Structure Probing of U4 snRNA: Investigations into the Mechanism of U4/U6 di-snRNP Formation

by

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ABSTRACT

Precursor messenger RNA splicing is catalyzed by a dynamic complex termed the spliceosome. An essential step in spliceosome assembly occurs when U4 snRNA base pairs with U6 snRNA to form the U4/U6 di-snRNP. Although failure to form the di-snRNP is lethal, the mechanism by which di-snRNP formation occurs is still unknown, in part due to lack of information about the structure of free U4 snRNP prior to binding U6. To investigate the contribution of U4 in di-snRNP formation, I carried out a series of structure probing experiments to determine the secondary structure of a Brr2 released U4 snRNP. The structural model establishes the presence of four stem loops in yeast U4 snRNA, including a novel short stem loop at the extreme 5' end of the molecule. To determine which nucleotides of U4 are required for base pair formation, I carried out a modification/interference experiment. Modification of the 5' stem loop uridines (U5, U6, and U8) interfered with di-snRNP formation, while modification of uridines within the central and 3' regions of U4 snRNA did not inhibit di-snRNP formation. Based on these results, I propose that intermolecular base-pairing between U4 loop nucleotides (U6 – A11) of the novel stem loop and U6 snRNA nucleotides (U70 – A75) may initiate di-snRNA formation. The U6 specific protein Prp24 would catalyze the subsequent annealing and stabilization of the U4/U6 intermolecular helices I and II.

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Chapter One - Introduction

Precursor messenger RNA splicing (pre-mRNA splicing) is the process by which non-coding introns are removed from transcribed pre-mRNA and coding exons are ligated to form mature mRNA, a template for protein synthesis. It is estimated that over 90% of human genes contain introns, and on average a gene is thought to contain 8.8 exons and 7.8 introns (Jurica 2008, Sakharkar et al. 2004). Thus in humans and other eukaryotes pre-mRNA splicing plays an important role in gene regulation and proteome diversification, and it is therefore important to understand how splicing is accomplished.

Of the five small nuclear ribonucleoprotein particles (snRNPs) that help to facilitate the removal of introns, U4 is the least well characterized. Due to the difficulty in purifying free U4 snRNP, the RNA secondary structure as well as the protein composition of this snRNP has not been determined biochemically. It is known that U4 snRNA must first base pair to U6 snRNA, then subsequently dissociate for splicing to occur, but it is unknown how these interactions are initiated and regulated. A major goal of this thesis was to determine the secondary structure of the U4 snRNA in the free snRNP form as an effort towards understanding how U4 can interact with and regulate U6.

1.1 Pre-mRNA Splicing

Pre-mRNA splicing is catalyzed by a large and dynamic complex known as the spliceosome, and occurs via two consecutive transesterification reactions (Figure 1). In the first, the 2' hydroxyl group of the bulged branchpoint adenosine attacks the 5' phosphoryl group of the 5' exon/intron junction (5' splice site). The result is the simultaneous formation of a free 5' exon and a lariat intron/exon 2 intermediate. The second step involves an attack by the 3' hydroxyl group of the free 5' exon on the 5' phosphoryl group of the 3' exon/intron

junction (3' splice site), resulting in ligation of the two exons and removal of the intron as a lariat (Ruskin et al. 1984, Padgett et al. 1984, Domdey et al. 1984).

The spliceosome is composed of five snRNPs and additional protein factors. Each snRNP consists of a uridine rich small nuclear RNA (snRNA) designated U1, U2, U4, U5, or U6, and a set of specific and tightly associated proteins. Assembly of a fully functional spliceosome and excision of an intron involves both the formation of snRNP particles, and the association and rearrangement of snRNPs and other protein components with respect to the pre-mRNA.

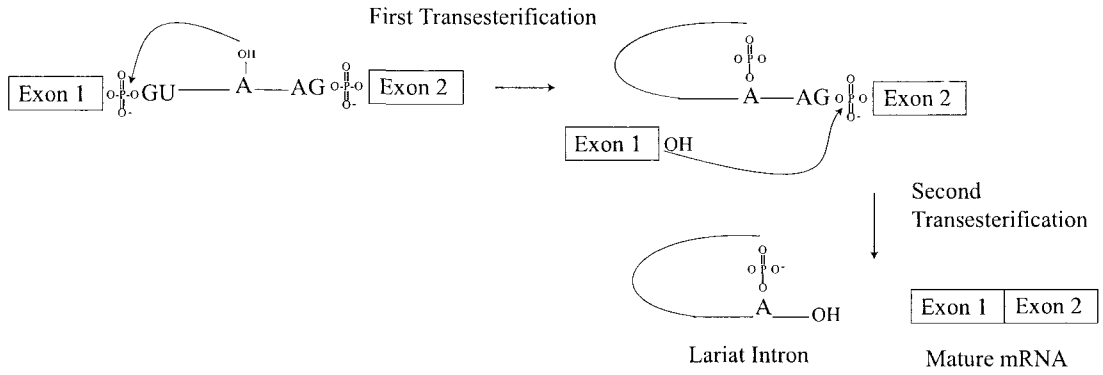


Figure 1. The excision of an intron from pre-mRNA by means of two consecutive transesterification reactions. The 5' and 3' consensus nucleotides are indicated along with the branchpoint adenosine.

Spliceosome assembly is initiated when U1 snRNP recognizes and base pairs to the 5' splice site of the pre-mRNA (Ruby and Abelson 1988; Figure 2). Concomitantly, U2 snRNP recognizes and binds to the branch point sequence, resulting in the branchpoint adenosine bulge. The U6 and U4 snRNPs base pair to form the U4/U6 di-snRNP, which subsequently interacts with U5 to form the U4/U6.U5 triple snRNP. The U4/U6.U5 triple snRNP is then integrated into a precatalytic complex where numerous RNA: RNA, RNA: protein, and protein: protein rearrangements occur to form the catalytic complex (Cheng and

Abelson 1987). During these structural rearrangements the U1 snRNP dissociates from the 5' splice site while the U4 snRNP is unwound from the U6 snRNP, allowing U6 and U5 to base pair with the 5' splice site, and U6 to base pair with U2 snRNA (Brow 2002).

