters are shown. TRR1: TBP recruitment region 1; TRR2: TBP recruitment region 2 and OIR: Oct-1 interaction region (modified from Ma and Hernandez, 2001) (Ford et al., 1998; Ma and Hernandez, 2001; Ma and Hernandez, 2002; Hinkley et al., 2003; Gu and Henry, unpublished data).


1
411
SNAP50
1
164
268
368
SNAP43
1
190
267
334
SNAP45
Myb Domain
1 33 83 133
Rh Ra Rb Rc Rd
869 912
1469
SNAP190
TRR1
263
TRR2
503
OIR
1281
1393
SNAP19
1
98

1998), while RcRd deletion is not, suggesting that the RcRd repeats contribute to recognition of the PSE on the promoter (Mittal et al. 1999). Moreover, a mini-$SNAP_C$ consisting of full-length SNAP43, full-length SNAP50, and a truncated SNAP190 (1-514) encompassing the Myb DNA binding domain can recognize the PSE and support snRNA *in vitro* transcription by both RNA pol II and pol III (Mittal et al., 1999). Thus, SNAP190 plays a central structural role by serving as a scaffold protein within $SNAP_C$ and contributes to the PSE recognition on snRNA gene promoters.

1.2.2.3 The TATA box binding factor

The TATA box on pol III-specific snRNA gene promoters is recognized by a TFIIIB-like complex (Waldschmidt et al. 1991; Murphy et al. 1992; Sadowski et al. 1993; Yoon et al. 1995; Schramm et al. 2000; Teichmann et al. 2000) designated Brf2-TFIIIB. This TFIIIB complex consists of the TATA binding protein (TBP), TFIIB-related factor 2 Brf2 (Willis 2002), originally called BRFU (Schramm et al. 2000; Cabart and Murphy, 2001) or TFIIIB50 (Teichmann et al. 2000), and a SANT domain (a putative DNA-binding domain in SWI-SNF, ADA, N-CoR and TFIIIB) containing protein Bdp1 (Schramm et al. 2000). Brf2 recognizes the TBP/TATA-box complex through its TFIIB-related core domain (Cabart and Murphy 2001). Another type of TFIIIB designated Brf1-TFIIIB functions specifically for tRNA and 5S rRNA transcription and is composed of TBP, Brf1, and Bdp1 (Kassavetis et al., 1992).

1.2.3 Composition of RNA pol II and III transcription initiation complexes

1.2.3.1 The basal transcription machinery for pol II-specific snRNA genes

The RNA pol II-specific snRNA promoters contain only a PSE in the core promoter region. $SNAP_C$ binding to the PSE is required for nucleating the assembly of a RNA pol II transcription preinitiation complex and directing basal RNA pol II transcription *in vitro*. TBP is also needed for the TATA-less snRNA gene transcription by RNA pol II (Sadowski et al., 1993). TBP cofractionates with $SNAP_C$ (Henry et al., 1995), and RNA pol II-specific U1 snRNA transcription *in vitro* can be reconstituted by addition of those $SNAP_C$ /TBP enriched fractions to an endogenous TBP depleted HeLa nuclear extract (Sadowski et al., 1993). The interaction between TBP and $SNAP_C$ is further confirmed by coimmunoprecipitation and GST-pulldown experiments (Henry et al., 1995; Sadowski et al., 1996; Ma and Hernandez, 2002; Hinkley et al., 2003). As the TBP used in U1 snRNA transcription is not part of the TBP-containing TFIID complex required for TATA box-containing pol II-specific mRNA promoters, or the TBP-containing TFIIIB complex required for TATA-less pol III-specific tRNA and 5S rRNA promoters (Sadowski et al., 1993; Yoon et al., 1996), it is likely that $SNAP_C$ serves as a TBP-associated factor (TAF) for the recruitment of TBP to the pol II-specific snRNA gene promoters (Henry et al., 1995). Other components required for pol II-specific snRNA transcription were also identified by reconstitution of transcription with recombinant factors or with purified factors from extracts. The general transcription factors (GTFs) TFIIA, TFIIB, TFIIF, and TFIIE have been determined to be parts of the pol II-specific snRNA transcription initiation complexes (Figure I-3, *top panel*). As these general

transcription factors and $SNAP_C$ are not sufficient to initiate transcription from the U1 promoter, it was speculated that additional factors are required for pol II-specific snRNA gene transcription (Kuhlman et al., 1999).

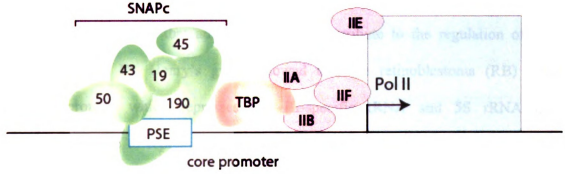
1.2.3.2 The basal transcription machinery for pol III-specific snRNA genes

The RNA pol III-specific snRNA promoters contain two core elements: a PSE and a TATA box that are recognized by $SNAP_C$ and Brf2-TFIIIB, respectively. Recombinant $SNAP_C$, Brf2-TFIIIB, and highly purified RNA pol III are sufficient for directing pol III-specific U6 snRNA gene transcription *in vitro* (Hu et al., 2003). The PSE-bound $SNAP_C$ plays a critial role for recruitment of the TATA box binding complex Brf2-TFIIIB (Ma and hernandez, 2002; Hinkley et al., 2003) (as illustrated in Figure I-3, *bottom panel*). Futher studies indicate that within $SNAP_C$, SNAP190 contributes to Brf2-TFIIIB recruitment (Ma and Hernandez, 2002; Hinkley et al, 2003). The SNAP190 RcRd repeats (also named TBP recruitment region 2, or TRR2) can stimulate TBP recognition and TFIIIB recruitment to the neighboring TATA box present in the U6 snRNA promoter (Hinkley et al, 2003). The region within SNAP190 from amino acids 34 to 83 (named TBP recruitment region 1, or TRR1) also contributed to cooperative binding with TBP in the context of mini-$SNAP_C$ (Ma and Hernandez, 2002) (Figure I-2).

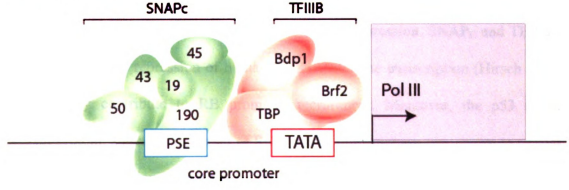
In summary, the $SNAP_C$ /PSE and Oct-1/DSE protein-DNA contacts and the SNAP190/Oct-1 protein-protein cooperative interactions are critical for RNA pol II-specific snRNA transcription. In the case of pol III-specific snRNA transcription, additional TFIIIB/TATA-box protein-DNA contact is reinforced by SNAP190/TBP protein-protein cooperative interaction. Thus, $SNAP_C$ plays a fundamental role in the

Figure I-3. Composition of snRNA gene transcription initiation complexes for transcription by RNA pol II and pol III. RNA pol II initiation complex assembled on the human U1 snRNA core promoter (top panel), and RNA pol III initiation complexes assembled on the human U6 snRNA promoter (bottom panel).

U1 snRNA gene promoter



U6 snRNA gene promoter



assembly of transcription initiation complexes for transcription by both RNA pol II and pol III.

1.2.4. Other factors involved in regulating human snRNA gene transcription

Human snRNA gene transcription is regulated during cell cycle progression (Diana et al., 2003; Hu et al., 2004) and in response to DNA damage (Gridasova and Henry, 2005), suggesting that additional factors contribute to the regulation of these genes. Studies by Dr. Henry's group showed that the retinoblastoma (RB) tumor suppressor protein, which represses pol III-specific tRNA and 5S rRNA gene transcription, is able to repress U6 snRNA gene transcription by RNA pol III as well (Hirsch et al., 2000), and may contribute to cell cycle regulation of these genes (Hirsch et al., 2004). The observation that RB and RNA pol III co-occupy the human U6 snRNA promoter suggested a unique mechanism that RB represses U6 snRNA gene through stable promoter association with the polymerase during repression. $SNAP_C$ and TBP are also important for RB repression of human U6 snRNA gene transcription (Hirsch et al., 2004) and may contribute to RB promoter recruitment. Moreover, the p53 tumor suppressor protein, a general repressor of RNA pol III transcription, was shown to repress snRNA gene transcription by both polymerases II and III. Endogenous p53 associates with U1 and U6 gene promoters, and p53 promoter occupancy is stimulated by ultraviolet (UV) light (Gridasova and Henry, 2005). In contrast, Simian virus (SV) 40 large T antigen, which interacts with SNAP43 and SNAP45, is capable of activating U6 snRNA gene transcription (Damania et al., 1998).

Additional factors involved in regulating RNA pol III-specific snRNA gene transcription were recently identified using a minimal RNA pol III system for human U6 transcription. This system utilizes recombinant $SNAP_C$, Brf2-TFIIIB, and a highly purified RNA pol III complex (Hu et al., 2003). Two RNA pol III associated factors, β-actin (Hu et al., 2004) and protein kinase CK2 (Hu et al., 2003), were shown to be involved in U6 snRNA gene transcription. CK2 co-purifies with RNA pol III and stimulates U6 snRNA gene transcription by phosphorylating RNA pol III. However, CK2 phosphorylation of the Bdp1 subunit of Brf2-TFIIIB complex during mitosis leads to Bdp1 dissociation from the endogenous U6 promoter and diminished transcription (Hu et al., 2004). These data suggest that CK2 has both positive and negative regulatory roles in pol III-specific U6 snRNA transcription. However, the role of CK2 in regulating the closely related pol II-specific snRNA gene transcription has been uncertain.

2. Protein Kinase CK2

Protein kinase CK2 (EC 2.7.1.37) (formly "casein kinase 2" or "casein kinase II") was first discovered in rat liver extracts using casein as phosphorylatable substrate (Burnett and Kennedy, 1954). Since then CK2 has been found in protozoa, yeast, plants, and animals (Faust and Montenarh, 2000; and references therein).

CK2 is the most conserved and the most functionally pleiotropic member among all protein kinases characterized thus far. More than 307 CK2 substrates have been found, many of which are involved in signal transduction, gene expression, cell proliferation, and DNA repair (Pinna, 2002; Meggio and Pinna, 2003). Consistently, CK2 is distributed

in the nucleus as well as the cytoplasm in all eukaryotic cells. CK2 is essential to cell viability (Pinna and Meggio, 1997; Ahmed et al., 2002; Unger et al., 2004). It has become clear that CK2 plays an important role in regulating numerous cellular processes both during normal growth (Litchfield et al., 1992; Litchfield and Lüscher, 1993; Hehn et al., 1997; Guerra and Issinger, 1999) and during cancer development (Seldin et al., 1995; Tawfic et al., 2001).

2.1 CK2 structure and chemical properties

2.1.1 CK2 holoenzyme

CK2 predominantly exists as a heterotetrameric complex consisting of two catalytic α subunits and two regulatory β subunits.

In humans, three catalytic isoforms, designated CK2α, CK2α′ and CK2α′′, have been identified. CK2α and CK2α′ are encoded from two distinct genes (Meisner et al., 1989; Lozeman et al., 1990) and amino acid sequences deduced from these two genes are about 85% identical. CK2α′′ is 91% identical to the CK2α. Its N-terminal sequence (1-353) is almost identical to CK2α but its carboxy (C)-terminal sequence (354-385) is unique. This unique C-terminal sequence is probably derived from an Alu-like exon (Wirkner et al., 1994) due to alternative splicing (Litchfield et al., 2001; Shi et al., 2001).

CK2α and CK2α′ have similar enzymatic activity (Litchfield, 2003), but are not functionally identical. First, these two isoforms show a difference in their localization. CK2α is found ubiquitously in all cell types of tissues in eukaroytic organisms, while CK2α′ is expressed in a tissue specific manner. For example, CK2α′ is detected only in mouse testis and brain (Guerra et al., 1999), and is much more abundant in rat neurons

than in glia cells (Diaz-Nido et al., 1994). Second, there are functional distinctions between CK2α and CK2α′. For instance, overexpression of the catalytically inactive CK2α′ dramatically inhibits cell proliferation in human osteosarcoma U2-OS cell lines, while overexpression of the inactive CK2α does not (Vilk et al., 1999). Furthermore, CK2α′ is essential for normal differentiation of male germ cells (Xu et al., 1999) and inactivation of CK2α′ causes infertility in male mice (Escalier et al., 2003). Thus far, CK2α′′ has been found only in the nuclei of liver tissue. CK2α′′ preferentially associates with the nuclear matrix (Hilgard et al., 2002) and the translated Alu sequence of CK2α′′ is necessary for directing the holoenzyme nuclear localization. CK2α′′ is specifically needed for hepatocellular membrane protein trafficking of some proteins (Hilgard et al., 2002). In addition, CK2α′′ appears to be involved in suppression of apoptosis mediated by a JNK signaling cascade (Hilgard et al., 2004). Thus, the three catalytic CK2α isoforms are functionally distinct from one another.

In humans, only a single regulatory subunit designated CK2β has been identified. Human CK2β is unusual because it does not share homology with any other regulatory subunit of protein kinases (Jakobi et al., 1989). In *Saccharomyces cerevisiae* two genes encoding CK2β subunits have been reported (Reed et al., 1994; Bidwai et al., 1995), and in *Arabidopsis* three genes have been identified (Collinge and Walker, 1994; Sugano et al., 1998).

In the CK2 holoenzyme, stable CK2 β-β dimerization mediated by the zinc-finger region Cys^{109} ~ Cys^{140} (Canton et al., 2001) is a prerequisite for the stable incorporation of CK2α into the CK2 tetramer (Graham and Litchfield, 2000; Canton et al., 2001;

Boldyreff et al., 1996; Chantalat et al., 1999; Niefind et al., 2001). Then, two identical or non-identical (Gietz et al., 1995) catalytic subunits join the complex through interaction with both β subunits, although the two catalytic subunits make no direct contact with one another in the complex (Niefind et al., 2001).

2.1.2. Regulation of CK2 enzymatic activity

Most protein kinases exist in an inactive form and their activities are turned on only in response to specific stimuli. For example, protein kinase A (PKA) becomes active after an inhibitory protein subunit is released upon cAMP binding. CK2 appears to be independent of any known secondary messengers, such as cAMP, cGMP, calcium or lipids (Kudlicki et al., 1978; Tuazon and Tuazon, 1991; Litchfield and Lüscher, 1993). Both the isolated CK2 catalytic subunits and CK2 holoenzyme exist predominantly in their active comformations. There are at least two independent mechanisms governing their activities. First, an intrinsic interaction between the activation loop (amino acids from 175 to 188) and its N-terminal segment (amino acids from 1 to 36) within the catalytic subunit ensures CK2α remains in its active form. Second, the association of the catalytic α subunits with the regulatory β subunits can compensate for the inactive mutants in which the contacts between the N-terminal and activation segments have been disrupted (Sarno et al., 2002), suggesting that an intermolecular interaction is important for CK2 holoenzyme activation. Within the holoenzyme, in addition to confering stability and stimulating the catalytic activity of CK2α with most protein substrates, CK2β is also responsible for docking and/or recruitment of CK2 partners, including substrates such as the nucleolar protein Nopp 140 (Li et al., 1997) and p53 (Fihol et al., 1992; Appel et al.,

1995), and potential CK2 regulators such as the cell surface receptor CD5 (Ramam et al., 1998) and the fibroblast growth factor-2 (FGF-2) (Bonnet et al.,1996).

Although the CK2 catalytic subunits and holoenzyme are predominantly in their active conformation, their activities can still be regulated. CK2 activity can be dramatically increased in response to hormones and growth factors (Sommercorn et al., 1987; Klarlund and Czech, 1988; Ackerman and Osheroff, 1989; Ackerman et al., 1990), which play an important role in transducing signals between extracellular growth factors and nuclear responses. CK2 is activated during cell division, cellular differentiation, and embryogenesis (Pinna and Meggio, 1997; Guerra and Issinger, 1999; Wilhelm et al., 1995). CK2 is sensitive to certain stresses, such as heat shock (Gerber et al., 2000) and UV light (Brenneisen et al., 2002). The mechanisms of CK2 up- or down-regulation are unclear, however posttranslational modification appears to be important for CK2 activation. For example, CK2β phosphorylation has been reported to increase CK2 activity (Ackerman et al., 1990). CK2α phosphorylation in the C-terminal domain by $p34^{Cdc2}$ inhibits CK2 holoenzyme activity (Messenger et al., 2002). In addition, CK2 activity can be regulated by other proteins through interactions with either its α subunit or β subunit. For example, bovine prion protein (bPrP) targets the CK2α subunit and stimulates CK2 holoenzyme activity (Meggio et al., 2000). FGF-2 can bind to CK2β and trigger the CK2 holoenzyme nuclear translocation (Bonnet et al., 1996). p53 binds to CK2β; however, the full length p53 inhibits CK2 activity while the C-terminus of p53 stimulates CK2 activity (Schuster et al., 2001). In addition, a number of external small molecules have been identified as CK2 inhibitors including 2,3-diphosphoglycerate (2,3-DPG) (Hathaway and Traugh, 1983), 3,3 ,4 ,5,7-pentahyroxyflavone (quercetin) (Davies

et al., 2000), emodin (Yim et al., 1999), and the ATP analogue 4,5,6,7-tetrabromobenzo-triazole (TBB) (Sarno et al., 2001) and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (Zandomeni et al., 1986). In contrast, basic polypeptides like polyamines (spermine and spermidine) and synthetic polylysine (Meggio et al., 1992; Song et al., 1994; Leroy et al., 1997), or proteins rich in lysine such as histones and protamine (Sacerdoti-Sierra and Jaffe, 1997) were identified as potential CK2 stimulators.

Accumulating evidence has indicated that individual CK2 catalytic subunits and the CK2 regulatory subunit can exist independently. The individual subunits of CK2 themselves may interact with a number of different proteins (for a review, see Guerra and Issinger, 1999; Korn et al, 1999). CK2β can interact with a large number of potential interacting partners independently of CK2α. For example, CK2β can interact with proteins involved in signal transduction including the cytoplasmic Raf serine/threonine kinase family member A-Raf (Boldyreff and Issinger, 1997; Hagemann et al., 1997), MAPK kinase kinase Mos (Lieberman and Ruderman., 2004), Src family protein tyrosine kinase Lyn (Lehner et al., 2004), and tumor suppressor protein checkpoint kinase 2 (Chk2) (Bjorling-Poulsen et al., 2005). CK2β can also interact with proteins involved in DNA damage repair including p53 (Götz et al., 1999) and $p21^{WAF1/CIP1}$ (Götz et al., 1996; Romero-Oliva and Allende, 2001), suggesting an additional physiological role of CK2β beside being the regulatory protein in the CK2 tetramer (for a review, see Bibby and Litchfield, 2005). More recently, the individual CK2 catalytic subunits have been found to complex with other proteins. Han et al. (2001) showed that CK2α can interact with phosphatidic acid-preferring phospholipase 1A (PA-PLA1α). CK2α phosphorylation of PA-PLA1α promotes stable PA-PLA1α/CK2α complex formation in an ATP/GTP-

dependent manner (Han et al., 2001). Moreover, the protein kinase 60S (PK60S) (Pilecki et al., 1992; Szyszka et al., 1996) in yeast 60S ribosomes consists of CK2α′ subunit and superoxide dismutase (SOD1) protein. Upon oxygen stress, CK2α′ catalytic activity is inhibited by the formation of inactive CK2α′/$(SOD1)_2$ complex, indicating that CK2α′ may be involved in regulating the ribosome activity of the protein synthesis machinery under stress conditions (Abramczyk et al., 2003).