Presently, it is unclear whether U1 and U4 snRNPs remain loosely associated with the activated spliceosome or whether they dissociate entirely from this complex, but splicing has been shown to occur in the absence of these snRNPs (Cheng and Abelson 1987, Yean and Lin 1991). The resultant active spliceosome consisting of U2, U5, and U6 snRNPs facilitates the transesterification reactions. From the current model of spliceosome assembly it can be seen that the snRNPs are structurally dynamic, and as the spliceosome is thought to be assembled *de novo* onto each intron that is removed, recycling of the snRNPs is critical.

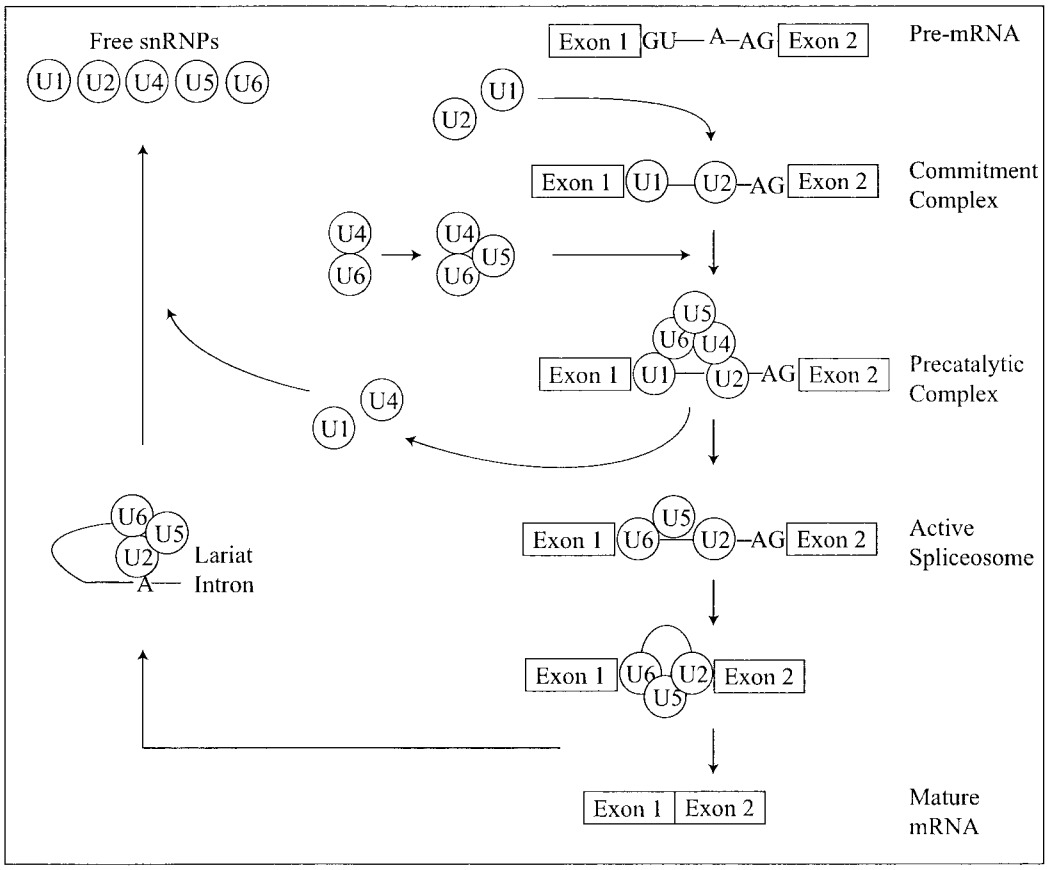


Figure 2. Spliceosome assembly and catalysis occurs in an ordered and sequential manner.

1.2 U6 snRNP

U6, the most highly conserved snRNA, is essential and is believed to play a central role in the catalysis of splicing (Brow and Guthrie 1988, Valadkhan and Manley 2001, Yean et al. 2000). In splicing extract, U6 is thought to be largely present in free snRNP form (~80%), where it is associated with the proteins Lsm2-Lsm8, and Prp24 (Li and Brow 1993, Jandrositz and Guthrie 1995, Achsel et al. 1999, Shannon and Guthrie 1991). Before incorporation into the active spliceosome, U6 must first base pair with U4 to form the U4/U6 di-snRNP. Di-snRNP formation is catalyzed by the U6 snRNP protein Prp24, and results in conformational changes in both U6 and U4. The conversion of free U6 snRNP into a di-snRNP is necessary for spliceosome assembly and subsequent catalysis, but the mechanism by which these conformational changes occur is still unknown.

To understand how di-snRNP initiation can occur between free U6 and free U4 snRNPs, the secondary structures of the free snRNPs must first be determined. Although a number of structural models for both the yeast and mammalian U6 snRNA in the free snRNP form have been proposed (Fortner et al. 1994, Vidaver et al. 1999, Ryan et al. 2002, Karaduman et al. 2006, Dunn and Rader 2010; Figure 3), and the RNA secondary structure has been characterized via chemical modification several times as both free RNA (Mougin et al. 2002), and in the free U6 snRNP both *in vitro* (Jandrositz and Guthrie 1995, Karaduman et al. 2006) and *in vivo* (Fortner et al. 1994), there is still debate over the structure of free U6 snRNP.

Until recently, published models of free U6 snRNP mostly agreed on the presence of 5' and 3' intramolecular stem loops (ISL), with discrepancies in the central portion of the models (Figure 3). Support for the 3' ISL comes from dimethylsulfate (DMS) probing *in*

vivo (Fortner et al. 1994). DMS can diffuse into living cells and methylate adenines and cytosines of single-stranded RNA. Two of the possible three nucleotides in the 3' loop were strongly modified by DMS, providing support for a free loop structure in U6 encompassing nucleotides G71 – A75. However, modification patterns obtained from this experiment cannot be unequivocally assigned to the free U6 snRNP, as U6 undergoes multiple interactions including formation of the U4/U6 di-snRNP, and the U6/U2 catalytic core where the 3' ISL is thought to be a critical structural feature.

U6 snRNP purified by TAP-tagged Prp24 and glycerol gradient centrifugation was analyzed by chemical modification and found to have a more accessible structure compared to *in vitro* transcribed U6 snRNA, especially in the bulge and 3' stem loop regions (Karaduman et al. 2006). The results showed that Prp24 and the Lsm proteins induce a conformational change in the U6 snRNP that allows the 3' stem loop and positions A79 and U80 to become available for interaction with U4 snRNA. This highlights the importance of protein binding inducing a conformational change in an snRNA. Also, this is in agreement with a number of biochemical studies suggesting that the Lsm proteins on the 3' end of U6 snRNA play a key role during U4/U6 biogenesis (Achsel et al. 1999, Verdone et al. 2004), and that Prp24 is necessary for di-snRNP formation (Raghunathan and Guthrie 1998).

Hydroxyl radical probing of the U6 snRNP suggested that the binding site of Prp24 occurs over the range of nucleotides 4-60 encompassing the 5' stem loop, but not the 3' stem loop, and UV-crosslinking indicated that Prp24 is bound to nucleotides 28, 29, 38, and 55 (Karaduman et al. 2006). Similarly, structure probing of glycerol gradient purified U6 snRNP indicated that Prp24 was likely bound to nucleotides A40-C43 (Jandrositz and

Guthrie 1995). However, it is unclear how Prp24 helps to facilitate the conformational rearrangements necessary for di-snRNP formation.

In spite of past models for the free U6 snRNP suggesting the presence of a 3' ISL, the secondary structure of the snRNA was not conserved among species, which lead to the proposal of a novel U6 secondary structure which is conserved among all known species (Dunn and Rader 2010; Figure 3). In the Dunn/Rader model the 3' ISL has been dissolved, and instead forms two intramolecular helices denoted Stem/loop A and B. Further experiments are needed to resolve the structure of the free U6 snRNP, however all models propose the U6 nucleotides C72, A73, U74, and A75 (*S. cerevisiae* numbering) to be unpaired in the free U6 as either an apical loop or a bulge, and consequently available for initial base pairing with the U4 snRNP.

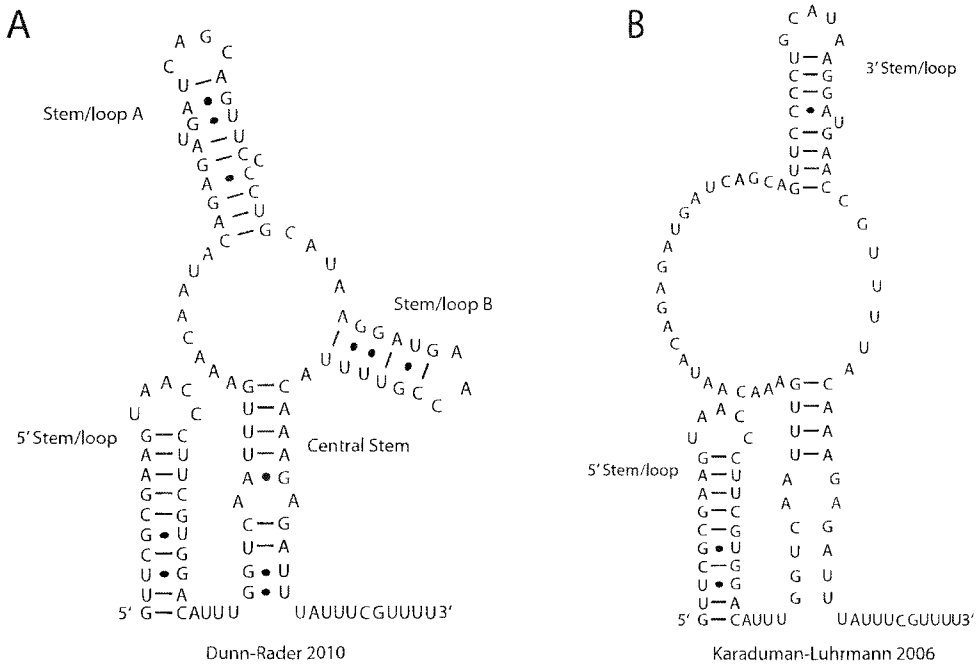


Figure 3. Secondary structure models of yeast U6 snRNA in free U6 snRNP. A) New model of free U6 snRNA secondary structure proposed by Dunn and Rader, 2010. B) Main model of free U6 snRNA secondary structure accepted as of 2009.

1.3 U4 snRNP

Contrary to the free U6 snRNP, in splicing extract the predominant species of U4 is found complexed to U6. Although U4 is essential, little is known about the structure and composition of this particle due to the minimal amounts of free U4, and the difficulty in isolating and purifying free U4 snRNP. It is unknown if any snRNP specific proteins associate with free U4, and if so, whether or not these proteins stabilize a particular conformation of the RNA. Currently it is believed that the U4 snRNP may function as a negative regulator of U6 by binding to and sequestering catalytically important regions of U6 snRNA (Brow and Guthrie 1988). It is also possible that U4 helps to activate U6 by positioning U6 in the pre-catalytic spliceosome in a manner that helps to facilitate the conversion to an active spliceosome (Dunn and Rader 2010).