2.1.3. Dual-substrate and dual-cosubstrate specificity of CK2

CK2 can phosphorylate not only seryl/threonyl residues but also tyrosyl residues. For example, the tyrosyl residue in yeast nucleolar immunophilin Fpr3 can be phosphorylated by CK2 both *in vivo* and *in vitro* (Marin et al., 1999). A CK2 consensus motif was derived from an analysis of 308 CK2 phosphorylation sites mapped within 175 CK2 substrates. CK2 recognizes a phosphorylatable residue that is followed by a series of acidic residues (n + 3 being preferred over n + 1, and/or n + 2) (Meggio and Pinna, 1984; Marin et al., 1986). The minimum consensus sequence of CK2 is S/T-X-X-D/E/pS/pY (Meggio et al., 1994)

Both CK2 holoenzyme and the isolated CK2α subunit from all sources examined to date show dual-cosubstrate specificity, meaning the ability to efficiently utilize either ATP or GTP as a phosphoryl donor. Water molecules are critical to switch the active site of CK2 from an ATP- to GTP-compatible state (Niefind et al.,1999). Mg^{2+} is required for CK2 activity. However, Mg^{2+} can be substituted by Mn^{2+} and Co^{2+}. CK2 prefers ATP as cosubstrate in the presence of Mg^{2+} while it prefers GTP in the presence of Mn^{2+} (Lasa and Pinna, 1997).

2.2. CK2 participates in various cellular processes

2.2.1. CK2 localization

CK2 is distributed in almost all tissues in various species at different stages of development. CK2 can associate with specific structures including the plasma membrane, cytoskeleton, ribosomes, nuclear matrix, nucleolus, centrosomes and nucleosome. CK2 has also been detected in various organelles such as the endoplasmic reticulum, mitochondria, and golgi (Faust and Montenarh, 2000). Studies also show that exogenous substrates such as fibrinogen, fibrin (Sonka et al., 1989), and vitronectin (Skubitz et al., 1991) can trigger CK2 release from the surface of intact cells (named ecto-CK2). Vitronectin phosphorylation by ecto-CK2 is required for urokinase (uPA)-dependent human vascular smooth muscle cell (VSMC) adhesion (Stepanova et al., 2002). Similarly, complement C9 protein phosphorylation by ecto-CK2 protects cells from complement-mediated lysis (Bohana-Kashtan et al., 2005). The level of CK2 appears to be tightly regulated in normal cells, however, CK2 activity and protein content have been shown to be elevated in proliferating cells (Issinger, 1993).

2.2.2. CK2 in cell proliferation

CK2 has been shown to phosphorylate a large number of proteins that are important regulators of cell division (Allende and Allende, 1995). CK2 is required at multiple transitions in the cell cycle including G_0 /G_1, G_1/S and G_2/M (Marshak and Russo, 1994; Pepperkok et al., 1994; Hanna et al., 1995). High levels of CK2 have been detected in cells undergoing mitosis (Issinger, 1993). In *Saccharomyces cerevisiae*, cell cycle progression is arrested in both G1 and G2/M phases of the cell cycle when

temperature-sensitive CK2 mutants are shifted to the nonpermissive temperature (Hanna et al., 1995). Similarly, the proliferation of primary human fibroblasts can be blocked by inactivation of CK2 through depletion of CK2 by either RNA interference (RNAi) or microinjection of CK2 specific antibodies. Furthermore, overexpression of the CK2α kinase-deficient mutant (K^{68}→A) induced a marked inhibition of cell proliferation in both NIH3T3 and CCL39 cells (Lebrin et al., 2001). These results indicate that CK2 plays an important role in controlling cell division and cell proliferation.

2.2.3. CK2 in cell survival and apoptosis

Biochemical and genetic evidence indicates that CK2 participates in the maintenance of cell viability. CK2 appears to exert an anti-apoptotic role by protecting regulatory proteins from caspase-mediated degradation (Litchfield, 2003). Inhibition of CK2 by its specific inhibitor TBB induces apoptosis in Jurkat cells (Ruzzene et al., 2002). Similarly, depletion of endogenous CK2α and/or CK2α′ catalytic subunits by RNAi also causes marked apoptosis when HeLa cells have been exposed to 6-thioguanine (6-TG) (Yamane and Kinsella, 2005a) or ionizing radiation (IR) (Yamane and Kinsella, 2005b). However, these responses can be suppressed by introducing ARC (apoptosis repressor with caspase recruitment domain) or by blocking caspase enzymatic activity (Yamane and Kinsella, 2005a; Yamane and Kinsella, 2005b), suggesting that CK2 participates in inhibition of apoptosis by negatively regulating caspase activity. A number of antiapoptotic proteins, such as Bid (Desagher et al., 2001), Max (Krippner-Heidenreich et al., 2001), ICB90 (Inverted CCAAT box Binding Protein of 90 kDa) (Bronner et al., 2004), and HS1 (haematopoietic lineage cell-specific protein 1) (Ruzzene et al., 2002) are

targeted by CK2 leading to downregulation of caspase-dependent degradation. Moreover, increased expression of CK2 protects cells from drug-induced apoptosis (Guo et al., 2001). Taken together, these observations indicate that CK2 plays a critical role in maintaining cell survival.

2.2.4. CK2 and cancer

The growth-promoting and anti-apoptotic properties of CK2 may contribute to its ability to participate in transformation. CK2 is abnormally active in a variety of human cancers including leukemias and solid tumors (Munstermann et al., 1990; Landesman-Bollag et al., 2001). The detection of higher CK2 nuclear translocation in tumor cells suggests that CK2 nuclear association plays an important role in tumorigenesis (Faust et al., 1999; Guo et al., 2001).

2.2.5. CK2 in stress response

Another important function of CK2 is that it acts as a downstream effector responsible for transducing genotoxic stress signals to either the transcriptional machinery or DNA-damage repair system. For example, in yeast, CK2 targets the TBP component of Brf1-TFIIIB for regulation of RNA pol III-specific tRNAs and 5S rRNA gene transcription. Transcription of these genes is repressed in cells that have experienced DNA damage, which causes the dissociation of the CK2α from the CK2/TFIIIB complex (Ghavidel and Schultz, 2001; Schultz, 2003). Thus, CK2 acts as a terminal effector in the signaling pathway that transduces DNA-damage signals to the RNA pol III

transcriptional machinery. In addition, disruption of the CK2β gene causes yeast cells to permanently arrest in G2/M at the DNA damage checkpoint when double-strand breaks (DSB) occur, while normal cells can override this block, suggesting a role of CK2 in controlling adaptation to the DNA-damage checkpoint (Toczyski et al., 1997). CK2 phosphorylation of histone H4 at serine 1 appears to promote DNA DSB repair (Cheung et al., 2005). Furthermore, in mammalian cells, CK2 facilitates DNA single-strand breaks (SSB) repair mediated by the X-Ray cross-complementing group 1 protein (XRCC1) (Loizou et al., 2004). CK2 can also control the activity of the DNA repair protein apurinic endonuclease (APE/Ref-1) (Fritz and Kaina, 1999). APE/Ref-1 phosphorylation by CK2 stimulates its redox capability towards the transcription factor AP-1, thus promoting AP-1 DNA binding activity and activating its target genes. Upon CK2 inhibition, cells become more sensitive to DNA-damage reagents (Fritz and Kaina, 1999). Moreover, CK2 has been shown to translocate to the nuclear matrix in response to heat shock (Gerber et al., 2000). In addition, CK2 regulates p53 activity (Keller et al., 2001), and p53 phosphorylation by CK2 is increased in response to UV irradiation (Blaydes and Hupp, 1998). In the case of nuclear factor kappa B (NFκB), its activation is dependent upon CK2 phosphorylation of IκBα, an inhibitory partner that associates with and sequesteres the NFκB in the cytoplasm. Upon oxidative stress, CK2 targets IκBαb for degradation, thereby allowing NFκB proteins to translocate to the nucleus and induce transcription (Schoonbroodt et al., 2000). These data indicate that CK2 is a key participant in the cellular response to various stress conditions.

2.3. The role of CK2 in transcription regulation

Protein phosphorylation appears to be one the most important post-translational modifications involved in the regulation of transcription factor activity. Protein phosphorylation also impinges upon most of the signal transduction pathways. Therefore protein phosphorylation provides a link between signal transduction and gene expression (Karin, 1994; Bohmann, 1990; Litchfield, 2003). Phosphorylation or dephosphorylation of transcription factors and/or other related proteins either positively or negatively regulate their activity to control gene expression.

2.3.1. CK2 phosphorylation controls transcription at different levels

2.3.1.1. Several mechanisms have been proposed for a role of CK2 phosphorylation of transcription factors in gene regulation.

(a) Phosphorylation by CK2 influences transcription factor subcellular localization. For example, the nuclear import of SV40 large T antigen is enhanced by CK2 phosphorylation at serine 112 (Xiao et al., 1998); while phosphorylation of muscle-specific transcription factor Myf-5 at serine 49 by CK2 reduces its nuclear localization (Winter et al., 1997). In contrast, CK2 phosphorylation of NFATc (nuclear factor of activated T cells) promotes NFATc nuclear export (Porter et al., 2000).

(b) Phosphorylation of transcription factors by CK2 governs their DNA-binding activities both positively and negatively. For example, CK2 phosphorylation of the myocyte enhancer factor-2C (MEF2C) at serine 59 enhances the DNA binding and

transcriptional activity of MEF2C (Molkentin et al., 1996). Heat shock factor-1 (HSF1) phosphorylation by CK2 at threonine 142 promotes it binding to heat shock elements and *trans*-activation of the HSP70 gene (Soncin et al., 2003). In addition, the high-mobility group domain protein SSRP1 (structure-specific recognition protein 1), which plays a role in transcription and DNA replication in the chromatin context, is targeted by CK2. SSRP1 phosphorylation at serine 510 by CK2 prevents the nonspecific DNA-binding activity of SSRP1 and FACT (facilitating chromatin-mediated transcription) complex (Li et al., 2005). CK2 phosphorylation of the c-Myb nuclear oncoprotein at serines 11 and 12 results in reduced DNA binding (Lüscher et al., 1990).

(c) CK2 phosphorylation can modulate protein-protein interaction. CK2 phosphorylation of TFIIF-associated CTD phosphatase (FCP1) enhances the interaction between TFIIF subunit RAP74 and FCP1 (Abbott et al., 2005). The *trans*-activation properties of some transcription factors are also regulated by CK2-mediated phosphorylation through influencing the recruitment of coregulators and/or RNA polymerase to the promoter. For instance, CK2 also targets the N-terminal transcriptional activation domain of CHOP/GADD153 (C/EBP homologous protein transcription factor/growth arrest and DNA damage inducible protein) for phosphorylation and downregulates its transcription activity (Ubeda and Habener, 2003). Normally, the zinc-finger transcription factor CTCF (CCCTC-binding factor) acts as a transcriptional repressor, but CK2 phosphorylation at serine 612 switches CTCF to an activator (El-Kady and Klenova, 2005). Phosphorylation by CK2 on threonine 142 of HSF1 promotes *trans*-activation of the HSP70 gene (Soncin et al., 2003). However, CK2 phosphorylation

at the C-terminal serine 386 of p53 is critical for p53-dependent repression (Hall et al., 1996).

(e) CK2 phosphorylation is also important for regulating the level of a transcription factor by either stabilizing it or marking it for destruction. For example, CK2 phosphorylation of Max at serine 11 protects Max from caspase-5 mediated degradation (Krippner-Heidenreich et al., 2001). Ubiquitin-directed proteasomal degradation of the c-Myc oncoprotein is protected by CK2 phosphorylation in its C-terminal proline-glutamic acid-serine-threonine (PEST) domain (Channavajhala and Seldin, 2002). In contrast, CK2 phosphorylation of IκBα at serine 283 and threonines 291 and 299 in its C-terminal PEST domain accelerates its degradation, which is important for NFκB nuclear translocation and transcription activation (Lin et al., 1996; McElhinny et al., 1996).

2.3.1.2. CK2 in chromatin remodeling processes

Evidence for CK2 function in chromatin remodeling processes is increasing. Firstly, histone cytoplasmic-nuclear translocation is influenced by CK2 phosphorylation. CK2 targets a histone chaperone protein called nucleosome assembly protein 2 (NAP-2) for phosphorylation and prevents histones/NAP-2 complex transport from the cytoplasm into the nucleus (Rodriguez et al., 2000). Secondly, chromatin associations and/or chromatin-related protein-protein interactions are affected by CK2-mediated phosphorylation. For example, a chromatin-associated phosphotyrosine phosphatase

PTP-S2 phosphorylation by CK2 results in its dissociation from chromatin (Nambirajan et al., 2000). DNA binding activity of the human chromatin protein DEK is also downregulated by CK2 phosphorylation (Kappes et al., 2004). Histone deacetylases (HDAC) 1 and HDAC2 phosphorylation by CK2 increases its binding affinity to interacting partners, including the Rb-associated protein (RbAp48), MTA-2 (multithreaded architecture), mSin3A, and CoREST (a corepressor for the repressor element 1 silencing transcription factor) (Pflum et al., 2001; Tsai and Seto, 2002; Sun et al., 2002). In addition, HDAC1 phosphorylation at serine 421 and 423 by CK2 promotes its enzymatic activity (Pflum et al., 2001), which is proposed to alter the balance of histone (de-)acetylation of its target genes. Furthermore, several high mobility group nonhistone chromosomal proteins (HMGs) and the heterochromatin-associated protein 1 (HP1) are phosphorylated by CK2, which then affect their DNA binding and their interaction with specific transcription factors (Wisniewski et al., 1999; Zhao et al., 2001; Krohn et al., 2002). Thirdly, CK2 function in chromatin remodeling processes is further supported by the observation that the level of CK2 is higher in the active than in inactive nucleosomes from normal prostate (Guo et al., 1998), and that CK2 co-localizes with productively transcribing RNA pol II on polytene chromosomes of Chironomus salivary gland cells (Egyhazi et al., 1999). CK2 can be recruited to the promoter by either complexing with histones and general chromatin remodeling factors (Gavin et al., 2002; Ho et al., 2002; Krogan et al., 2002), or interacting with other transcription factors such as ATF1, CREB, c-Fos or c-Jun through the basic leucine-zipper domains (Yamaguchi et al., 1998). Thus, CK2 may participate in promoting the conformational transition of

inactive nucleosomes to the active form and/or in the function of transcriptionally active nucleosomes.

2.3.2. The role of CK2 in protein-coding gene expression

Numerous lines of evidence have established that CK2 plays an important role in regulating protein-coding gene transcription by RNA pol II. A comparative genome-wide expression analysis of *Saccharomyces cerevisiae* CK2 deletion strains and corresponding wild types were performed by Barz and coworkers (2003). Their studies showed that of roughly 900 gene products proposed to be involved in cell cycle regulation, the expression of 283 protein-coding genes depend on or are affected by CK2 (Barz et al. 2003). CK2 not only phosphorylates a large number of transcription factors or effectors of DNA/RNA structure or translational elements (Meggio and Pinna, 2003), which are important for regulating protein-coding gene expression, but also directly targets the basal transcriptional machinery. For example, CK2 phosphorylates two subunits of RNA pol II [214,000 and 20,500 Daltons (Da)], and these modifications are required for transcriptional activity (Dahmus, 1981). The general transcription factors TFIIA, TFIIE, and TFIIF are also phosphorylated by CK2. Pre-initiation complex (PIC) formation on the Ad-MLP promoter was stimulated by CK2 phosphorylation of TFIIA, TFIIF, and RNA pol II. However, the mRNA synthesis was dramatically inhibited by CK2 phosphorylation of RNA pol II (Cabrejos et al., 2004). One possibility is that CK2 phosphorylation of RNA pol II increases the formation of a PIC but inhibits RNA pol II recycling. Indeed, CK2 also functions to recruit a CTD-specific phosphatase FCP1 to the transcriptional machinery to facilitate RNA pol II recycling. CK2 phosphorylation of

FCP1 stimulates its phosphatase activity and enhances the interaction between TFIIF subunit RAP74 and FCP1 (Egyhazi et al., 1999; Abbott et al., 2005). CK2 has been shown to associate with the RNA pol II elongation complex (Krogan et al., 2002). Also, a study with TATA-less DPE (downstream promoter element)-dependent transcription indicates that CK2 along with the coactivator PC4 is required for DPE-specific transcription (Lewis et al., 2005). Moreover, activation of pre-mRNA splicing by human RNPS1 (RNA-binding protein prevalent during S phase) is regulated by CK2 phosphorylation (Trembley et al., 2005). Taken together, these data strongly suggest that CK2 plays a role in regulating protein coding gene expression.

2.3.3. The role of CK2 in non-coding gene transcription

Non-coding RNAs trancribed by RNA polymerases I, II, and III play important catalytic and structural roles in the RNA processing and protein synthesis. Research from numerous labs shows that CK2 is involved in controlling transcription of non-coding genes, such as RNA pol I-specific rRNA (Saez-Vasquez et al., 2001), RNA pol III-specific tRNA and 5S rRNA (Hockman and Schultz, 1996; Ghavidel et al., 1999; Johnston et al., 2002), and RNA pol III-specific U6 snRNA (Hu et al., 2003; Hu et al., 2004).

RNA pol I holoenzyme is a multi-subunit complex with at least 2MD in mass and is composed of 30 or more polypeptides (Seither et al., 1998; Albert et al., 1999; Hannan et al.,1999). RNA pol I transcribes the tandemly repeated genes that encode the precursor of 18S, 5.6S and 28S rRNAs. Initially, transcription factor UBF (Upstream Binding Factor) binds to upstream promoter element (UPE), and SL1/TIF-IB (promoter selectivity

factor or transcription initiation factor IB) recognizes the core promoter element (CPE). SL-1 and UBF function cooperatively in the formation of the initiation complex which then facilitates RNA pol I transcription. It has been reported that CK2 copurifies with epitope-tagged RNA pol I from mammalian cells (Hannan et al., 1998). Also, the CK2 activity associates stably with the rRNA promoter in plants (Saez-Vasquez et al., 2001). CK2 targets the key DNA-binding transcription factor UBF in the C-terminal hyperacidic tail, which is essential for transactivation (O'Mahony et al., 1992; Voit et al., 1992; Voit et al., 1995).