Although, no snRNP specific proteins have been identified in the free U4 snRNP, it is possible that the U4/U6 specific proteins may be brought to the di-snRNP complex by association with the free U4 snRNP. In humans, proteins 15.5K, 61K, 20K, 60K, and 90K have been shown to interact with the U4/U6 di-snRNP in a hierarchical manner (Nottrott et al. 2002). Except for the 20K protein, which does not have a homologue in yeast, the proteins Snu13 (15.5K), Prp4 (60K), Prp3 (90K), and Prp31 (61K) have also been shown to interact with the yeast U4/U6 particle. Analysis of the interactions of these proteins in humans has led to the observation that 15.5K binding to the U4 5' kink turn stem loop is necessary for the subsequent binding of 61K and the triple protein complex of 20/60/90K (Nottrott et al. 1999, Nottrott et al. 2002, Schultz et al. 2006). In addition, binding of 20/60/90K appears to occur through interactions of the 90K protein with stem II of the U4/U6 duplex (Nottrott et al. 2002). In humans all of the U4/U6 di-snRNP specific proteins

are destabilized or released in the rearrangement to an activated spliceosomal complex (Makarov et al. 2002). These proteins may be released bound to U4 snRNA, and given the putative binding site locations in the di-snRNP, 15.5K (Snu13) and 61K (Prp31) appear to be the strongest candidates for binding the U4 snRNA in the free snRNP.

It is known that the U4 snRNA interacts with the Sm proteins B/B' (alternatively spliced protein products that differ by only 11 residues at the C-termini), D1, D2, D3, E, F, and G. The Sm proteins are common to U1, U2, U4 and U5 snRNAs, and these proteins interact with a uridine rich Sm binding site, which in U4 is located at the 3' end of the snRNA (Reviewed in Nagai et al. 2001). Anti-Sm antibodies have been shown to immunoprecipitate U4/U6 and U4/U6.U5 particles, but the Sm proteins do not bind directly to the U6 snRNA in the free U6 snRNP (Luhrmann et al. 1990, Seraphin 1995). Therefore they are not brought to the U4/U6 particle by free U6, and may be associated with the free U4 snRNP, or associate after U4/U6 formation.

The structure of phenol/chloroform extracted U4 snRNA from yeast U4/U6.U5 tri-snRNP has been analyzed by chemical and enzymatic probing, and fit onto a previously proposed secondary structure by the Branlant lab (Mougin et al. 2002, Krol and Branlant 1981, Myslinski and Branlant 1991; Figure 4). The data shows support for a 5' (kink-turn) stem loop structure encompassing nucleotides 13-60, a central stem loop (61-82), and a 3' stem loop structure (91-142). Human and other eukaryotic U4 snRNAs can also adopt this secondary structure confirmation, and many eukaryotes encompass an additional stem loop 3' of the Sm binding site that is not present in yeast. Notably, the structure of the first 10 nucleotides has not been analyzed by structure probing techniques, and this region has been proposed to be single stranded, despite the high degree of sequence conservation (Myslinski

and Branlant 1991). In addition, the structure of U4 snRNA has yet to be analyzed in the snRNP form, and it is conceivable that association with proteins might stabilize an alternate conformation of the RNA.

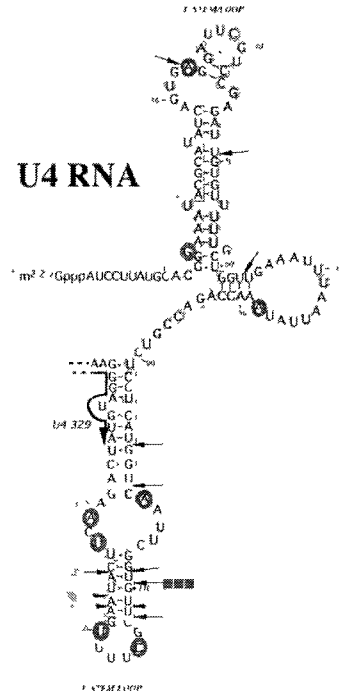


Figure 4. Chemical modification and enzymatic cleavage data of *Saccharomyces cerevisiae* U4 snRNA phenol/chloroform extracted from U4/U6.U5 triple-snRNP. Circles indicate sites of chemical modifications, and squares indicate sites of cleavage by ribonuclease V_1: red indicates strong, yellow indicates moderate, and green indicates weak reactions. Blue boxes indicate sites of no modification, and black nucleotides indicate areas of primer binding, or reverse transcriptase stops (Mougin et al. 2002).

1.4 U4/U6 di-snRNP

To be activated for assembly into the spliceosomal complex, U6 snRNA must first base pair with U4 snRNA to form the U4/U6 di-snRNP. This complex is obligatory for U6 incorporation into the active spliceosome, and for subsequent spliceosomal function. Prior to the first step of splicing, U4 must dissociate to allow U6 to form mutually exclusive base pairs with U2 in the catalytically active spliceosome.

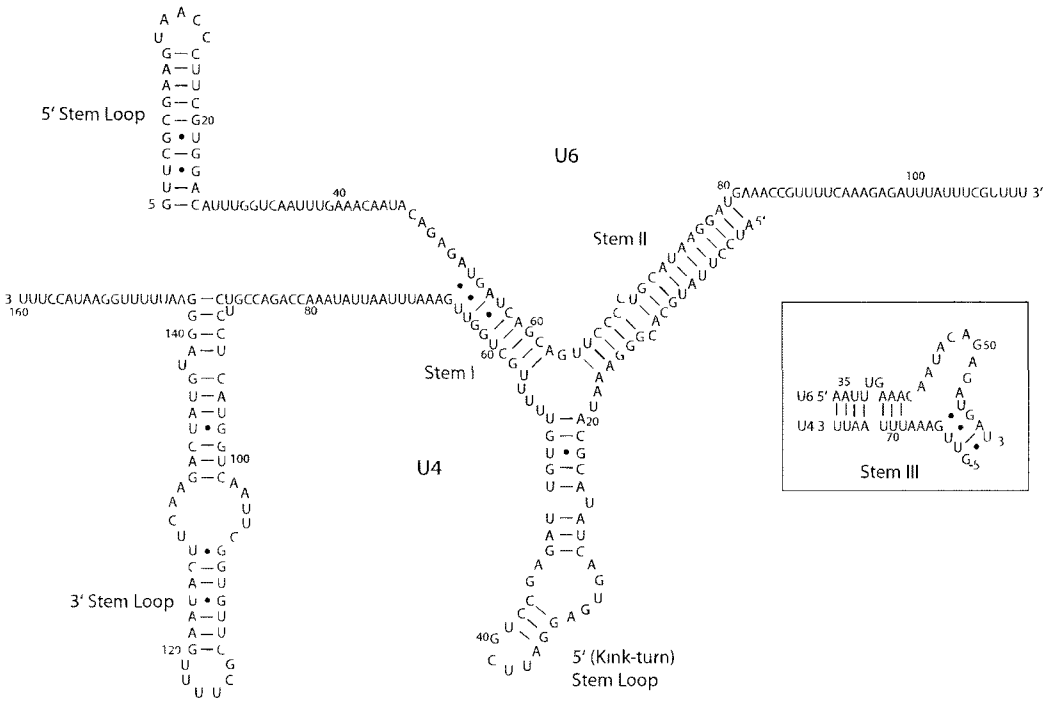


Figure 5. Proposed secondary structure for yeast U4/U6 di-snRNA (Brow and Guthrie 1988). Inset – Stem III proposed by Jakab et al. 1997.

The mechanism by which the di-snRNP forms is poorly elaborated but central to our understanding of snRNP biogenesis. The proposed secondary structures of both U4 and U6 snRNAs in the singular form differ substantially from the secondary structure of the U4/U6 RNA in the di-snRNP (Figure 5). In the di-snRNP, U4 and U6 snRNAs are stably associated with each other in two regions that base pair to form intermolecular helices denoted stems I and II, and which are separated by the 5' (kink-turn) stem loop of U4 (Brow and Guthrie 1988, Mougin et al. 2002, Shannon and Guthrie 1991). Biochemical, phylogenetic, and genetic experiments including psoralen cross-linking of HeLa cells (Rinke et al. 1985), and chemical modification of yeast and human tri-snRNPs (Mougin et al. 2002), support the U4/U6 interaction domain of Stem I and II (Brow and Guthrie 1988). In addition, a putative stem III region has been proposed based on sequence comparison between organisms (Jakab

et al. 1997) in which nucleotides 34-37 and 40 - 42 of U6 base pair with nucleotides 75 – 72 and 71 - 69 of U4 (Figure 5 inset).

1.5 Kissing Loop Model of U4/U6 di-snRNP Formation

An RNA-RNA kissing loop is an interaction in which base pairing occurs between two complementary sequences in the apical loops of two RNA hairpins (Li et al. 2006). Kissing loop complexes have been observed in a variety of RNAs such as tRNAs (Ladner et al. 1975), and ribozymes (Lehnert et al. 1996, Rastogi et al. 1996, Anderson and Collins 2001), and are essential in biological processes including dimerization of retroviral RNAs (Kim and Tinoco 2000, Reviewed in Paillart et al. 1996). A well-known kissing loop complex controls replication of the ColE1 plasmid in *Escherichia coli*. Replication is initiated by a primer known as RNAII, and negatively regulated by plasmid encoded antisense RNAI. RNAI interacts with RNAII in a kissing loop complex that is stabilized by ColE1 encoded protein RNA one modulator (ROM) (Reviewed in Brunel et al. 2002).

U6 and U4 snRNAs may initiate intermolecular base pairing by means of an RNA-RNA kissing loop interaction, in which two intramolecular stem loops form intermolecular base pairs between the loops (Figure 6). Stabilization of the initial RNA base pairing contact between the loops would facilitate formation of the intermolecular helices. Karaduman et al. 2006 suggested that the U6 3' stem loop resembled a kissing loop structure, yet the corresponding U4 nucleotides are predicted to be single stranded in the current model (Mougin et al. 2002). In the kissing loop model the U4 stem loop should be directly opposite the U6 stem loop in the base-paired di-snRNA, and encompass loop nucleotides A7, U8, G9, and C10. We have conducted a sequence comparison analysis that demonstrates that the corresponding 5' end of U4 snRNA can form a short stem loop in all species examined

(unpublished results). However, the putative stem may encompass 2 to 5 base pairs, and 0 or 1 bulged nucleotides, making covariation analysis difficult.

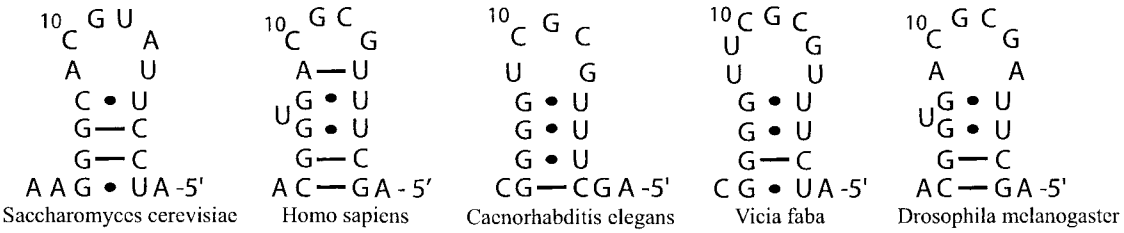


Figure 6. The short kissing stem loop can be formed at the 5' end of U4 snRNA from the organisms *Saccharomyces cerevisiae*, *Homo sapiens* (NCBI Accession Number X59361), *Caenorhabditis elegans* (X07828), *Vicia faba* (X07112), and *Drosophila melanogaster* (K03095).