CK2 is also involved in RNA pol III transcription of tRNA and 5S rRNA (Hockman and Schultz, 1996; Ghavidel et al., 1999; Johnston et al., 2002). The general transcriptipn factors TFIIIA, Brf1-specific TFIIIB, and TFIIIC are required for tRNA and 5S rRNA gene transcription by RNA pol III. It has been reported that endogenous human CK2 associates stably with TFIIIB and phosphorylates the BRF component of Brf1-specific TFIIIB (Johnston et al., 2002). CK2 can also target the TBP subunit of Brf1-specific TFIIIB for phosphorylation (Ghavidel and Schultz, 1997; Ghavidel et al., 1999; Ghavidel and Schultz, 2001). Furthermore, recent studies showed that RNA pol III-specific U6 snRNA gene transcription is also regulated by CK2 (Hu et al., 2003, Hu et al., 2004). CK2 occupies U6 snRNA gene promoters, and CK2 phosphorylation of the RNA pol III holoenzyme stimulates U6 transcription (Hu et al., 2003). CK2 also targets the Bdp1 component of Brf2-specific TFIIIB complex during mitosis. Phosphorylated Bdp1, in turn, disassociates from the U6 promoter, and thus downregulating U6 transcription in a cell cycle dependent fashion (Hu et al., 2004). Taken together, these data strongly

suggest that CK2 plays an important role in regulating non-coding gene transcription by RNA pol I and pol III.

As mentioned previously, some non-coding snRNA genes are transcribed by RNA pol II. Whether CK2 plays a role in regulating RNA pol II-specific snRNA gene transcription is unknown. Because CK2 appears to globally regulate non-coding gene transcription and human U1 and U6 snRNA genes share similar promoter elements and their gene products have related functions, it is reasonable to hypothesize that CK2 also regulates U1 snRNA gene transcription by RNA pol II. Thus, the major focus of this thesis has been to investigate the role of CK2 in U1 snRNA gene transcription.

3. Main hypothesis and experimental design

The main objective of this doctoral thesis research is to explore the role of CK2 for snRNA gene transcription and to investigate the mechanism of CK2 regulation. To directly test this idea, CK2 promoter occupancy was detected by a chromatin immunoprecipitation (ChIP) assay. Nascent U1 snRNA transcripts in Hela cells were monitored by reverse transcriptase polymerase chain reaction (RT-PCR) following endogenous CK2 depletion by RNAi. U1 snRNA gene transcription in the presence of CK2 and/or CK2 inhibitors was employed *in vitro* to understand the mechanism of CK2 regulation. The general transcription factor $SNAP_C$ was identified as a potential CK2 target by an *in vitro* kinase assay and mass spectrometry (MS). The ability of $SNAP_C$ to bind DNA and to recruit TBP in the presence of CK2 was tested using an electrophoretic mobility shift assay (EMSA).

In the current studies, we demonstrated that CK2 downregulates U1 snRNA gene transcription by RNA pol II. Decreased levels of endogenous CK2 correlates with increased U1 expression while CK2 associates with U1 gene promoters, indicating that it plays a direct role in U1 gene regulation. CK2 phosphorylates the general transcription factor $SNAP_C$ that is required for both RNA pol II and III transcription. $SNAP_C$ phosphorylation by CK2 inhibits binding to snRNA gene promoters. However, restricted promoter access by phosphorylated $SNAP_C$ can be overcome by cooperative interactions with TBP at the U6 promoter but not at a U1 promoter. We have further determined that within SNAP190, CK2 phosphorylates the N-terminal half of SNAP190 at two regions that contain multiple consensus CK2 sites (amino acid 20-63 and 518-557) by mass spectrometric analysis. To test whether these regions are important for $SNAP_C$ function, partial $SNAP_C$ complexes containing full length SNAP43, full length SNAP50, and various truncated SNAP190 molecules [SNAP190 (1-719), SNAP190 (63-719), SNAP190 (1-505), or SNAP190 (63-505)] were co-expressed and tested for DNA binding and function in transcription. All four complexes maintain normal DNA binding activity in electrophoretic mobility shift assays. Furthermore, these complexes all support U1 and U6 snRNA gene transcription using $SNAP_C$ depleted HeLa nuclear extracts *in vitro*, indicating that these regions are not essential for $SNAP_C$ activity. However, the SNAP190 (1-719)-containing $SNAP_C$ complex shows less DNA binding and transcription activity upon CK2 treatment, while the SNAP190 (1-505)-containing $SNAP_C$ complex shows no change under this condition. Thus, SNAP190 phosphorylation by CK2 plays a negative regulatory role on $SNAP_C$ function.

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CHAPTER TWO

COOPERATION BETWEEN $SNAP_C$ AND TBP ANTAGONIZES PROTEIN KINASE CK2 INHIBITION OF DNA BINDING BY $SNAP_C$

Abstract

Protein kinase CK2 regulates RNA polymerase III transcription of human U6 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 while CK2 associates with U1 gene promoters, indicating that it plays a direct role in U1 gene regulation. CK2 phosphorylates the general transcription factor $SNAP_C$ that is required for both RNA polymerase II and III transcription, and $SNAP_C$ phosphorylation inhibits its binding to snRNA gene promoters. However, restricted promoter access by phosphorylated $SNAP_C$ can be overcome by cooperative interactions with TBP 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.

This work was published as the following manuscript: Gu L, Esselman WJ, Henry RW. (2005) Cooperation between $SNAP_C$ and TBP antagonizes protein kinase CK2 inhibition of DNA binding by $SNAP_C$. J Biol Chem. 2005 Jun 14; [Epub ahead of print]

Introduction

Protein kinase CK2 is an important regulator of cellular growth (Guerra and Issinger, 1999; Meggio and Pinna, 2003; Pinna, 2002; Pinna and Meggio, 1997) and abnormal CK2 activity may contribute to tumor progression (Tawfic et al., 2001). CK2 is a tetrameric enzyme composed of two catalytic subunits α and α' and two copies of the regulatory β subunit (Niefind et al., 2001). 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 CK2α' subunit is shifted to a restrictive temperature (Hanna et al., 1995). This decline in total RNA synthesis also suggests that expression of highly transcribed genes encoding ribosomal (r), transfer (t), and small nuclear (sn) RNAs is sensitive to levels of functional CK2.

In yeast, CK2 is important for active RNA polymerase III transcription (Hockman and Schultz, 1996), and yet paradoxically, CK2 has been proposed to be the terminal effector in a DNA damage response pathway that represses RNA polymerase III transcription (Ghavidel and Schultz, 2001). 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 (Hu et al., 2004). The nature of the regulation is dictated by CK2 target selection. One key target for CK2 is the general transcription factor TFIIIB (Ghavidel and Schultz, 1997; Hu et al., 2003; Johnston et al., 2002). There are at least two versions of human TFIIIB that function for transcription of distinct classes of genes (Schramm and Hernandez, 2002). The Brf1-TFIIIB complex functions for 5S rRNA and tRNA transcription and is composed of the TATA-box binding protein (TBP) and the TBP associated factors, Bdp1

and Brf1. The Brf2-TFIIIB complex functions for U6 snRNA transcription and is composed of TBP plus Bdp1 but Brf2 instead of Brf1. Brf1-TFIIIB phosphorylation during M-phase results in the selective release of Bdp1 from tRNA promoters (Fairley et al., 2003). Hernandez and colleagues further demonstrated that Bdp1 is the critical CK2 target within Brf2-TFIIIB for mitotic repression of U6 transcription (Hu et al., 2004). As Bdp1 is a shared component of both TFIIIB complexes, CK2 may target this factor to repress global RNA polymerase III transcription. However, CK2 inhibitors also interfere with Brf1-TFIIIB binding to the TFIIIC complex (Johnston et al., 2002), which itself recognizes intragenic promoter elements of 5S 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 (Johnston et al., 2002), and in this process may additionally phosphorylate RNA polymerase III itself (Hu et al., 2003). 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 (Henry et al., 1998a; Hernandez, 2001; Lobo and Hernandez, 1994). 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 TFIIIB and thus rely on other factors for regulatory intervention. Regardless of polymerase specificity, all human snRNA genes contain a distal sequence element (DSE) encompassing an octamer element that is

recognized by Oct-1. Additional sites for the Sp1 (Ares et al., 1987) and STAF (Schaub et al., 1997) transcriptional activator proteins are adjacently located to the DSE at some snRNA genes (Hernandez, 1992). Oct-1 activates snRNA transcription by direct protein contacts (Ford et al., 1998; Hovde et al., 2002; Mittal et al., 1999) with the basal transcription factor called the snRNA activating protein complex ($SNAP_C$) (Sadowski et al., 1993), which is also referred to as the proximal sequence element transcription factor (PTF) (Murphy et al., 1992). $SNAP_C$ 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 (Henry et al., 1998a; Henry et al., 1996; Henry et al., 1998b; Henry et al., 1995; Sadowski et al., 1996; Sadowski et al., 1993; Wong et al., 1998). $SNAP_C$ contains at least five proteins called SNAP190 (PTFα), SNAP50 (PTFβ), SNAP45 (PTFδ), SNAP43 (PTFγ), and SNAP19 (Bai et al., 1996; Henry et al., 1996; Henry et al., 1998b; Henry et al., 1995; Sadowski et al., 1996; Wong et al., 1998; Yoon and Roeder, 1996). 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 $SNAP_C$ (Ma and Hernandez, 2001; Ma and Hernandez, 2002). Once the complex is assembled, SNAP190 further recognizes the PSE through its Myb DNA binding domain (Wong et al., 1998) and is also the direct target for Oct-1 (Ford et al., 1998; Mittal et al., 1996). In an unexpected twist, SNAP190 can make DNA contacts within the U1 DSE 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 (Hovde et al., 2002).

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 (Lobo and Hernandez, 1989). The TATA box is recognized by the TBP component of the Brf2-TFIIIB complex (Cabart and Murphy, 2001; Cabart and Murphy, 2002; Hinkley et al., 2003; Ma and Hernandez, 2002; Zhao et al., 2003). $SNAP_C$, through its SNAP190 subunit, stimulates TBP binding to the U6 TATA box as an early critical step in RNA polymerase III transcription (Hinkley et al., 2003; Ma and Hernandez, 2002).

TBP is also required for human snRNA gene transcription by RNA polymerase II (Sadowski et al., 1993), 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 $SNAP_C$ (Henry et al., 1995), 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 (Sadowski et al., 1993). 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 pre-initiation 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 (Kuhlman et al., 1999). 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 while TFIIF phosphorylation can stimulate RNA polymerase II elongation. In

contrast, CK2 phosphorylation of RNA polymerase II inhibits transcription, potentially by impairing elongation (Cabrejos et al., 2004).

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 architectures.

Materials and Methods

Chromatin immunoprecipitation assays

ChIP assays from HeLa cells were performed using the anti-CK2α (Ab245), anti-CK2β (Ab278) antibodies (Yu et al., 1991) as well as anti-SNAP43 (CS48) (Henry et al., 1995) and anti-TBP antibodies described previously (Hirsch et al., 2004). Enrichment of genomic sequences in the immunoprecipitation reactions was measured by PCR as previously described (Hirsch et al., 2004).

RNAi

CK2α and CK2α′ cDNA were generated with a T7 promoter at both ends by RT-PCR using total RNA from HeLa cells as a template. The primers for CK2α are CK2α

forward 5 -GCGTAATACGACTCACTATAGGAAATAATGAAAAAGTTGTTG-3 , and CK2α reverse 5 -GCGTAATACGACTCACTATAGGCTCTTGCAGTAAGCCGT GAC-3 . The primers for CK2α′ are CK2α′ forward 5 -GCGTAATAC GACTCACTAT AGGCAACAATGAGAGAGTGGTTG-3 , and CK2α′ reverse 5 -GCGTAATACGACT CACTATAGGTCTGTTGATGGTCGTATCGC-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 - TTAATACGACTCACTATAGGGAGACGATAACCACCACGCT CATCG-3 , and lacZ reverse 5 - TTAATACGACTCACTATAGGGAGAGCGTTACC CAACTTAATCGCC-3 . Resultant cDNAs were subjected to *in vitro* transcription with T7 polymerase to produce double stranded (ds) RNA. After DNase I treatment, dsRNA was incubated with recombinant Dicer and resultant Dicer generated small interfering RNA (d-siRNA) were purified according to the manufacturer's instructions (Invitrogen). Approximately 250 ng of d-siRNA for lacZ, CK2α, or CK2α plus 250 ng CK2α' d-siRNA were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen). Cells were harvested 30 hr later and total RNA was extracted using TriZol (Gibco). Reverse transcription (RT)-PCR was carried out using Titan One Tube RT-PCR System (Roche). The primers used to amplify U1 primary transcript are U1prim forward 5 - ACTTGCTGCTTCACCACGAA-3 , and U1prim reverse 5 - ACAGCCTCATACGCCTCACT-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 (Lobo and Hernandez, 1989), with the following modifications. The HeLa cell nuclear extracts were pre-incubated with Dignam buffer D either with or without recombinant CK2 and kinase inhibitors for 60 min at 30°C prior to initiating transcription by addition of transcription buffers, nucleoside triphosphates, and DNA templates. The amounts of recombinant CK2, and kinase inhibitors used are indicated in the figure legend. Transcripts were separated by denaturing PAGE and visualized by PhosphorImager analysis (Molecular Dynamics).

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 Figure II-3 A, 60 and 180 μL of HeLa cell nuclear extract (~10 mg/mL) was incubated with 20 μL of rabbit anti-SNAP43 (CS48) (Henry et al., 1995), anti-SNAP190 (CS398, CS402) (Henry et al., 1995), anti-CK2α (AB245) (Yu er al., 1991) or pre-immune antibodies covalently coupled to protein-G agarose beads. Recovered proteins were analyzed by Western blot using a mouse mAb against CK2α (Transduction Laboratories). For Figure II-3 B, 40 μL 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 $MgCl_2$, 10% glycerol, 0.5% Tween-20, 150 mM KCl), the beads were suspended in 40 μL of HEMGT-150 buffer containing 2 μL of $\gamma[^{32}P]$-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 Figure II-3 C, 100 μL of HeLa cell nuclear extracts were used for immunoprecipitation. After kinase reactions, proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. Radiolabeled proteins were detected first by autoradiography. Subsequently, Western blot analyses were performed using anti-SNAP190 (CS402) antibodies.

For Figure II-5B and 5C, approximately 5 μg of GST-SNAP190 (1-719) was bound to glutathione agarose beads (10 μL). Immobilized GST-SNAP190 (1-719) was incubated with 10 μL 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 $\gamma[^{32}P]$-ATP or $\gamma[^{32}P]$-GTP with or without D-ribofuranosyl benzimidazole (DRB; Sigma) or 3,3 ,4 ,5,7-pentahyroxyflavone (quercetin; Sigma).

Tryptic phosphopeptide mapping

To obtain material for thin layer chromatography (TLC) analysis, approximately 1 μg GST-SNAP190 (1-719) was labeled with $\gamma[^{32}P]$-ATP by using HeLa cell nuclear extracts or recombinant CK2. Phosphorylated GST-SNAP190 (1-719) was gel purified prior to 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/dH_2O, 25:78:897, v/v/v) and in the second dimension by chromatography in chromatography buffer (n-butanol/pyridine/ glacial acetic acid/dH_2O, 15:10:13:12, v/v/v/v) prior to detection by PhosphorImager analysis (van der Geer and Hunter, 1994).

Phosphoamino Acid Analysis

Endogenous SNAP190 was immunoprecipitated, phosphorylated *in vitro* in the presence of 2 μL of $\gamma[^{32}P]$-ATP (6000 Ci/mmol, 150 mCi/mL), separated by 7.5% SDS-PAGE, and transferred to nitrocellulose membrane. The ~190 kDa radioactive protein corresponding to SNAP190 was excised and hydrolyzed in 5.7N HCl for 1 hr at 100°C.

Recovered amino acids were vacuum dried and dissolved in 10 μL pH 1.9 buffer containing unlabeled phosphoserine, phosphothreonine and phosphotyrosine mixture. The mixture was separated by one-dimensional electrophoresis (500V) on cellulose thin layer chromatography (TLC) plates (Eastman Kodak Co.) for 1 hr 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 TBP were preincubated alone or with 15 or 150 units CK2 and/or 7 mM ATP for 30 min at 30°C. EMSAs 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 (Hinkley et al., 2003; Mittal and Hernandez, 1997).

Results

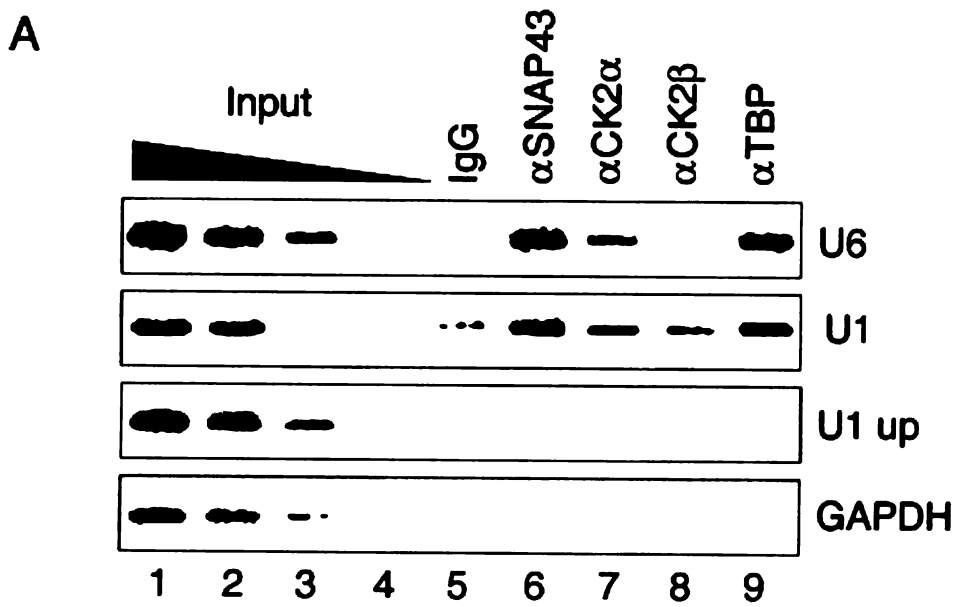
CK2 inhibits U1 snRNA gene transcription.

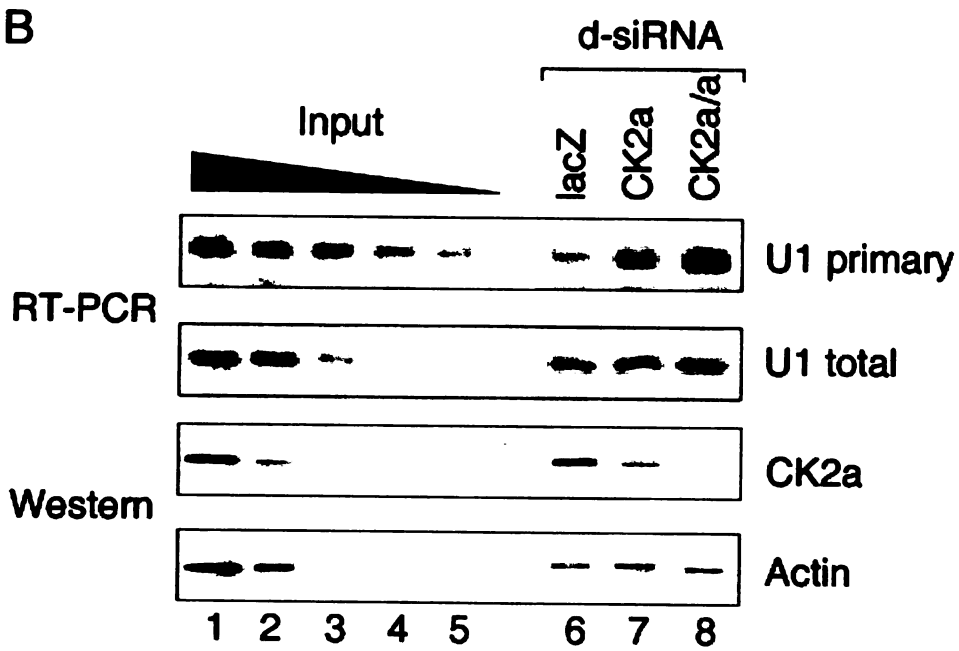
The conservation of similar promoter architectures among the human snRNA gene family suggests that these genes could be coordinately regulated. That CK2 regulates human U6 snRNA gene transcription by RNA polymerase III (Hu et al., 2003)

prompted us to examine whether CK2 similarly regulates human snRNA gene transcription by RNA polymerase II. First, chromatin immunoprecipitation (ChIP) experiments were performed to determine whether endogenous CK2 could associate with the promoter regions of both U6 and U1 snRNA genes. As shown in Figure II-1 A, both U6 and U1 promoter regions were enriched in immunoprecipitation reactions using anti-CK2α antibodies (lane 7), while U1 but not U6 promoter regions were enriched in the anti-CK2β immunoprecipitation reactions (lane 8). Similar results were obtained in experiments performed with different antibodies directed against CK2α and CK2β (data not shown). Possibly the epitopes recognized by the CK2β antibodies may be occluded by other transcription factors at the U6 promoter. However, this result stands in contrast with that noted previously (Hu et al., 2003), wherein a more robust CK2β 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 GAPDH 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 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 RNA interference (RNAi), and the effect on U1

Figure II-1. CK2 inhibits U1 snRNA in vivo 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 was compared to recovery of the U1 upstream regions (U1 up) and GAPDH exon 2 (GAPDH), as negative controls. (B) U1 primary transcripts accumulate after CK2 reduction. CK2 levels were reduced by transient transfection of dicer generated small interfering RNA (Myers et al., 2003) corresponding to CK2α (lane 7), or CK2α plus CK2α' (lane 8). Cells were also treated with LacZ d-siRNA as a reference (lane 6). Levels 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 two-fold decreasing increments of material harvested from untreated cells to serve as a standard curve for each assay.


A
Input
IgG
αSNAP43
αCK2α
αCK2β
αTBP
U6
U1
U1 up
GAPDH
1 2 3 4 5 6 7 8 9


B
d-siRNA
Input
lacZ
CK2a
CK2a/a
RT-PCR
U1 primary
U1 total
Western
CK2a
Actin
1 2 3 4 5 6 7 8

snRNA production was monitored by RT-PCR (Figure II-1 B). As a negative control, RNAi was also performed using lacZ-specific RNA. As it was demonstrated that phosphorylation of the carboxy terminal domain (CTD) of RNA polymerase II contributes to 3 processing of human U2 snRNA (Jacobs et al., 2004; Medlin et al., 2003), 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) shows 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 CK2α RNAi (lane 7) and this effect was enhanced by RNAi directed against both CK2α and CK2α' (lane 8) as compared to 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 approximately 2-4 fold relative to the levels observed in the LacZ-RNAi treated 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.

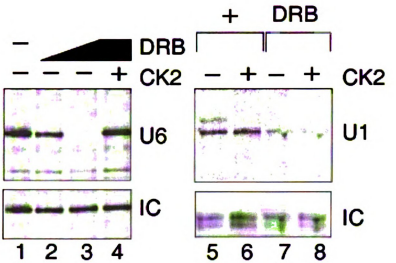
To determine whether CK2 plays a direct role in U1 snRNA gene transcription, recombinant CK2 and commonly used inhibitors of CK2 were tested for their effect using in vitro U1 transcription assays. One characteristic of CK2 is that it can be inhibited by both DRB and quercetin, and therefore, these inhibitors were selected. As a positive

control for CK2 activity, in vitro U6 transcription was also examined (Figure II-2 A). First, U6 transcription was inhibited with increasing amounts of the kinase inhibitor DRB (lanes 2 and 3) and DRB action was reversed by addition of CK2 (lane 4). Together, these data suggest that CK2 has an overall positive role in U6 transcription, consistent with observations previously described (Hu et al., 2003). No measurable effect on U1 transcription was observed by 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 addition of CK2 (lane 8), suggesting, 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 CTD of RNA polymerase II (Price, 2000), also contributes positively to efficient U1 transcription in vitro (data not shown).

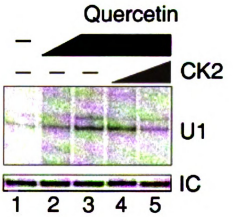
Next, the effect of quercetin in U1 transcription was tested. As shown in Figure II-2 B, and in contrast with DRB, quercetin addition stimulated U1 transcription (lanes 2 and 3). The increased background in reactions containing quercetin indicates this inhibitor may have non-specific positive effects on transcription possibly from cryptic promoters on the reporter plasmid. While quercetin can inhibit a variety of kinases, the increase in U1-specific transcription was reversed by 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 (Jacobs et al., 2004; Medlin et al., 2003), suggesting that CK2 is not involved in the transcription of these

Figure II-2. CK2 represses in vitro 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 μM DRB; lanes 3 and 4, 7 μM 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 μM DRB (lanes 7 and 8). (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 μM (lanes 2) and 7 μM quercetin (lanes 3-5), respectively, while an additional 10 and 100 units of CK2 were added to reactions shown in lanes 4 and 5, respectively.

A



B



genes. The possibility remains that U1 and U2 gene transcription are differentially sensitive to regulation by CK2. Nonetheless, as 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.

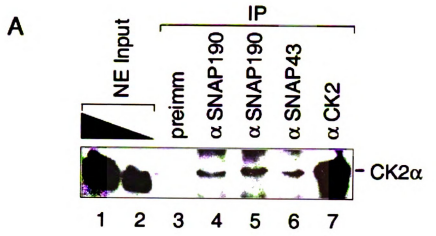
Endogenous CK2 targets SNAP190 for phosphorylation at multiple sites.

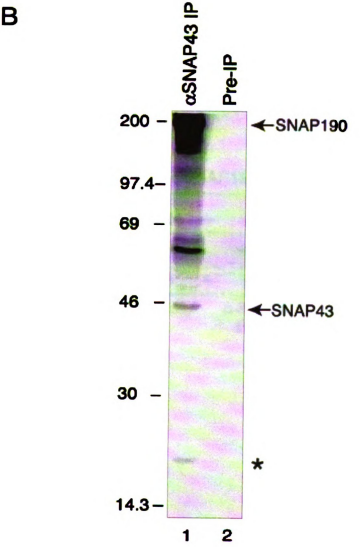
$SNAP_C$ recognizes the core promoters of human snRNA genes and plays an important early role in coordinating transcription of snRNA genes by both RNA polymerases II and III. The findings that CK2 can affect both human U1 and U6 transcription (Hu et al., 2003) implicates $SNAP_C$ as a potential target for CK2. First, we examined whether CK2 co-purifies with $SNAP_C$. As shown in Figure II-3 A, 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 CK2α (lane 7), suggesting that only a minor proportion of CK2 is associated with $SNAP_C$ 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 immuno-precipitated

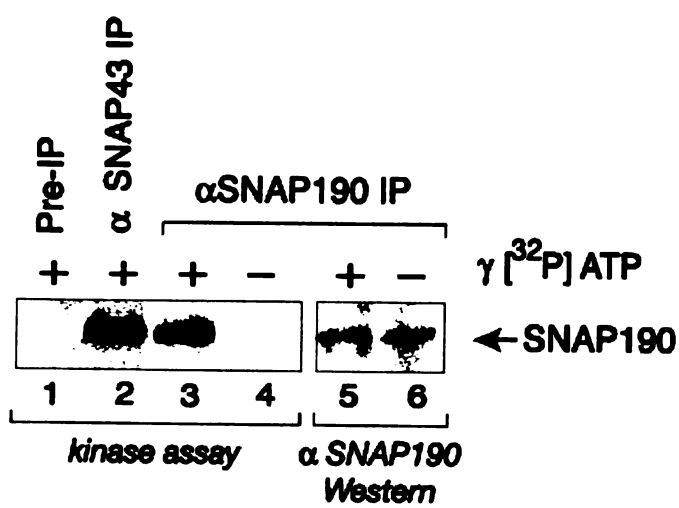
Figure II-3. Endogenous CK2 associates with $SNAP_C$. (A) HeLa cell nuclear extracts were immunoprecipitated with pre-immune (lane 3), anti-SNAP190 (lane 4, CS398; lane 5, CS402), anti-SNAP43 (lane 6, CS48), or anti-CK2α (lane 7, Ab245) antibodies, as indicated. Recovered proteins were analyzed by Western blot analysis using antibodies directed against the α subunit of CK2. Lanes 1 and 2 contain 3 and 1 μL 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 pre-immune (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 2 μL of $\gamma[^{32}P]$-ATP (6000 Ci/mmol, 150 mCi/mL). 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 pre-immune (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 $\gamma[^{32}P]$-ATP. Proteins were separated by 7.5% SDS-PAGE and transferred to nitrocellulose membrane. Radiolabeled proteins were detected by autoradiography for 1 hour (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 seconds. The position of SNAP190 is indicated.

*The experiment in B was performed by RW Henry.


A
IP
NE Input
preimm
α SNAP190
α SNAP190
α SNAP43
α CK2
CK2α
1 2 3 4 5 6 7


B
αSNAP43 IP
Pre-IP
200
97.4
69
46
30
14.3
SNAP190
SNAP43
*
1 2

C


Pre-IP
α SNAP43 IP
αSNAP190 IP
+ + + − + − γ [32P] ATP
←SNAP190
1 2 3 4 5 6
kinase assay
α SNAP190 Western

proteins were directly assayed for kinase activity by incubation with 2 μL of γ[^{32}P]-ATP (6000 Ci/mmol, 150 mCi/mL). As shown in Figure II-3 B, 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 Figure II-3 B), proteins of 60 kDa, 43 kDa (labeled SNAP43) and 19 kDa (labeled *) 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 (Figure II-3 C). 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[^{32}P]-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[^{32}P]-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 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 assay. Taken together, these results demonstrate that a $SNAP_C$-associated kinase phosphorylates SNAP190 and SNAP43.

To determine whether endogenous $SNAP_C$ is phosphorylated, anti-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 Figure II-4, a protein of approximately 190 kDa was detected in the anti-SNAP43 immunoprecipitated sample (lane 5) that was not observed in a similarly immuno-precipitated sample treated with phosphatase (lane 6), indicating that this protein is phosphorylated. The 190 kDa protein was not detected in the sample recovered by immunoprecipitation with non-specific antibodies (lane 4). Furthermore, the 190 kDa protein co-migrated with HA-tagged SNAP190 recovered by anti-HA immuno-precipitation 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 immuno-precipitated 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

Figure II-4. Endogenous $SNAP_C$ is phosphorylated. HeLa cell nuclear extracts were immuno-precipitated by either pre-immune (lanes 1 and 4) or anti-SNAP43 antibodies (lanes 2-3 and 5-6). The protein complex recovered by anti-SNAP43 immunoprecipitation was left untreated (lanes 2 and 5) or dephosphorylated by calf intestine alkaline phosphatase (lanes 3 and 6). As an additional size marker, HA-SNAP190 was over expressed in HeLa cells by transient transfection and was immuno-precipitated 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).

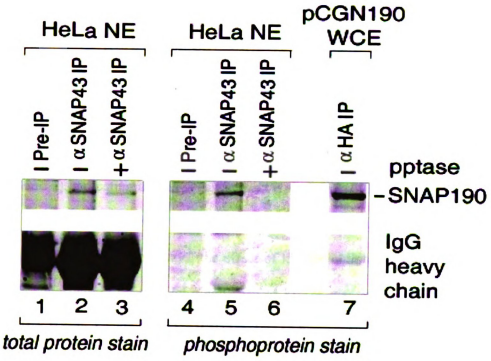
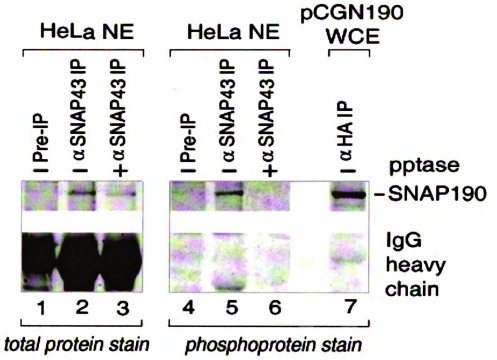

HeLa NE
HeLa NE
pCGN190
WCE
I Pre-IP
I α SNAP43 IP
+α SNAP43 IP
I Pre-IP
I α SNAP43 IP
+α SNAP43 IP
I α HA IP
pptase
-SNAP190
IgG
heavy
chain
1 2 3
4 5 6 7
total protein stain
phosphoprotein stain

Figure II-4. Endogenous $SNAP_C$ is phosphorylated. HeLa cell nuclear extracts were immuno-precipitated by either pre-immune (lanes 1 and 4) or anti-SNAP43 antibodies (lanes 2-3 and 5-6). The protein complex recovered by anti-SNAP43 immunoprecipitation was left untreated (lanes 2 and 5) or dephosphorylated by calf intestine alkaline phosphatase (lanes 3 and 6). As an additional size marker, HA-SNAP190 was over expressed in HeLa cells by transient transfection and was immuno-precipitated 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).


HeLa NE
HeLa NE
pCGN190
WCE
I Pre-IP
I αSNAP43 IP
+αSNAP43 IP
I Pre-IP
I αSNAP43 IP
+αSNAP43 IP
I αHA IP
pptase
-SNAP190
IgG
heavy
chain
1 2 3
4 5 6 7
total protein stain
phosphoprotein stain

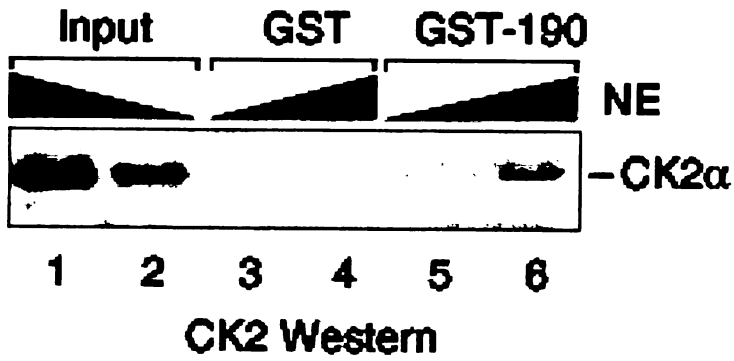
phosphorylated in vivo or the recovered levels of phosphorylated protein in these assays were below the threshold of detection using the Pro-Q diamond stain.

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 (Figure II-5 A). Significant amounts of CK2 associated with GST-SNAP190 (1-719) 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.

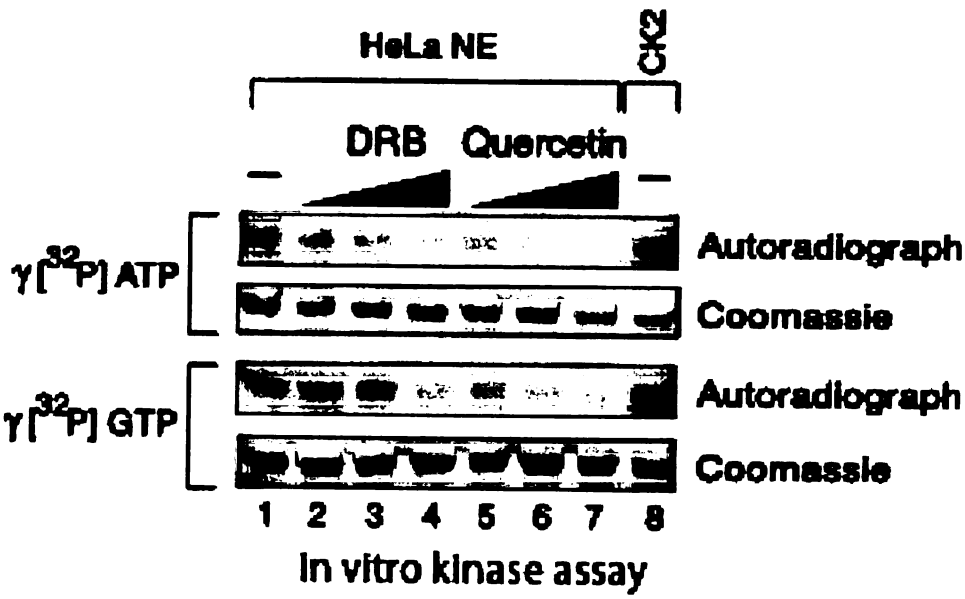
To investigate whether CK2 is the predominant kinase that associates with GST-SNAP190 (1-719) in these assays, the effect of kinase inhibitors on GST-SNAP190 (1-719) phosphorylation by the HeLa cell-derived kinase was investigated. As shown in Figure II-5 B (top panel), both DRB (lanes 2-4) and quercetin (lanes 5-7) were effective in limiting the extent of GST-SNAP190 (1-719) phosphorylation as compared to 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.