Support for a U4/U6 kissing loop interaction comes from modification/interference analysis of human U6 snRNA with affinity purified U4 snRNP indicating that the U6 3' loop nucleotides G65-A70 (analogous to yeast nucleotides G71-A76) were essential for initiating base pair formation with U4 (Wolff and Bindereif 1993). These nucleotides are located in the middle of stem II in the U4/U6 di-snRNP. In addition, when nucleotides A53, C55, and A56 (located in stem I of U4/U6) were modified they were found to weakly interfere with the formation of the di-snRNP. This study suggests that the location of primary nucleation in di-snRNA formation is in the center of stem II, and that an auxiliary site of di-snRNP nucleation may be in the center of stem I.

In an *in vivo* study using *Xenopus* oocytes substitution of the proposed kissing loop nucleotides in the U4 snRNA blocked di-snRNP formation, while substitution of surrounding nucleotides allowed formation of the di-snRNP to continue (Vankan et al. 1990). The results demonstrate strong support for an initial interaction between U4 and U6 that leads to propagation of stem II, though it is unclear if this region in U4 forms a stem-loop structure.

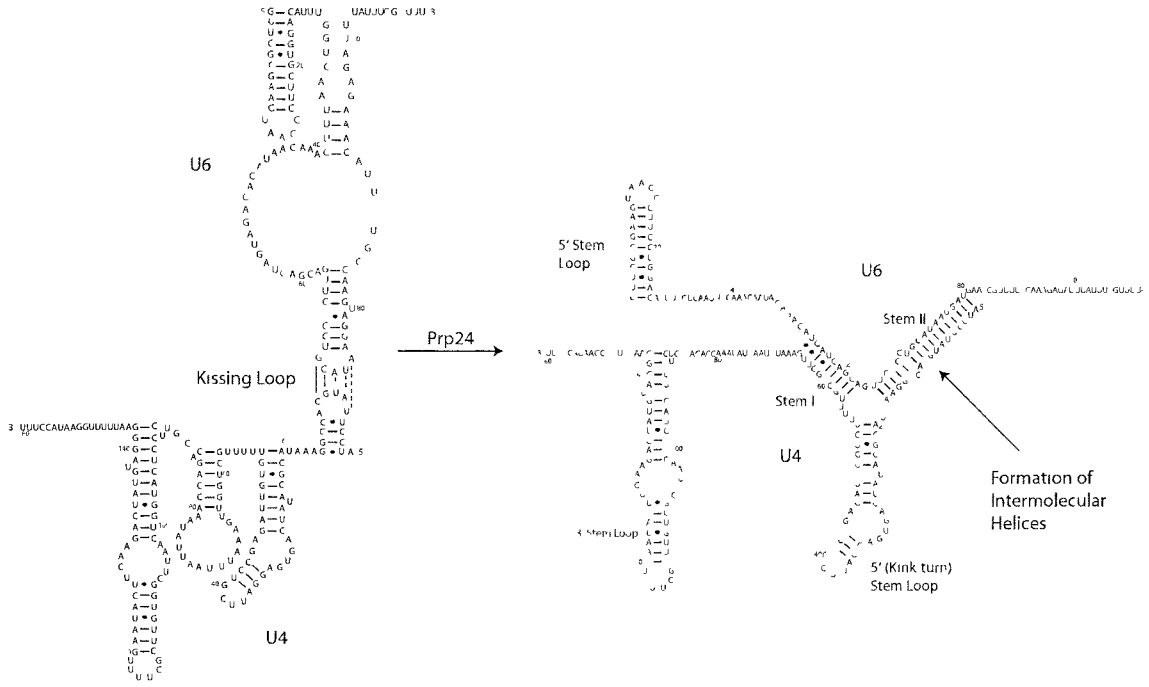


Figure 7. The kissing loop interaction may initiate formation of U4/U6 intermolecular base pairing.

1.6 Overall Research Objective

Understanding the mechanism of formation of the U4/U6 di-snRNP will allow us to propose better hypotheses for how spliceosome assembly is regulated, as well as for the functional significance of U4. My overall research objective was to establish biochemically which nucleotides of U4 are important in U4/U6 di-snRNP formation *in vitro*. To understand how U4 can interact with U6 I first required information about the secondary structure in the snRNP form, therefore my initial objective was to elucidate the structure of U4 snRNA in the free snRNP by means of chemical and enzymatic probing. This allowed me to investigate whether a short 5' stem loop was present and available for a kissing loop interaction. Genetic modification of U4 snRNA at the C12 position was employed to determine if stabilization of a supposed base pair in the 5' stem loop would affect yeast growth, or U4/U6 di-snRNP formation *in vivo*. Finally, modification/interference

experiments were done to determine whether U6 and U4 snRNAs could potentially initiate intermolecular base pairing by means of an RNA-RNA kissing loop interaction.

Chapter Two – Generation of Free U4 snRNP via the Brr2 Release Preparation

In order to determine which nucleotides are sequestered within the free U4 snRNP, and which nucleotides are available for intermolecular base-pairing with U6, the free U4 snRNP had to be generated. In splicing extract, the predominant species of U4 snRNP is bound to U6 in the U4/U6 di-snRNP. Therefore free U4 snRNP was generated from duplex U4/U6 di-snRNP by a Brr2 release preparation (Raghunathan and Guthrie 1998). Brr2 is a snRNP specific protein associated with U5 snRNA (Lauber et al. 1996) that contains two RNA helicase domains, and has been shown to unwind U4/U6 di-snRNAs. Based on independent identification of Brr2 *in vitro* by four different labs and the fact that only Brr2 has been shown to unwind U4/U6 duplex *in vivo* (Kim and Rossi 1999), the evidence strongly supports the assumption that Brr2 facilitates unwinding of the U4/U6 di-snRNP in the conversion to an active spliceosome (Laggerbauer et al. 1998, Raghunathan and Guthrie 1998).