Figure II-5. Endogenous CK2 phosphorylates the N-terminal region of SNAP190. (A) Endogenous CK2 can interact with SNAP190 (1-719). Approximately 3 μg 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 and 180 μL of HeLa cell nuclear extract. Associated proteins were separated by 12.5% SDS-PAGE for anti-CK2α Western analysis. Lanes 1 and 2 contain 3 and 1 μL 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 pre-treated with HeLa cell nuclear extracts, washed, and was then incubated with either γ[^{32}P]-ATP (*top panel*) or γ[^{32}P]-GTP (*bottom panel*) in the presence of 1, 7, 50 μM DRB (lanes 2, 3 and 4, respectively) or 1, 7, 50 μM 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 prior to 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, phosphothreonine and phosphotyrosine. The mixture was separated by one dimensional thin layer electrophoresis 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).

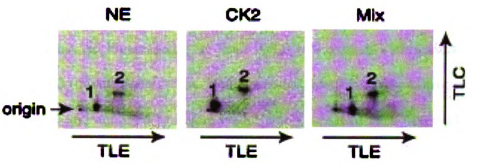
A


Input
GST
GST-190
NE
CK2α
1 2 3 4 5 6
CK2 Western

B

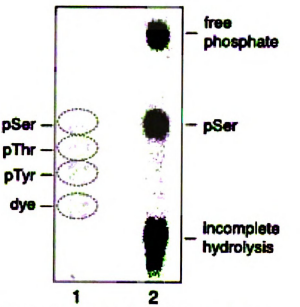

HeLa NE
CK2
DRB
Quercetin
γ[32P] ATP
Autoradiograph
Coomassie
γ[32P] GTP
Autoradiograph
Coomassie
1 2 3 4 5 6 7 8
In vitro kinase assay

C



2D phosphopeptide analysis

D



TLE phosphoamino acid analysis

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 (Figure II-5 C). 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 predominant 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 (Figure II-5 D), which revealed that phosphorylation was occurring predominantly 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.

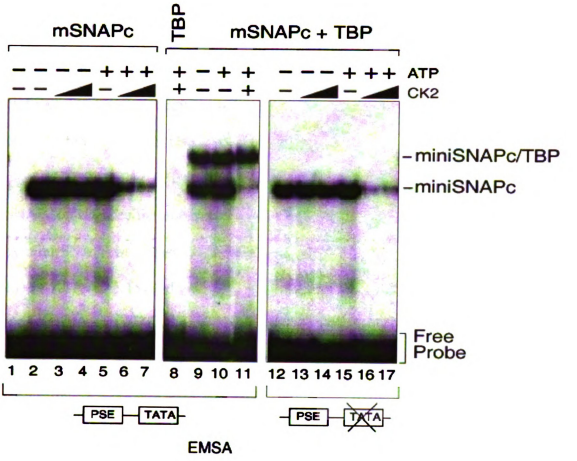
An examination of the amino acid sequence of SNAP190 (1-719) revealed a total of 13 CK2 consensus motifs containing serines, most of which are clustered around regions involved in cooperative promoter recognition by $SNAP_C$ in the presence of TBP (Hinkley et al., 2003; Ma and Hernandez, 2002). Other CK2 sites are contained within

the Myb DNA binding domain of SNAP190, in particular, the Rh and Ra Myb repeats (Wong et al., 1998). As CK2 had previously been shown to inhibit DNA binding by other Myb-domain proteins (Luscher et al., 1990), 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 $SNAP_C$, EMSAs 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 is competent for both U1 and U6 promoter binding. As shown in Figure II-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 (Mittal and Hernandez, 1997). 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,

Figure II-6. TBP and $SNAP_C$ cooperate at U6 but not at U1 promoter probes to overcome CK2 inhibition of DNA binding by $SNAP_C$. EMSA was performed using U6-like (wtPSE, wtTATA) or U1-like (wtPSE, muTATA) 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 mini-$SNAP_C$/DNA and mini-$SNAP_C$/TBP/DNA complexes are indicated.


mSNAPc
TBP
mSNAPc + TBP
ATP
CK2
miniSNAPc/TBP
miniSNAPc
Free
Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
PSE
TATA
PSE
TATA

EMSA

formation of a super shifted complex is observed with mini-$SNAP_C$ and TBP in reactions performed without CK2 and ATP (lane 9). 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). Therefore, 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 (Henry et al., 1996; Henry et al., 1998b; Henry et al., 1995; Sadowski et al., 1996; Wong et al., 1998). Our data demonstrates 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 (Hu et al., 2003), 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 (Hu et al., 2004), 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 (Hu et al., 2004). 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 non-productive pre-initiation 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 (Wong et al., 1998) and CK2 was shown previously to inhibit DNA binding by the c-Myb nuclear oncoprotein (Luscher et al., 1990). That phosphorylated $SNAP_C$ can bind DNA cooperatively with TBP in reactions wherein $SNAP_C$ is unable to bind DNA alone (Figure II-6) argues that most $SNAP_C$ in these reactions is phosphorylated and 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 (Ma and Hernandez, 2002). 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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CHAPTER THREE

CK2 PHOSPHORYLATION OF SNAP190 NEGATIVELY REGULATES $SNAP_C$ FUNCTION

Abstract

Human small nuclear RNAs play fundamental roles in regulating protein-coding gene transcription and in processing of other RNAs. The general transcription factor snRNA activating protein complex ($SNAP_C$) is required for all human snRNA gene transcription. In our previous study discussed in chapter 2, we showed that SNAP190, the largest subunit of $SNAP_C$, is phosphorylated *in vivo* and can be phosphorylated *in vitro* by the protein kinase CK2. In this report we investigate further the role of CK2 in regulating the transcriptional properties of $SNAP_C$ and demonstrate that $SNAP_C$ DNA binding and transcription activity is inhibited by CK2 phosphorylation. Within SNAP190, CK2 phosphorylates the N-terminal half of SNAP190 at two regions that contain multiple CK2 consensus sites (amino acid 20-63 and 514-545) as determined by mass spectrometric analysis. Partial SNAP complexes containing full-length SNAP43, full-length SNAP50, and various truncated SNAP190 molecules [SNAP190 (1-719), SNAP190 (63-719), SNAP190 (1-505), SNAP190 (63-505)] were co-expressed and tested for function in DNA binding and transcription. All four complexes maintain normal DNA binding activity in electrophoretic mobility shift assays. Furthermore, these complexes all support U1 and U6 snRNA gene transcription using $SNAP_C$ depleted HeLa nuclear extracts *in vitro*, indicating that these regions are not essential for $SNAP_C$ activity.

Moreover, we have further determined that the region of SNAP190 (506-719) harboring one set of the CK2 sites is required for CK2 inhibition, suggesting that CK2 phosphorylation of SNAP190 in the region of amino acid (514-545) negatively regulates $SNAP_C$ function by inhibiting DNA binding by the adjacent DNA binding domain within SNAP190.

Introduction

Human snRNA activating protein complex ($SNAP_C$) is a basal transcription factor required for snRNA gene transcription by both RNA pol II and pol III (Sadowski et al., 1993; Waldschmidt et al., 1991; Meissner et al., 1995; Murphy et al., 1992). $SNAP_C$ consists of at least five subunits called SNAP190 (PTFα), SNAP50 (PTFβ), SNAP45 (PTFδ), SNAP43 (PTFγ), and SNAP19 (Wong et al., 1998; Henry et al., 1996; Bai et al., 1996; Sadowski et al., 1996; Yoon and Roeder, 1996; Henry et al., 1995; Henry et al., 1998). The largest subunit SNAP190 plays a central structural role within $SNAP_C$ for interacting with SNAP45, SNAP43 and SNAP19, while SNAP50 joins the complex by interacting with SNAP43 (Henry et al., 1996; Henry et al., 1998a; Wong et al., 1998; Mittal et al., 1999; Ma and Hernandez, 2001). Upon complex assembly, the Myb DNA binding domain within SNAP190 further recognizes the proximal sequence element (PSE) located in the core promoter region of snRNA gene promoters. This Myb DNA binding domain consists of four complete repeats (RaRbRcRd) and a half repeat (Rh) (Wong et al., 1998) and the RcRd repeats alone have been shown to be sufficient for this recognition (Mittal et al., 1999). Furthermore, SNAP190 is an activation target of a human enhancer-binding factor Oct-1, which binds to an octamer sequence in the distal

sequence element (DSE) located in the regulatory region. The cooperative binding of Oct-1 and $SNAP_C$ is mediated by direct protein-protein contact between SNAP190 and the POU-specific domain of Oct-1 (Mittal et al., 1996; Ford et al., 1998) and protein-DNA contact between SNAP190 and the DNA phosphate backbone within the enhancer (Hovde et al., 2002). A positioned nucleosome that resides between the DSE and the PSE in the natural snRNA promoters is proposed to enhance interaction between Oct-1 and SNAP190 (Zhao et al., 2001). In addition, SNAP190 interacts with TBP to stimulate TBP recognition and TFIIIB recruitment to the neighboring TATA box present in the human U6 snRNA promoter (Ma and Hernandez, 2002; Hinkley et al., 2003). Together, these results suggest that SNAP190 plays a pivotal role in snRNA gene transcription by facilitating direct core promoter recognition, serving as a target for the Oct-1 activator, and coordinating TBP activity and preinitiation complex assembly for both RNA pol II and pol III.

In our previous studies we demonstrated that SNAP190 is phosphorylated both *in vivo* and *in vitro* (Figure II-4 and Figure II-3 B). Protein kinase CK2 associates with and phosphorylates the N-terminal half of SNAP190 at multiple serine residues (Figure II-5). CK2 is distributed ubiquitously in eukaryotic organisms, and predominantly exists in tetrameric complexes consisting of two catalytic subunits and two regulatory subunits. CK2 is an important regulator of cellular growth and proliferation (reviewed in Guerra and Issinger, 1999; Meggio and Pinna, 2003; Pinna, 2002; Pinna and Meggio, 1997). One role for CK2 is to function as a downstream effector of signaling pathways to regulate gene expression. CK2 phosphorylation of transcription factors may govern their DNA-binding activities and transcription properties (Bieker et al., 1998; Armstrong et al.,

1997; Tsutsui et al., 1999). Specifically, CK2 has been shown to directly target the basal transcription machinery. For example, CK2 has been shown to phosphorylate two subunits of RNA pol II (Dahmus, 1981) and the general transcription factors TFIIA, TFIIE, and TFIIF (Cabrejos et al., 2004). CK2 copurifies with epitope-tagged RNA pol I (Hannan et al., 1998) and RNA pol III (Hu et al., 2003). Furthermore, CK2 forms a stable complex with Brf1-specific TFIIIB which is required for tRNA and 5S rRNA gene transcription by RNA pol III. CK2 targets two components of Brf1-TFIIIB including BRF1 (Johnston et al., 2002) and TBP (Ghavidel and Schultz, 1997; Ghavidel et al., 1999; Ghavidel and Schultz, 2001). CK2 phosphorylation of TBP and BRF1 can activate tRNA and 5S rRNA gene transcription by RNA pol III (Johnston et al., 2002; Ghavidel and Schultz, 1997; Ghavidel et al., 1999). In addition, CK2 phosphorylation of RNA pol III holoenzyme stimulates U6 snRNA gene transcription (Hu et al., 2003); however, CK2 downregulates U6 transcription by phosphorylating the Bdp1 component of Brf2-TFIIIB during mitosis (Hu et al., 2004).

The observation that CK2 occupies human U1 gene promoters and decreased levels of endogenous CK2 correlates with increased U1 expression suggests that CK2 plays a direct role in U1 gene regulation (Figure II-1). CK2 targets the general transcription factor $SNAP_C$ for phosphorylation. It is unclear, however, whether CK2 phosphorylation of $SNAP_C$ contributes to U1 and probably U6 snRNA gene regulation.

Here, the role of CK2 in regulating the transcriptional properties of $SNAP_C$ was investigated. Our findings revealed that CK2 phosphorylation of $SNAP_C$ inhibits snRNA gene transcription by both RNA pol II and pol III *in vitro*. CK2 target sites within SNAP190 (1-719) have been identified and are located in the serine rich region (Wong et

al., 1998) downstream of the Myb-DNA binding domain, as well as in the N-terminal region overlapping with TBP-recruitment region 1 (TRR1) (Ma and Hernandez, 2002). Mini-SNAP complexes containing full-length SNAP43, full-length SNAP50, and truncated SNAP190 lacking the N-terminal region (Δaa 1-62) and/or the serine rich region (Δaa 506-719) are capable of directing snRNA gene transcription, indicating that CK2 plays a regulatory role. Further analysis showed that the region of SNAP190 (506-719) is critical for CK2 inhibition, suggesting that CK2 phosphorylation of SNAP190 in the region of amino acid (514-545) plays a negative role in regulating $SNAP_C$ function.

Materials and Methods

Expression and purification of recombinant proteins

The GST-SNAP190 (1-719) protein used for experiments described in Figures III-2 and III-3 was overexpressed in *E. coli* BL21 (DE3) using the vector pSBet-GST-SNAP190 (1-719) and purified by affinity chromatography using glutathione-sepharose beads (Amersham Biosciences) followed by extensive washing in HEMGT-150 buffer (20 mM HEPES [pH 7.9], 0.5 mM EDTA, 10 mM $MgCl_2$, 10% glycerol, 0.1% Tween 20) containing protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium bis-sulfate, 1 mM benzamidine, 1 □M pepstatin A) and 1 mM dithiothreitol. Bound protein was then used directly for *in vitro* kinase assays and phosphopeptide mapping.

The various truncated versions of GST-SNAP190 fusion protein [GST-SNAP190 (1-719), GST-SNAP190 (63-719), GST-SNAP190 (1-505), and GST-SNAP190 (63-505)]

used for experiments described in Figure III-4 B were expressed in *E. coli* BL21 (DE3) using the vectors pSBet-GST-SNAP190 (1-719), pSBet-GST-SNAP190 (63-719), pSBet-GST-SNAP190 (1-505), and pSBet-GST-SNAP190 (63-505), respectively. The proteins were purified for *in vitro* kinase assays by affinity chromatography using glutathione-sepharose beads. Bound proteins were then used directly for *in vitro* kinase assays.

Recombinant mini-SNAP complexes containing full-length SNAP43, full-length SNAP50, and various truncated SNAP190 molecules [SNAP190 (1-719), SNAP190 (63-719), SNAP190 (1-505), and SNAP190 (63-505)] were co-expressed in *E. coli* using the vector combination pET21-His-SNAP43-HA-SNAP50 and pSBet-GST-SNAP190 (1-719), pSBet-GST-SNAP190 (63-719), pSBet-GST-SNAP190 (1-505), or pSBet-GST-SNAP190 (63-505), respectively. Various recombinant mini-SNAP complexes [SNAP190 (1-719)/43/50, SNAP190 (63-719)/43/50, SNAP190 (1-505)/43/50, and SNAP190 (63-505)/43/50, hereafter designated mS, mSΔN, mSΔC, mSΔ(N+C), respectively] were purified by GST tag affinity chromatography and released from the GST-tag by thrombin cleavage. The protein complexes were then dialyzed against Dignam buffer D (Dignam et al., 1990) containing 80 mM KCl prior to *in vitro* transcription assay and EMSAs.

In vitro kinase assays

For the experiment presented in Figure III-4 B, approximately 3 μg of various truncated GST fusion SNAP190 molecules [GST-SNAP190 (1-719), GST-SNAP190 (63-719), GST-SNAP190 (1-505), and GST-SNAP190 (63-505)] bound to glutathione-

sepharose beads (10 μL) were washed with HEMGT-150 buffer. *In vitro* kinase assays were performed directly on the beads by incubation with 10 units of recombinant CK2 (New England Biolabs) in the presence of 8 nM $\gamma[^{32}P]$ATP for 15 minutes at room temperature. The beads were then washed extensively in HEMGT-150 buffer and proteins were separated by 12.5% SDS-PAGE. The amount of GST-SNAP190 proteins used in each reaction was detected by staining with Coomassie Blue. Radiolabeled proteins were visualized by autoradiography.

For the experiments presented in Figure III-1 and Figure III-5, *in vitro* kinase assays were performed by incubating mini-SNAP complexes with recombinant CK2 in the absence or presence of unlabeled ATP for 30 minutes at 30°C (EMSA) or at room temperature (*in vitro* transcription assay). The amounts of CK2 and ATP used are indicated in the figure legends.

EMSAs

PSE-specific DNA binding by $SNAP_C$ was assayed by EMSA as described previously (Sadowski et al., 1993) using a DNA probe containing a wild-type mouse U6 PSE with mutant human U6 TATA box (Hinkley et al., 2003; Mittal and Hernandez, 1997) with the following modifications. For the experiments presented in Figure III-5 B (*panel a*), approximately 20 ng of purified mini-SNAP complexes mS or mSΔC were preincubated alone or with 5 or 50 units recombinant CK2 and/or 1 mM ATP for 30 min at 30°C. EMSAs were then performed by addition of the DNA probes and incubated for an additional 30 min at 30°C. For the experiments presented in Figure III-5 B (*panel b*), EMSAs were performed by incubating mS or mSΔC with the DNA probes for 30 min at

30°C. Subsequently, 5 or 50 units of recombinant CK2 and/or 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).

Immunodepletions and in vitro transcription assays

To deplete $SNAP_C$ from HeLa nuclear extracts (Henry et al., 1995), 150 μL of extract was incubated with 50 μL of protein A agarose beads pre-coupled to pre-immune or anti-SNAP190 antibodies (CS402) (Wong et al., 1998) for 1 hour at room temperature. *In vitro* transcription of human U1 and U6 snRNA genes was performed as described previously (Lobo and Hernandez, 1989) with the following modifications.

For the reconstitution reactions whose results are depicted in Figure III-4 C, the transcription reactions were performed in a total volume of 36 μL containing 1 μg of the DNA templates, 8 μL of HeLa nuclear extracts or 14 μL immuno-depleted extracts. The amounts of recombinant mini-SNAP complexes are indicated in the figure legend. U6 transcripts were analyzed by RNase T_1 protection as described previously (Lobo and Hernandez, 1998). The RNAs were separated by denaturing PAGE and visualized by autoradiography or by PhosphorImager analysis (Molecular Dynamics).

For Figure III-1 and Figure III-5 A, recombinant mini-$SNAP_C$ mS or mSΔC was preincubated with Dignam buffer D either with or without recombinant CK2 and ATP, and/or quercetin as indicated in the figure legends. *In vitro* transcriptions were initiated by addition of transcription buffers, nucleoside triphosphates, 1 μg of DNA template, and

8 μL of HeLa nuclear extracts or 14 μL immuno-depleted extracts, and incubated for additional 60 min (U6) or 90 min (U1) at 30°C. The amounts of each protein and ATP used are indicated in the figure legends.

Phosphopeptide mapping by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and by β-elimination coupled with quadrupole time of flight mass spectrometry (Q-TOF MS/MS)

To map the sites of SNAP190 phosphorylation, approximately 1 μg of GST-SNAP190 (1-719) immobilized on glutathione sepharose beads was treated with 50 μL HeLa cell nuclear extract in a total volume of 200 μL adjusted with HEMGT-150 buffer. After 1 hr 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 $\gamma[^{32}P]$ATP and 1 mM unlabeled ATP. The phosphorylated GST-SNAP190 (1-719) was digested with trypsin followed by phosphopeptide purification through a Gallium spin column (Posewitz and Tempst, 1999) according to the manufacturer's instructions (Pierce Biotechnology). The purified peptides were directly analyzed or were dephosphorylated with calf intestine alkaline phosphatase (New England Biolabs) prior to analysis by MALDI-TOF mass spectrometry (Perseptive Biosystems, Inc., Farmingham, MA) in a positive ion reflector mode using α-cyano-4-hydroxycinnamic acid as a matrix (Liao et al., 1994). The purified peptides were also subjected to $Ba(OH)_2$ treatment for β-elimination (Jiang and Wang, 2004) prior to liquid chromatography coupled with tandem mass spectrometric analysis using the Waters CapLC system (Waters Corp., Milford, MA)

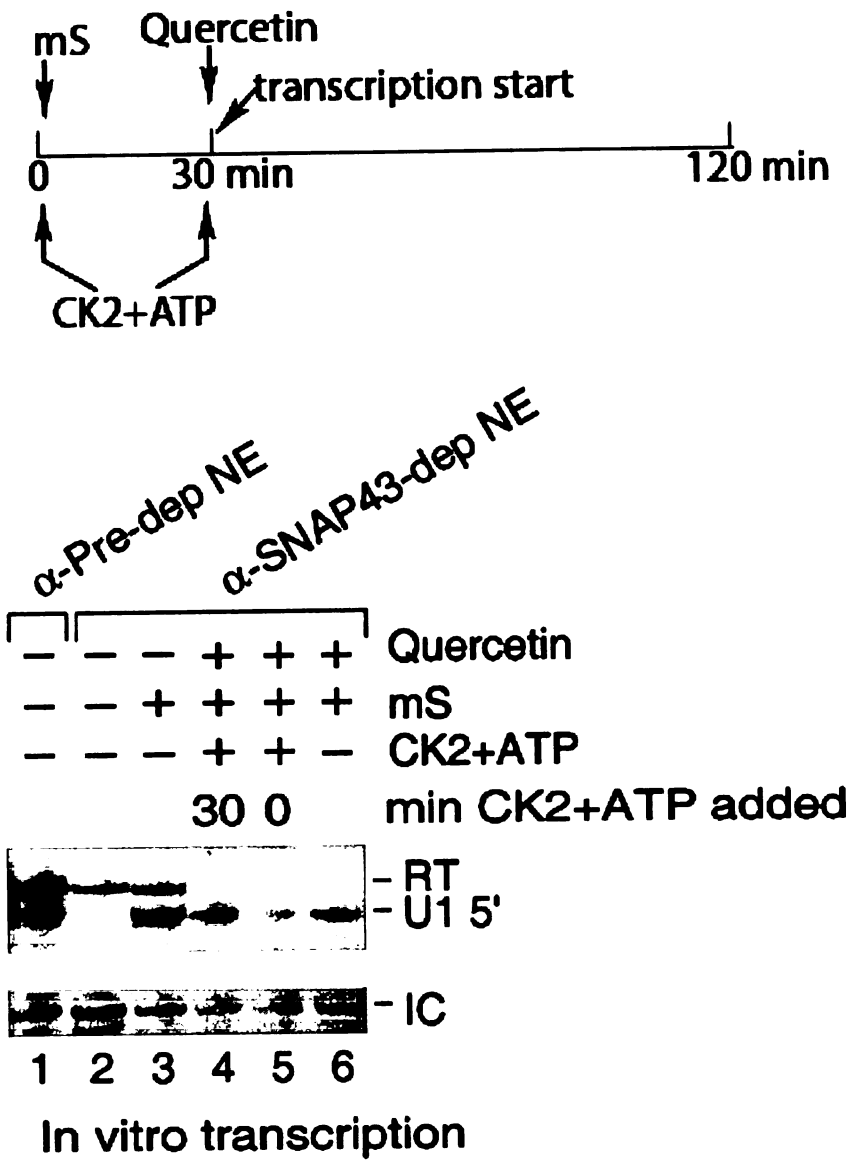
coupled to an LCQ DECA quadrupole ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) through the Picoview nanospray source (New Objectives, Cambridge, MA).

Results

$SNAP_C$ function is modulated by CK2.

The observation that CK2 occupies human U1 and U6 snRNA gene promoters and CK2 phosphorylates the general transcription factor $SNAP_C$ prompted us to investigate whether CK2 regulates the transcriptional properties of $SNAP_C$. First, we depleted endogenous $SNAP_C$ from a transcription extract with anti-SNAP190 antibodies and tested the abilities of mini-$SNAP_C$ (mS) to restore transcription in the $SNAP_C$-depleted HeLa nuclear extract. As shown in Figure III-1, U1 transcription was dramatically diminished upon depletion with anti-SNAP190 antibodies beads (lane 2) comparing to the signal from depletion with preimmune antibodies (lane 1). With addition of recombinant mS to the depleted extract, similar levels of U1 transcription were observed compared to lane 1, suggesting that mS is competent for U1 transcription. To assess the roles of CK2 phosphorylation of $SNAP_C$ in U1 transcription, recombinant mS was preincubated with CK2 in the presence of ATP. CK2 activity was then inhibited by addition of CK2 inhibitor quercetin prior to initiation of transcription by addition of $SNAP_C$-depleted extracts, the transcription buffers, nucleoside triphosphates, and DNA template. Although CK2 inhibitor quercetin inhibits overall U1 transcription (comparing lane 6 to lane 3), decreasing amounts of U1 transcripts with 30 minute preincubation of CK2 and mS were observed (comparing lane 5 to lane 4), indicating that $SNAP_C$ phosphorylation by CK2 inhibits its transcription activity.

Figure III-1. $SNAP_C$ function is modulated by protein kinase CK2. 20 ng recombinant mS was preincubated with either Dignam buffer D (lanes 3, 4 and 6) or Dignam buffer D containing 20 units of recombinant CK2 and 1 mM ATP (lane 5) for 30 min at room temperature, then complemented with 20 units of CK2 and 1 mM ATP (lane 4). 2 mM quercetin was added to the reactions in lanes 4 and 5 to block CK2 activity or to the reaction in lane 6 as a control. The U1 transcription was initiated by addition of either a preimmune-depleted HeLa nuclear extract (lane 1) or an anti-SNAP43 immuno-depleted HeLa nuclear extract (lanes 2 ~ 6). The correctly initiated transcripts are labeled U1 5'. Readthrough transcripts (RT) from cryptic mRNA-type promoters in the U1 transcription experiment (Sadowski et al., 1993) and an internal control (IC) included in the reaction mixtures to monitor RNA handling and recovery are indicated to the right of the gel.

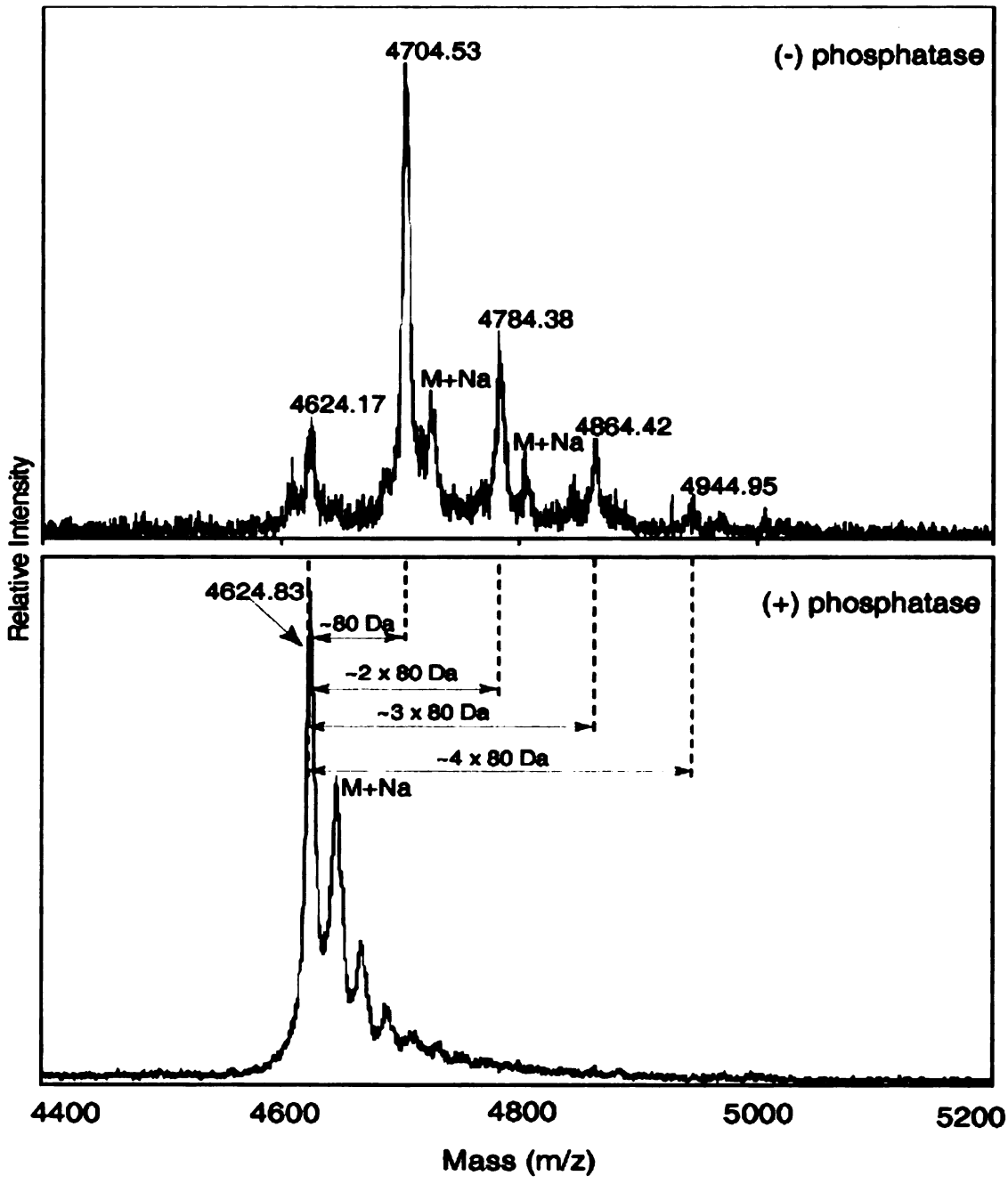

mS
Quercetin
transcription start
0
30 min
120 min
CK2+ATP
α-Pre-dep NE
α-SNAP43-dep NE
– – – + + + Quercetin
– – + + + + mS
– – – + + – CK2+ATP
30 0 min CK2+ATP added
RT
U1 5'
IC
1 2 3 4 5 6
In vitro transcription

Multiple phosphorylation sites in SNAP190 are identified by mass spectrometry.

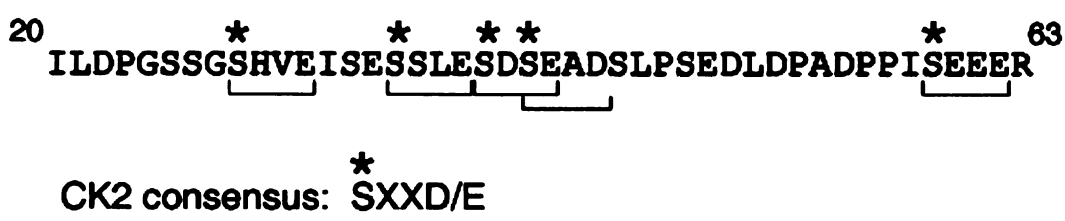
As an initial step towards investigating the function of SNAP190 phosphorylation by CK2, phosphopeptide mapping of SNAP190 was performed by mass spectrometry. First, GST-SNAP190 (1-719) was used as the bait to recover endogenous CK2 from HeLa cell nuclear extracts prior to performing an *in vitro* kinase assay in the presence of $\gamma[^{32}P]$ ATP. Phosphorylated GST-SNAP190 (1-719) was then gel purified, digested with trypsin, followed by phosphopeptide purification through a Gallium column (Posewitz and Tempst, 1999). The resultant peptides 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 to the mass calculated from its sequence and the mass should be converted back to the calculated value after treatment of the peptide with phosphatase. As shown in Figure III-2 A, five peaks with approximately 80 Da or multiples of 80 Da differences are observed (*top panel*), indicating that these masses may represent a population of one peptide with different phosphorylated status. Phosphatase treatment reveals only one peak at *m/z* 4624.83 Da (*bottom panel*), further confirming that the mass spectral peaks at *m/z* 4704.53, 4784.38, 4864.42, and 4944.95 (*top panel*) correspond to the peptide with *m/z* 4624.17 Da bearing 1, 2, 3, and 4 PO_3^- group(s), which can be removed by phosphatase treatment. These data strongly suggest that a non-phosphopeptide with approximately *m/z* 4624 Da can be phosphorylated at multiple sites. By careful comparison with the predicted masses of GST-SNAP190 (1-719)

Figure III-2. Endogenous CK2 phosphorylates the N-terminus of SNAP190 in the region within amino acids 20-63 containing multiple CK2 consensus sites. (A) Tryptic phosphopeptide of SNAP190 identified by MALDI-TOF MS. GST-SNAP190 (1-719) was incubated with HeLa cell nuclear extracts to recover associated CK2. Samples were then used for an *in vitro* kinase assay to radiolabel GST-SNAP190 (1-719). Proteins were digested with trypsin followed by affinity purification of phosphopeptides through a Gallium column. The purified tryptic fragments were analyzed by MALDI-TOF MS before (*top panel*) and after calf intestine alkaline phosphatase treatment (*bottom panel*). The peaks present in the top MALDI-TOF MS spectrum 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 4624.07-4626.79 Da, observed MH^+ *m/z* is 4624.17 Da) with 1, 2, 3, 4 phosphate groups. After phosphatase treatment, these peaks shift to the peak at *m/z* 4624.83 Da. (B) Sequence of the SNAP190 tryptic peptide containing amino acids 20-63. CK2 consensus sites within these peptides are bracketed. Serine residues that match CK2 consensus motif within this peptide are highlighted with asterisks.

A


4704.53
(-) phosphatase
4784.38
M+Na
4624.17
4864.42
M+Na
4944.95
Relative Intensity
4624.83
(+) phosphatase
~80 Da
~2 x 80 Da
~3 x 80 Da
~4 x 80 Da
M+Na
4400
4600
4800
5000
5200
Mass (m/z)

B


20
63
ILDPGSSGSHVEISESSLESDSEADSLPSEDLDPADPPISEEER
CK2 consensus: SXXD/E

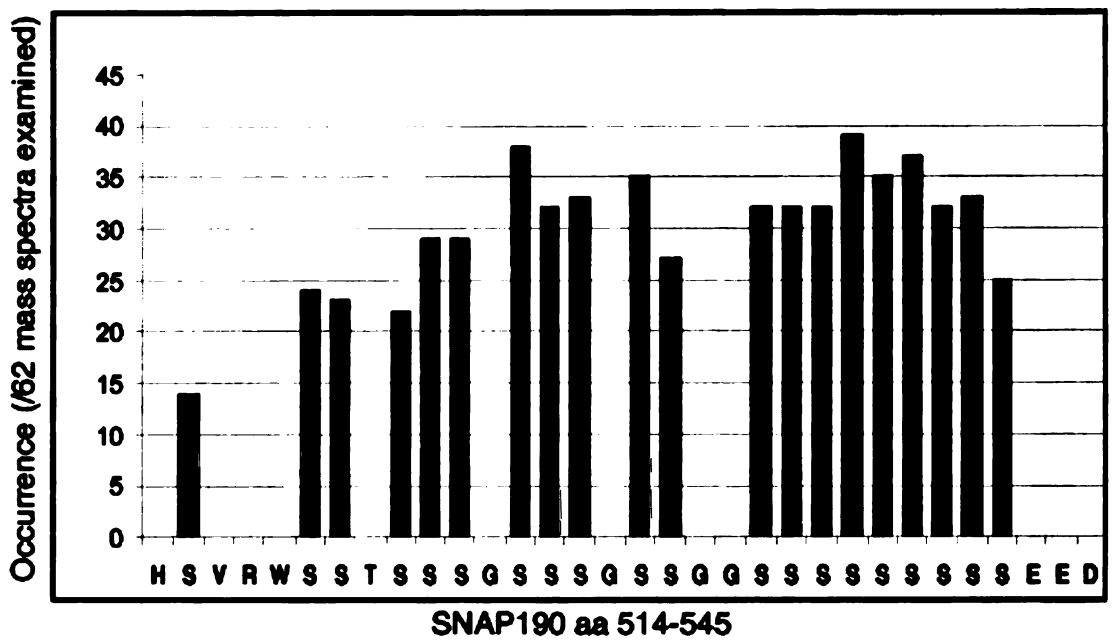
tryptic peptides, only SNAP190 (20-63) matches this criterion (calculated MH^+ at *m/z* 4624.07~4626.79). The amino acid sequence of SNAP190 (20-63) is given in Figure III-2 B. Five CK2 consensus sites are found in this 44 amino acid region of SNAP190, indicating that this peptide is likely phosphorylated by CK2.