The mechanism by which Brr2 initiates unwinding of the duplex is currently unknown. It has been suggested that a number of splicing factors, including U5 snRNP proteins Prp8 and Snu114, may play an important role in signalling Brr2 activity (Kuhn et al. 1999, Small et al. 2006, Reviewed in Frazer et al. 2008 and references within), and that Brr2 and Prp24 may have antagonistic properties, whereby an annealing and dissociation reaction may be constantly occurring in kinetic preparation for entry into the splicing cycle (Raghunathan and Guthrie 1998). This is not surprising considering that U4/U6 dissociation is critical to initiate splicing, thus the timing must be precise, and Brr2 may be tightly regulated by other splicing factors.

Importantly, the Brr2 release is thought to generate snRNP particles, which for di-snRNP formation is advantageous compared to *in vitro* transcribed RNA. Formation of the di-snRNP has been found to be more efficient in the presence of the U6 Lsm proteins than with naked U6 snRNA (Rader and Guthrie 2002, Verdone et al. 2004). It is currently unknown how the presence of the U4 Sm or snRNP specific proteins affects di-snRNP formation.

2.1 Materials and Methods

Splicing Extract Preparation

Whole cell extract was prepared from a polyoma tagged Brr2 yeast strain (YSR193) as previously described (Ansari and Schwer 1995). Briefly, 4L of yeast culture was grown to an OD_{600} between 1.5 and 2. Cells were harvested, washed, and resuspended in cold AGK buffer (10mM Hepes-KOH pH 7.9, 1.5mM $MgCl_2$, 200mM KCl, 0.5mM DTT, 10% glycerol), dripped through a 21 gauge needle, and frozen in liquid nitrogen. Frozen cell pellets were lysed and homogenized using a mortar and pestle. Cellular debris was removed from the slurry and the supernatant was dialyzed into Buffer D (20mM Hepes-KOH pH 7.9, 0.2mM EDTA, 50mM KCl, 0.5mM DTT, 20% glycerol), snap frozen and stored at -80°C.

Brr2 Release Preparation

Free U4 and U6 snRNPs were generated with the Brr2 release preparation as previously described (Raghunathan and Guthrie 1998). Two and a half milliliters of splicing extract (YSR 193) was centrifuged for 10 minutes at 16,100 RCF, 4°C to remove cellular debris. Seventy-five micrograms of anti-polyoma antibodies (Glu-Glu monoclonal antibody that recognizes a six amino acid sequence EY/FMPME, Covance) was added to the splicing extract and nutated at 4°C for at least 30 minutes. One hundred microliters of Protein G

Sepharose slurry (GE Healthcare) was washed 3 times with 1mL of cold Net50 (50mM Tris-HCl pH 7.5, 50mM NaCl, 5mM EDTA, 0.05% Igepal) for 1 minute at 500RCF, 4˚C. Splicing extract/antibodies were added to the washed resin and nutated overnight at 4˚C.

The resin was gently pelleted for 1 minute at 500RCF, 4˚C to remove the flowthrough, and washed 3 times with 14mL of cold Net50 (3 minutes, 500RCF, 4˚C). The washed resin was then incubated with splicing mix and ATP (40% Buffer D, 2.5mM $MgCl_2$, 60mM K-phosphate pH 7.2, 3% PEG 8000, 2mM ATP) for 10 minutes at room temperature. The resin was pelleted at 500RCF for three minutes at room temperature, and the supernatant was snap frozen and stored at -80˚C. This elution procedure was repeated 2 to 4 times, and all subsequent elution fractions and the resin were snap frozen and stored at -80˚C.

Phenol/Chloroform Extraction

Samples were diluted with 0.3M NaOAc to a total volume of 500µL, extracted twice with 25:24:1 phenol/chloroform/isoamyl alcohol (Sigma), and back extracted once with chloroform (Sigma). Twenty micrograms of glycogen and 100% ethanol was added to the aqueous layer to a total volume of 1.5mL, and the RNA was precipitated for 30 minutes at 13,200RPM, 4˚C. Pellets were washed with 70% ethanol and resuspended in 10µL of 1X native gel loading buffer (12.5% glycerol, xylene cyanol, bromophenol blue) for Northern blot analysis.

5'End Labeling of DNA Oligonucleotides

Two and a half microliters of 10X T4 Polynucleotide Kinase Buffer (NEB) and 14.8µL of ddH_2O was added to 7 picomoles of DNA oligonucleotide. Five microliters of [γ-^{32}P] ATP (PerkinElmer) was added to this along with 20 units of T4 Polynucleotide Kinase (NEB) to a total volume of 25µL and incubated at 37°C for one hour. The reaction was

diluted to 50μL with ddH_2O and unincorporated [γ-^{32}P] ATP was removed with a G25 or G50 microspin column (GE Healthcare) according to the manufacturer's instructions.

Northern Blot

Samples were run on a pre-chilled 4.5% non-denaturing gel at 400V for 40 minutes at 4°C. The gel was then transferred to Amersham Hybond-N+ nylon transfer membrane (GE Healthcare) for 15 minutes at 450mA (32mA per cm^2) using a semi-dry electroblotter (Owl Panther Hep-3). The RNA was crosslinked to the membrane in a UV Stratalinker 1800 (Stratagene) with 120,000J of ultraviolet radiation. The membrane was then blocked with 10mL of Rapid-Hyb Buffer (GE Healthcare) for at least 30 minutes at room temperature, followed by incubation with labeled DNA oligonucleotide (14b) for at least 1 hour at room temperature. The membrane was washed 3 times for 3 minutes with 10mL of Wash Buffer (6X SSC, 0.2% SDS), and then exposed to a phosphor screen (PerkinElmer) overnight. The autoradiogram was visualized and quantified with Cyclone © phosphorimager and OptiQuant © Software (Packard Instruments).

Solution Hybridization (Brow Gel)

Following RNA extraction samples were resuspended in 9μL of ddH_2O and 1μL of 10X Brow hybridization buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA), incubated with 100,000 counts of labeled oligonucleotide 14b, and heated to 42°C for 8 minutes. Two microliters of 4X native gel loading buffer was added to the sample and loaded onto a pre-chilled 6% non-denaturing gel, run at 300V for 45 minutes at 4°C, and exposed and quantified (as above).