The phosphorylated GST-SNAP190 (1-719) tryptic peptides were also analyzed by β-elimination coupled with Q-TOF MS/MS. In this assay, phosphorylation of numerous fragments encompassing the serine rich region within aa 514-545 was observed. Figure III-3 A shows one example of MS/MS ion after β-elimination of *m/z* 627.11 Da, which corresponds to amino acid residues 519-543 [(^{519}SSTSSSGSSSGSSGGSSSSSSSSSE^{543}+3H)$^{3+}$] according to MS/MS data. β-elimination removes the phosphoric acid groups from phosphorylated serine and leaves unmodified serine unattacked, which leads to a net 18 Da loss relative to unmodified serine (Byford, 1991; Resing et al., 1995). A total of 20 y-series product ions (ions from the C-terminal end) and 6 b-series product ions (ions from the N-terminal end) was observed within this spectrum (Figure 3A, *top panel*), the $y(5)^{++}$, $y(6)^{++}$, $y(7)^{++}$, $y(9)^{++}$, $y(10)^{++}$, $y(13)^{++}$, $y(16)^{++}$, $y(17)^{++}$, and $y(18)^{++}$ ions were highlighted in the enlarged spectrum (*bottom panel*). The mass differences between $y(5)^{++}$ and $y(6)^{++}$ is 43.5 (87/2) Da, indicating serine 538 is not phosphorylated, while the mass differences between $y(6)^{++}$ and $y(7)^{++}$, $y(9)^{++}$and $y(10)^{++}$, $y(16)^{++}$and $y(17)^{++}$, $y(17)^{++}$and $y(18)^{++}$ are 34.5 [(87-18)/2] Da due to β-elimination of phosphoserine. Within this peptide, a total of fourteen different serine residues has a net mass 18 Da loss, suggesting that fourteen serines are phosphorylated. We analyzed a total of 62 spectra data encompassing the

Figure III-3. Endogenous CK2 phosphorylates the N-terminus of SNAP190 in the serine rich region within amino acids 514-545 containing multiple CK2 consensus sites. (A) Q TOF MS/MS of the ion of [(^{519}SSTSSSGSSSGSSGGSSSSSSSSSE^{543}+3H)$^{3+}$] with *m/z* 627.11 Da after β-elimination. The sequence of the peptide segment corresponding to amino acid residues 519-543, the b-series and y-series fragment ions were shown above the spectrum. The y(5)$^{++}$, y(6)$^{++}$, y(7)$^{++}$, y(9)$^{++}$, y(10)$^{++}$, y(13)$^{++}$, y(16)$^{++}$, y(17)$^{++}$, and y(18)$^{++}$ ions were highlighted in the enlarged spectrum in the mass range of *m/z* 200-700 (*bottom panel*). "S - 18" designates a dehydroalanine, which results from the β-elimination of phosphoserine (labeled as pS in the peptide sequence). (B) Each of the 20 serines in the serine rich region could be phosphorylated. A total of 62 MS/MS spectra data from numerous phosphopeptides encompassing the serine rich region aa 514-545 were analyzed. The occurrence of phosphorylation at each amino acid residue is shown in y-axis; the corresponding amino acid sequence is indicated in x-axis. (C) Sequence of the SNAP190 peptide containing amino acids 514-545. CK2 consensus sites within these peptides are bracketed underneath. Serine residues that match CK2 consensus motif within this peptide are highlighted with asterisks. Other sites that are bracketed above indicate the possible CK2 sites generated by the nascent phosphoserines.

y24 y23 y21 y19 y18 y17 y16 y13 y10 y9 y7 y6 y5 y4 y2
519 pS pS T pS pS pS G pS pS pS G pS S G G pS S pS pS S S pS pS S E 543
b7 b13 b14 b16 b19
Relative intensity
y0(2) y(5)++ y(6)++ y(7)++ y0(4) y(9)++ y0(10)++ y0(10)++ y0(5) b0(13)++ b(7) y(13)++ y0(16)++ y(16)++ y(17)++ y0(8) y(18)++ y0(19)++ y0(21)++ y(21)++ y(23)++ y(24)++ b0(14) b(14) b(16) b(19)
200 400 600 800 1000 1200 1400
S/2 (S-18)/2 (S-18)+S/2 (S-18)/2 G+G+(S-18)/2 (S-18)+G+(S-18)/2 (S-18)/2 (S-18)/2
Relative intensity
y0(2) y(5)++ y(6)++ y(7)++ y0(4) y(9)++ y0(10)++ y(10)++ y0(5) b0(13)++ b(7) y(13)++ y0(16)++ y(16)++ y(17)++ y0(8) y(18)++
200 300 400 500 600 700
Mass (m/z)

B


Occurrence (/62 mass spectra examined)
45
40
35
30
25
20
15
10
5
0
H S V R W S S T S S S G S S S G S S G G S S S S S S S S S S E E D
SNAP190 aa 514-545

C

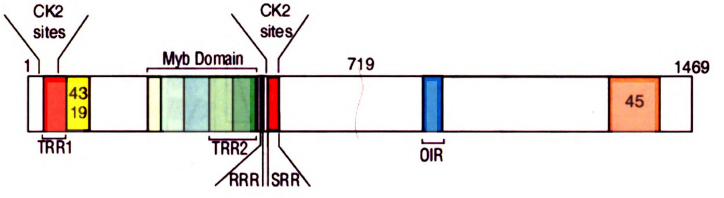

514
***
545
HSVRWSSTSSSGSSSGSSGGSSSSSSSSSEED
*
CK2 consensus: SXXD/E

serine rich region and the numbers are summarized in Figure III-3 B, showing that each of the 20 serines in this serine rich region was phosphorylated. Although other kinases derived from HeLa nuclear extracts could contribute to phosphorylation in this region, CK2 is likely the major kinase responsible for phosphorylation in this region (Figure II-5). SNAP190 (514-545) has three CK2 consensus substrate recognition sequences at the C-terminal side of multiple blocks of contiguous serine residues, that is, Glu^{543}, Glu^{544}, and Asp^{545} (illustrated in Figure III-3 C). As a result, Ser^{540}, Ser^{541}, and Ser^{542} are the first sites that can be phosphorylated. Phosphorylation introduces a negative charge to that residue, which potentially creates a new CK2 site at a nearby serine residue located at the N - 3 position. Therefore, the initial phosphorylation at $Ser^{540-542}$ leads to propagation of upstream serine phosphorylation. This observation is further supported by recent study that CK2 can phosphorylate seven contiguous serine residues (Ser^{78-84}) in the serine tract ^{78}SSSSSSSEDD^{87} in dehydrin (DHN1) protein (Jiang and Wang, 2004).

A schematic representation of SNAP190 and the position of the CK2 sites relative to other functional regions are shown in Figure III-4 A (*top panel*). Interestingly, the CK2 phosphorylated region aa (514-545) is adjacent to the Myb DNA binding domain, and both CK2 phosphorylated regions are adjacent to the TBP-recruiting regions (TRR1 and TRR2) in SNAP190 that are involved in cooperative promoter recognition by $SNAP_C$ and TBP, suggesting a potential role for CK2 in controlling DNA binding and/or TBP recruitment. Our previous data demonstrated that PSE-specific DNA binding by mS was inhibited by CK2, and that cooperation between $SNAP_C$ and TBP can antagonize CK2 inhibition of DNA binding by $SNAP_C$ (Figure II-6). The mechanism of the inhibitory effect of CK2 phosphorylation on $SNAP_C$ DNA binding is under investigation.

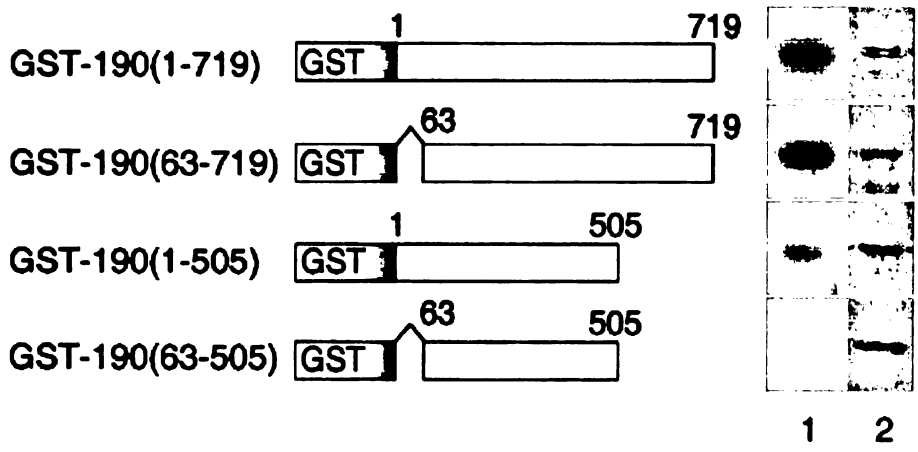
Figure III-4. Deletion of CK2 phosphorylation regions within SNAP190 did not affect the transcription properties of $SNAP_C$. (A) A schematic representation of full-length SNAP190 and the position of the CK2 sites relative to other functional regions are shown (*top panel*). Myb domain: Myb DNA binding domain; SRR: the serine rich region; RRR: the arginine rich region; 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 Ma and Hernandez, 2001). The wavy vertical line around position 719 indicates the C-terminus of the truncated SNAP190 protein used for these studies. Sequence of the SNAP190 amino acids 1-719 is also given and thirteen CK2 sites are highlighted (*bottom panel).* (B) SNAP190 phosphorylation was diminished in the deletion mutants. Schematic representation of various GST fusion proteins containing truncated SNAP190 is shown on the left side. The GST fusion proteins were overexpressed in *E. coli* and purified for *in vitro* kinase assays by affinity chromatography using glutathione-sepharose beads. 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). (C) SNAP190 deletion mutants are competent for both U1 and U6 transcription. Partial SNAP complexes mS, mSΔN, mSΔC, and mSΔ(N+C), were assembled by co-expressed in *E. coli* and purified through affinity chromatography. The titrations of the mini-SNAP complexes were over a threefold range. The correctly initiated transcripts are labeled U6 and U1 5'.

A

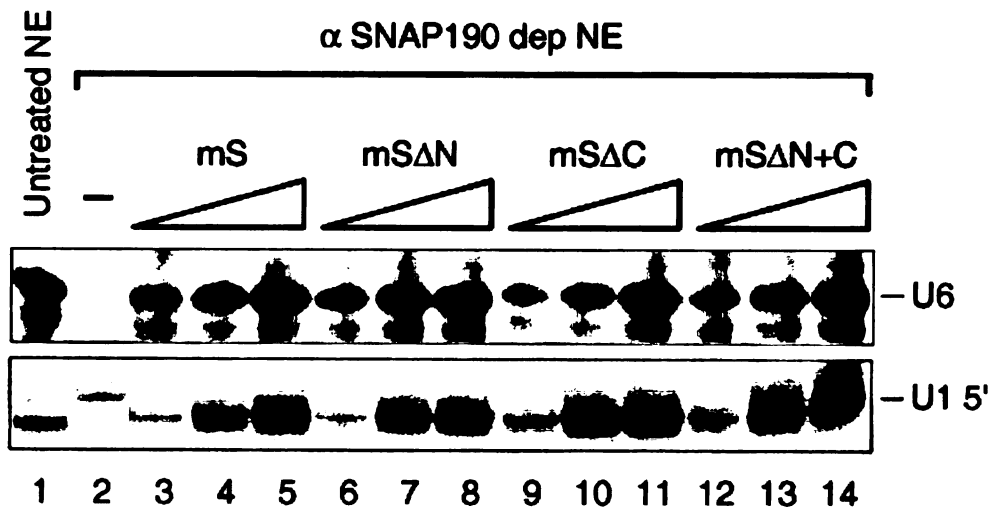



```
  1 MDVDAERERKI TQEIKELERI LDPGSSGSHV EISESSLESD SEADSLPSED LDPADPPISE
 61 EERWGEASND EDDPKDKTLP EDPETCLQLN MVYQEVIQEK LAEANLLLAQ NREQQEELMR
121 DLAGSKGTKV KDGKSLPPST YMGHFMKPYF KDKVTGVGPP ANEDTREKAA QGIKAFEELL
181 VTKWKNWEKA LLRKSVVSDR LQRLLQPKLL KLEYLHQKQS KVSSELERQA LEKQGREAEK
241 EIQDINQLPE EALLGNRLDS HDWEKISNIN FEGSRSAEEI RKFWQNSEHP SINKQEWSRE
301 EEERLQAIAA AHGHLEWQKI AEELGTSRSA FQCLQKFQQH NKALKRKEWT EEEDRMLTQL
361 VQEMRVGSHI PYRRIVYYME GRDSMQLIYR WTKSLDPGLK KGYWAPEEDA KLLQAVAKYG
421 EQDWFKIREE VPGRSDAQCR DRYLRRLHFS LKKGRWNLKE EEQLIELIEK YGVGHWAKIA
481 SELPHRSGSQ CLSKWKIMMG KKQGLRRRRR RARHSVRWSS TSSSGSSSGS SGGSSSSSSS
541 SSEEDEPEQA QAGEGDRALL SPQYMVPDMD LWVPARQSTS QPWRGGAGAW LGGPAASLSP
601 PKGSSASQGG SKEASTTAAA PGEETSPVQV PARAHGPVPR SAQASHSADT RPAGAEKQAL
661 EGGRRLLTVP VETVLRVLRA NTAARSCTQK EQLRQPPLPT SSPGVSSGDS VARSHVQWL
```

B


GST-190(1-719)
GST
1
719
GST-190(63-719)
63
719
GST-190(1-505)
505
GST-190(63-505)
1
2

C


Untreated NE
α SNAP190 dep NE
–
mS
mSΔN
mSΔC
mSΔN+C
U6
U1 5'
1 2 3 4 5 6 7 8 9 10 11 12 13 14

SNAP190 deletion mutants diminish phosphorylation but remain functional for transcription.

Computer-assisted screening (Blom et al., 1999) of the amino acid sequence of SNAP190 (1-719) reveals a total of thirteen putative CK2 phosphorylation sites containing serines (highlighted in Figure III-4 A, *bottom panel*). There are six sites clustered in the N-terminal region (Ser^{28}, Ser^{35}, Ser^{39}, Ser^{41}, Ser^{59} and Ser^{68}) and three sites in the serine rich region (Ser^{540}, Ser^{541}, and Ser^{542}). Because mass spectrometric analysis of SNAP190 (1-719) revealed that at least 4 serines are phosphorylated in the N-terminal region and up to 20 in the serine rich region. We therefore assumed that a truncated SNAP190 lacking the N-terminal region and/or the serine rich region would be a poor substrate for CK2 phosphorylation. The GST fusion deletion mutants containing SNAP190 (63-719), SNAP190 (1-505), and SNAP190 (63-505) were expressed in *E. coli*, purified through glutathione sepharose beads (Figure III-4 B lane 2, *right panel*), and tested for CK2 phosphorylation in an *in vitro* kinase assay by recombinant CK2. As shown in Figure III-4 B lane 1, as compared to GST-SNAP190 (1-719), GST-SNAP190 (63-719) phosphorylation was slightly decreased, while GST-SNAP190 (1-505) phosphorylation was dramatically reduced, indicating that the serine rich region we mapped harbors the majority of CK2 phosphorylation sites within SNAP190 (1-719). The truncated polypeptide lacking both the N-terminal region and the serine rich region GST-SNAP190 (63-505) was phosphorylated less well than GST-SNAP190 (1-719) and GST-SNAP190 (1-505). Collectively, these findings demonstrate that CK2 phosphorylates SNAP190 (1-719) predominantly at two regions aa (20-63) and aa (514-545).

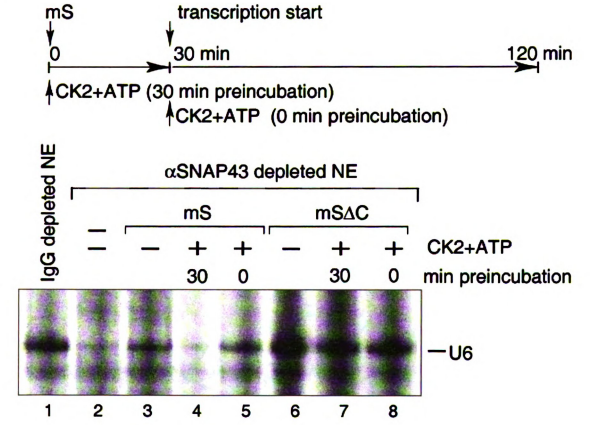
Next, we asked whether these SNAP190 deletion mutants are capable of reconstituting snRNA gene transcription in an *in vitro* transcription system. Partial SNAP complexes containing full-length SNAP43, full-length SNAP50, and various truncated SNAP190 molecules mS, mSΔN, mSΔC, and mSΔ(N+C) were assembled in *E. coli* and tested for function in transcription. In our experiments, a titration for each complex was performed and showed that all four complexes can support both U1 and U6 snRNA gene transcription equally well using $SNAP_C$ depleted HeLa nuclear extracts *in vitro* (Figure III-4 C). Thus, these recombinant mini-SNAP complexes are capable of mediating transcription by both RNA polymerases II and III. Thus, the regions of aa (1-62) and/or aa (506-719) are not essential for $SNAP_C$ activity and may play a regulatory role on $SNAP_C$ function.

The region of SNAP190 (506-719) is required for CK2 inhibition.

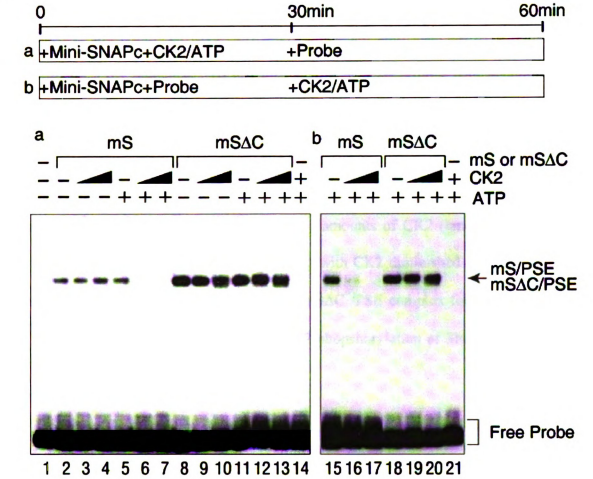
To further investigate a role of CK2 phosphorylation in snRNA transcription, we tested the $SNAP_C$ function in the presence of recombinant CK2 using the partial SNAP complexes mS and mSΔC. As shown in Figure III-5 A, both mini-SNAP complexes mS and mSΔC were able to reconstitute U6 transcription in the $SNAP_C$-depleted HeLa nuclear extracts (comparing lanes 3 and 6 to lane 2). However, when mS was preincubated with CK2 at 30°C for 30 minutes in the presence of ATP, U6 transcription was decreased remarkably (lane 3), while addition of same amount of CK2 and ATP without preincubation did not affect U6 transcription (lane 5), indicating that allowing mS phosphorylation by CK2 causes U6 transcription downregulation. Moreover, when

Figure III-5. A functional comparison between mini-SNAP complexes mS and mSΔC. (A) U6 transcription of mS and mSΔC showed differential sensitivity upon CK2 treatment. Approximately 100 ng of purified mS and mSΔC were used in each reaction. $SNAP_C$ were preincubated with either Dignam buffer D (lanes 3, 5, 6, and 8) or Dignam buffer D containing 20 units of recombinant CK2 and 1 mM ATP for 30 min. The reactions in lane 5 and 8 were then complemented with equal concentration of CK2 and ATP prior to initiation of transcription. The correct transcripts are labeled U6. (B) PSE-specific DNA binding by mS and mSΔC was assayed by EMSA. Approximately 20 ng of purified mini-SNAP complex mS (lanes 2 ~ 7) and mSΔC (lanes 8 ~ 13) were preincubated alone (lanes 2 and 8) or with 5 units (lanes 3, 6, 9, and 12) or 50 units (lanes 4, 7, 10, 13, and 14) of CK2 and/or 1 mM ATP (lanes 5 ~ 7 and 11 ~ 14) for 30 min at 30°C. EMSA was then performed using a DNA probe containing a wild-type mouse U6 PSE with mutant human U6 TATA box as described previously (Mittal and Hernandez, 1997), and incubated for an additional 30 min at 30°C (*left panel*). Alternatively, EMSA was performed by preincubating mS (Lanes 15 ~ 17) and mSΔC (Lanes 18 ~ 20) with the DNA probe for 30 min at 30°C (lanes 8 ~ 10). Then, 5 units (lanes 16 and 19) or 50 units (lanes 17, 20 and 21) of CK2 and/or 1 mM ATP (Lanes 8 ~ 10) were added and reactions were incubated for an additional 30 min at 30°C (*right panel*). The reaction shown in lane 1 contains probe alone, and the reaction in lanes 14 and 21 contained only 50 units CK2 and 1 mM ATP. The positions of the protein/DNA complexes are indicated.

A


mS
transcription start
0
30 min
120 min
CK2+ATP (30 min preincubation)
CK2+ATP (0 min preincubation)
IgG depleted NE
αSNAP43 depleted NE
mS
mSΔC
CK2+ATP
min preincubation
30
0
30
0
U6
1 2 3 4 5 6 7 8

B


0
30min
60min
a +Mini-SNAPc+CK2/ATP
+Probe
b +Mini-SNAPc+Probe
+CK2/ATP
a
mS
mSΔC
b
mS
mSΔC
mS or mSΔC
CK2
ATP
mS/PSE
mSΔC/PSE
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

mSΔC and CK2 were preincubated and tested for U6 transcription, no difference was observed compared to the same sample without preincubation (comparing lane 7 to lane 8), suggesting that the region of SNAP190 (506-719) is critical for CK2 inhibition.

As mentioned previously, the CK2 phosphorylated region aa (514-545) is adjacent to the Myb DNA binding domain (Figure III-4 A), and CK2 phosphorylation of SNAP190 at the region aa (514-545) might block the ability of the Myb domain to recognize the PSE sequence. To test this, EMSA was performed with approximately equal amounts of mS or mSΔC that was preincubated with either CK2 or ATP or both for 30 minutes, followed by addition of a DNA probe. As shown in Figure III-5 B (*panel a*), robust DNA binding was observed by mS (lane 2) and mSΔC (lane 8), even in the presence of ATP (lanes 5 and 11) or increased amounts of CK2 (lanes 3-4 and 9-10). However, preincubation of mS with ATP along with CK2 diminished mS/PSE complex formation (lanes 6 and 7), but did not affect mSΔC /PSE complex formation (lanes 12 and 13). These data strongly suggest that CK2 phopshorylation of SNAP190 (506-719) plays a negative role in $SNAP_C$ DNA binding.

$SNAP_C$ DNA binding can be prevented by CK2 phosphorylation of $SNAP_C$ (Figure III-5 B, *panel a*), thus, whether the $SNAP_C$/DNA complex can be disrupted by CK2 phosphorylation of $SNAP_C$ after DNA binding had occurred was tested. To address this, EMSAs was performed by allowing $SNAP_C$/DNA complexes to form followed by CK2 phosphorylation. As shown in Figure III-5 B (*panel b*), when mS bound to PSE, subsequent addition of CK2 and ATP leads to less complex formation (comparing lanes 16 and 17 to lane 15). A similar response for mSΔC mutant was not observed. Thus,

CK2 phosphorylation in the serine rich region of SNAP190 can inhibit $SNAP_C$ PSE binding both before and after DNA binding has occurred.

Taken together, we conclude that the region of SNAP190 (506-719) is critical for CK2 inhibition. CK2 targets SNAP190 for phosphorylation in the serine rich region, which in turn inhibits $SNAP_C$ DNA binding activity and transcription properties.

Discussion

Previously I have demonstrated that CK2 inhibits overall U1 snRNA gene transcription in human HeLa cells. CK2 targets the snRNA activating protein complex ($SNAP_C$) for phosphorylation. Here, I provide additional evidence that CK2 may downregulate snRNA gene transcription by inhibiting $SNAP_C$ DNA binding and transcription activity. The CK2 phosphorylation sites within SNAP190 were identified by mass spectrometry, and the region in SNAP190 that is required for CK2 inhibition was narrowed down to the region between aa 506-719 encompassing a serine rich region with multiple CK2 consensus sites.

It has been determined that $SNAP_C$ PSE-specific DNA binding activity was inhibited by CK2 (Figure II-6). The mechanism of the inhibitory effect of CK2 phosphorylation on $SNAP_C$ DNA binding is not known; however, it is unlikely due to phosphorylation at a critical residue within the SNAP190 Myb domain, because this same complex is capable of binding DNA when TBP is present (Figure II-6). For the same reason, that phosphorylation outside the Myb DNA binding domain would affect intermolecular interactions between SNAP190 and SNAP43 is unlikely. Instead, we

favor the idea that CK2 phosphorylates SNAP190 outside the Myb DNA binding domain, leading to intramolecular interactions in SNAP190 that block SNAP190 Myb domain PSE recognition. Two CK2 target regions within SNAP190 (1-719) have been identifed by mass spectrometric analysis. One region (aa 20-63) is located in the N-terminal region upstream of the SNAP43 interacting region (Ma and Hernandez, 2001), consistent with our prediction that the SNAP43 interacting region within SNAP190 is not a CK2 target. Another region (aa 514-545) is located in the serine rich region downstream of the Myb-DNA binding domain. Deletion mutant of SNAP190 (1-505) lacking the serine rich region was generated and tested for transcriptional properties in the presence of CK2. Both DNA binding and transcription activities of mSΔC were not affected by CK2 (Figure III-5), while mS was sensitive to CK2 treatment, suggesting that CK2 phosphorylation of SNAP190 in the serine rich region downregulates $SNAP_C$ DNA binding and transcription activity. It is interesting that the serine rich region with CK2 consensus sites in SNAP190 is conserved between mouse (accession number: AK077522.1) and human, thus, if CK2 phosphorylation of this serine rich region has a physiological function, it could be restricted to those organisms.

It has been proposed previously that the full-length SNAP190 contains a built-in damper located in the region from aa 506-1469, which downregulates SNAP190 binding to DNA (Mittal et al., 1999). It will be interesting to determine the exact location of this damper. In our experiments, when comparing the DNA binding activities of SNAP complexes mS and mSΔC using equal concentrations, we observed that mSΔC has higher DNA binding affinity than mS (Figure III-5 B), raising the possibility that the region of SNAP190 aa (506-719) acts as a damper of DNA binding. Within SNAP190, there is an

arginine rich region located between the Myb domain and the serine rich region (illustrated in Figure III-4 A) and we speculate that a conformational change in SNAP190 mediated by the interaction between the negatively charged residues ($Glu^{543, 544, and 546}$, and Asp^{545}) and the positively charged arginines is unfavorable for $SNAP_C$/PSE complex formation. CK2 phosphorylation introduces more negative charges in the serine rich region, which will strengthen the interaction between the serine rich region and the arginine rich region, and make it more unfavorable for the PSE recognition. The observation that the weak DNA binding activity of mS was further diminished by CK2 treatment (Figure III-5 B) further supports this charge effect mechanism for inhibition of DNA binding.

Another interesting finding in this study is that $SNAP_C$ DNA binding inhibited by CK2 occurred both before and after $SNAP_C$/PSE complex had formed (Figure III-5). This observation raises the possibility that the CK2 phosphorylation sites within $SNAP_C$ might be exposed so that CK2 can reach them even when SNAP190 is complexed with the PSE. CK2 phosphorylation not only prevents the free $SNAP_C$ from binding DNA, but it can also increase the off-rate of $SNAP_C$ from the promoter. This double checkpoint mechanism ensures a maximum CK2 inhibition on snRNA gene transcription.

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CHAPTER FOUR

SUMMARY AND FUTURE PLAN

CK2 is a highly conserved, ubiquitous kinase that appears to influence non-protein coding gene transcription, such as RNA pol I-specific rRNA and RNA pol III-specific tRNA, 5S rRNA and U6 snRNA gene transcription. Whether CK2 regulates RNA pol II-specific snRNA gene transcription has been uncertain. Here we report that CK2 regulates U1 snRNA gene transcription by RNA pol II. Inhibition of CK2 by either depleting it using RNAi in cells (Figure II-1 B) or blocking CK2 activity with its specific inhibitor in human cell extracts (Figure II-2 B) stimulates U1 snRNA synthesis. The promoter occupancies of both CK2α and CK2β subunits on the U1 gene in human cells (Figure II-1 A) provide further evidence that CK2 is in close proximity with U1 transcriptional machinery. Thus, these observations demonstrate that CK2 is involved in regulating RNA pol II-specific snRNA transcription.

Previously, it has been demonstrated that CK2 targets TFIIIB and RNA pol III for RNA pol III-specific U6 snRNA transcription regulation (Hu et al., 2003; Hu et al., 2004). However, TFIIIB or RNA pol III are not required for U1 transcription by RNA pol II. One candidate target for CK2 regulation is the basal transcription factor $SNAP_C$, which is used for all snRNA gene transcription by both RNA pol II and pol III. $SNAP_C$ 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 thereby promoting preinitiation complex assembly for both RNA pols II and III. Indeed,

endogenous CK2 associates with $SNAP_C$ and phosphorylates the SNAP190 subunit (Figure II-3). SNAP190 likely plays a central role in coordinating $SNAP_C$ phosphorylation by recruiting CK2 to the complex. This idea is supported by the observation that GST-SNAP190 (1-719) can recruit CK2 from HeLa cell nuclear extracts (Figure II-5 A).

Although CK2 is involved in regulating snRNA gene transcription by both RNA pol II and III, the mechanism of its function is unclear. Hu et al. (2003; 2004) discovered that CK2 stimulates U6 snRNA gene transcription by targeting RNA pol III. On the other hand, CK2 inhibits U6 transcription by phosphorylating Bdp1. Notably, CK2 treatment of recombinant $SNAP_C$ did not affect U6 transcription in their minimal RNA pol III system (Hu et al., 2003). Our experiments demonstrate that mini-$SNAP_C$ phosphorylation by CK2 inhibits its DNA binding activity and TBP is capable of counteracting this inhibition. As shown in Figure II-6, CK2 inhibition of $SNAP_C$ DNA binding is not altered by the addition of TBP for the U1 like probe (Figure II-6, *right panel*). This observation supports the proposal that downregulation of $SNAP_C$ DNA binding activity by CK2 may contribute to reduced U1 snRNA gene transcription. However, CK2 inhibition can be reversed by the addition of TBP for the U6 like probe (Figure II-6, *middle panel*), which can explain why U6 snRNA gene transcription was not affected by CK2 phosphorylation of $SNAP_C$ (Hu et al., 2003). These findings indicate that cooperation between $SNAP_C$ and TBP antagonizes CK2 inhibition of DNA binding by $SNAP_C$.

It has been demonstrated that CK2 targets $SNAP_C$ for phosphorylation. Whether CK2 phosphorylation of $SNAP_C$ directly contributes to CK2 downregulation of U1 snRNA gene transcription was tested. As a first, I have established an *in vitro*

transcription system wherein the endogenous $SNAP_C$ was immuno-depleted and reconstituted with recombinant E. coli assembled mini-$SNAP_C$ to support transcription (Figure III-1 and Figure III-4 B). In this system, mini-SNAP complexes alone are capable of reconstituting snRNA gene transcription by both RNA pol II and pol III in the $SNAP_C$-depleted HeLa nuclear extracts without addition of recombinant TBP and ATP mix (0.3 M ATP, 10 □g of phosphocreatine kinase per ml, and 10 mM creatine kinase) (Ma and Hernandez, 2002). Next, this system was used to directly test the effect of $SNAP_C$ phosphorylation by CK2 for transcription. I observed that preincubation of $SNAP_C$ with CK2 and ATP exhibited less U1 (Figure III-1) and U6 snRNA transcripts (Figure III-5 A), suggesting that CK2 phosphorylation inhibits $SNAP_C$ transcription activity.

To further investigate the mechanism of $SNAP_C$ phosphorylation by CK2, phosphopeptide mapping of SNAP190 (1-719) was performed by mass spectrometry. The MS data revealed that SNAP190 was phosphorylated at two regions that contain multiple CK2 consensus sites [amino acid (aa) 20-63 and 514-545]. As the serine rich region from amino acids 514-545 represents the majority of CK2 phosphorylation within SNAP190 (1-719) (Figure III-3 B and Figure III-4 B), the importance of this region for CK2 regulation of $SNAP_C$ function was tested. Mini-SNAP complexes containing SNAP43, SNAP50, and SNAP190 (1-719) (mS) or SNAP190 lacking the serine rich region SNAP190 (1-505) (mSΔC) were compared for DNA binding and transcription activity in the presence of CK2. As described in Figure III-5, mS showed lower DNA binding affinity than mSΔC and it was sensitive to CK2 treatment. Thus, SNAP190 (506-719) plays an important role in downregulating $SNAP_C$ DNA binding activity and CK2 phosphorylation enhances this negative regulation.

It has been determined that $SNAP_C$ PSE-specific DNA binding activity was inhibited by CK2 (Figure II-6) and the region of SNAP190 (506-719) is required for CK2 inhibition (Figure III-5). The mechanism of the inhibitory effect of CK2 phosphorylation on $SNAP_C$ DNA binding is not known. We favor the idea that CK2 phosphorylates SNAP190 outside the Myb DNA binding domain, leading to intramolecular interactions in SNAP190 that masks PSE recognition by the SNAP190 Myb domain. An alignment of the SNAP190 Myb domain with the human c-Myb protein showed that SNAP190 Rd repeat (aa 451-503) and c-Myb R3 repeats have 30% identity (Wong et al., 1998). NMR studies of c-Myb indicate that the R3 repeat is composed of three helices (Jamin et al., 1993). Computational modeling of SNAP190 (1-719) revealed that within the SNAP190 Myb domain, Rd repeat may also contain three helices with the last helix extended to residue 510, meaning that the arginine rich region (aa 506-511) is part of the helix-turn-helix (HTH) motifs important for DNA binding. Therefore, I hypothesized a charge-directed mechanism in which the heavily negative charges in the serine rich region introduced by CK2 phosphorylation can attract the positively charged arginines and may influence the HTH motif in the DNA binding domain. To test this idea, I have made the non-phosphorylatable mutant complex by disrupting the CK2 consensus sites in SNAP190 ($EED^{543\text{-}545}\rightarrow AAA$) and the phospho-mimetic mutant with S→D substitution ($SSSS^{549\text{-}542}\rightarrow DDDD$). My preliminary data shows that DNA binding activity of the non-phosphorylatable mutant mS ($EED^{543\text{-}545}\rightarrow AAA$) is less sensitive to CK2 treatment, while the phospho-mimetic mutant mS ($SSSS^{549\text{-}542}\rightarrow DDDD$) itself exhibites weak DNA binding compared to wild type mS, suggesting a charge effect mechanism. If so, then a mutant with R→Q substitution ($RRRRR^{506\text{-}511}\rightarrow QQQQQ$) in SNAP190 will deactivate

the CK2 inhibition by phophorylating the serine rich region. This mutant has been constructed. The ability of DNA binding and transcription of this mutant can be tested in an EMSA and in vitro reconstitution transctiption system.

To explore whether the N-terminal region (aa 20-63) of SNAP190 also contributes to CK2 inhibition, the non-phosphorylatable mutant with SNAP190 ($Ser^{28, 35, 39, 41, 59}\rightarrow$AAAAA) was generated and tested for DNA binding activity. My preliminary data shows that DNA binding activity of the non-phosphorylatable mS ($Ser^{28, 35, 39, 41, 59}\rightarrow$AAAAA) is less sensitive to CK2 treatment compared to wild type mS. However, the double mutant mS ($Ser^{28,35,39,41,59}\rightarrow$AAAAA /$EED^{543\text{-}545}\rightarrow$AAA) DNA binding did not alter upon CK2 treatment. These results suggest that CK2 phosphorylation at both regions (aa 20-63 and aa 514-545) of SNAP190 negatively regulates $SNAP_C$ function. Further experiments to test mutant $SNAP_C$ DNA binding activity and their abilities to direct snRNA transcription are needed to understand the mechanism.

It has been proposed that in the full length $SNAP_C$, SNAP190 had a built-in damper located within the C-terminal two-thirds of SNAP190 aa (506-1469) to downregulate its binding to DNA, which was deactivated by a direct protein-protein contact between SNAP190 (aa 869-912) and the Oct-1 POU domain (Mittal et al., 1999). In the context of mini-SNAP complexes, upon CK2 treatment, DNA binding activity of mS containing SNAP190 aa (1-719) was greatly reduced, while mSΔC lacking the serine rich region did not change (Figure III-5), however, mS($EED^{543\text{-}545}\rightarrow$AAA) with the non-phosphorylatable serine rich region was also reduced, but to a lesser degree comparing to mS. These observations imply that the region of SNAP190 aa 506-719 probably acts as a damper of DNA binding. As the cooperative binding between TBP and $SNAP_C$ can

overcome the CK2 inhibition (Figure II-6), it is not clear whether TBP functions through direct contact with the CK2 phosphorylated region of SNAP190 (aa 20-63), or TBP has higher affinity to interact with SNAP190 RcRd than the CK2 phosphorylated region of SNAP190 (aa 514-545) in order to release CK2 inhibition. To test this, TBP recruitment to the promoter DNA by the various deletions or point-mutations within mini-SNAP complexes can be measured by EMSA. The region within SNAP190 that is targeted by CK2 to inhibit DNA recognition can be determined by comparing the deletions or mutations in SNAP190 in the context of mini-$SNAP_C$. Thus, we might be able to distinguish which region contributes to DNA recognition inhibition and the mechanism for TBP antagonizing function.

Previously, we had demonstrated that SNAP190 is phosphorylated in vivo (Figure II-4). Endogenous SNAP190 has been reported to be phosphorylated in HeLa cells (Beausoleil et al., 2004). It is unclear, however, whether CK2 sites in SNAP190 we mapped in vitro are also phosphorylated in the cells. Raising antisera specifically recognize phosphoserines in SNAP190 (anti-phospho-SNAP190 antibody) will help us to answer the questions about whether the CK2 sites in SNAP190 are also phosphorylated in the cells. It will also be interesting to know whether SNAP190 phosphorylation by CK2 is regulated in a cell cycle dependent manner and/or whether the level of CK2 phosphorylation correlates with snRNA gene transcription.

In conclusion, CK2 negatively regulates human U1 snRNA gene transcription by inhibiting the basal transcription factor $SNAP_C$ DNA binding and transcription activity. The cooperation between $SNAP_C$ and TBP can antagonize CK2 inhibition of DNA

binding by $SNAP_C$, implying that CK2 may differentially regulate snRNA gene transcription by different RNA polymerases.

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