Labeled Oligonucleotide Used for Northern Blots and Solution Hybridization Gels

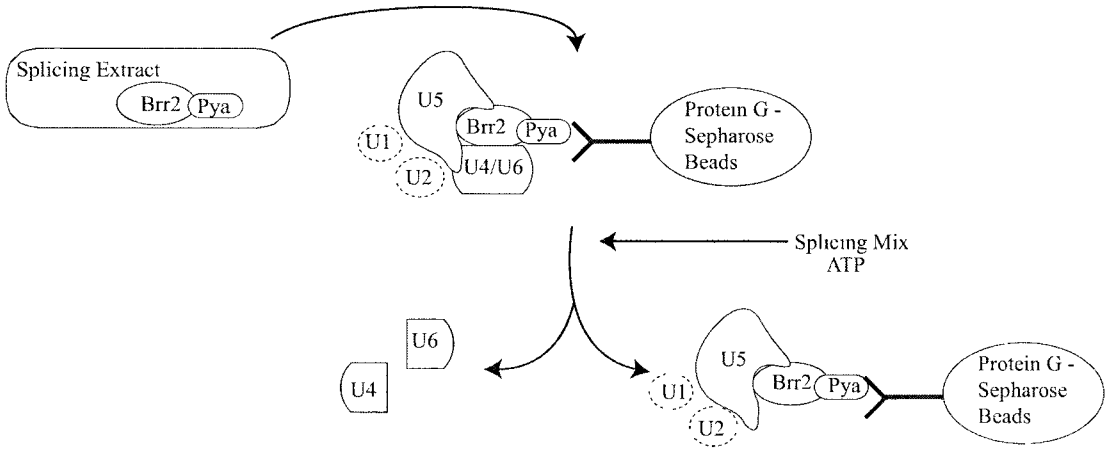
14b: 5' – AGGTATTCCAAAAATTCCCTAC – 3' (binds U4 nucleotides 158 – 137)

2.2 Results and Discussion

In the Brr2 release, U4/U6.U5 triple-snRNPs and possibly higher orders of snRNP complexes (that may include U1 and U2 snRNPs) bind to the resin through an interaction with the polyoma tagged Brr2 and the monoclonal polyoma antibodies linked to Protein G Sepharose resin. Binding of these snRNP complexes to the resin is characterized by decreased amounts of U4/U6 di-snRNP in the flowthrough (material that has not bound to the resin) fraction. After washing the resin to disrupt proteins and RNA that bind non-specifically, ATP is used to promote dissociation of the di-snRNP via Brr2. Elution fractions should ideally contain only free U4 and free U6, and any remaining di-snRNP should be retained on the resin. If the Brr2 release is optimized to generate maximal amounts of free U4 and U6 snRNPs, only a small amount of di-snRNP should remain associated with the resin.

Extracted RNA from splicing extract, flowthrough, elution fractions and the remaining resin can be visualized by means of Northern blot, or solution hybridization. For Northern blots, the extracted RNA is run on a non-denaturing gel, transferred to a membrane, and the membrane is probed with labeled oligonucleotides complementary to U4 or U6. In solution hybridization, the labeled oligonucleotide is added to a fraction prior to separation on a non-denaturing gel. The advantage of a Northern blot is that the membrane can be stripped of labeled oligonucleotide, and re-probed with different oligonucleotides complimentary to another species, allowing for easy determination of different molecules in the same fraction. Solution hybridization is a less time consuming technique, and I have found solution hybridization to be a more sensitive technique for visualizing small amounts of RNA.

A)



B)

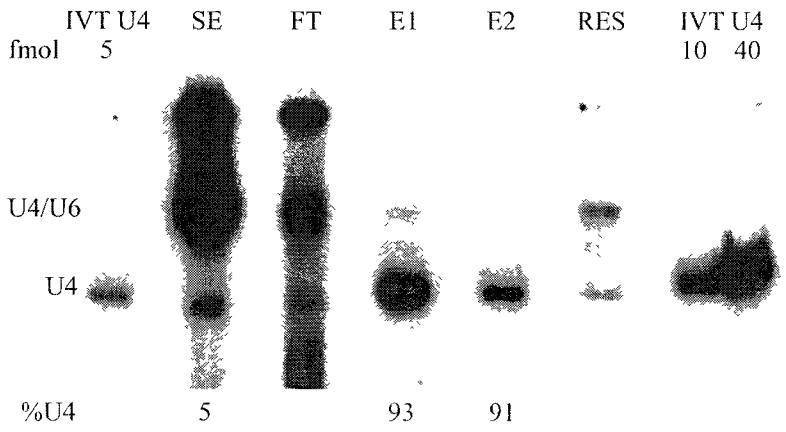


Figure 8. The Brr2 release preparation generates free U4 snRNP. A) Schematic diagram of the Brr2 release preparation. U1 and U2 snRNPs that may or may not associate with the U4/U6.U5 triple-snRNP are indicated with a dotted outline. B) Solution hybridization gel probed for U4 with labeled oligonucleotide 14b, with 1.5% of the total loaded in each lane. (SE – splicing extract, FT – flowthrough, E1 and E2 – elutions, RES – resin).

The Brr2 release preparation consistently enriched for free U4 snRNP in the elution fractions compared to splicing extract (Figure 8). A small amount of U4/U6 di-snRNP was found to be present in the elutions, likely from leaching off of the resin. At maximum the U4/U6 di-snRNP accounted for 16% of the U4 snRNP species present in an elution, but it

was frequently found to be between 2% and 10%. Any elution that was found to contain more than 16% U4/U6 di-snRNP was not used for subsequent structure probing.

A number of Brr2 release conditions were tested to attain elution fractions with a high concentration of free U4 snRNP. While addition of GTP is thought to stimulate the ATPase activity of Brr2 via U5 associated GTP binding protein Snu114 (Small et al. 2006), the addition of GTP to a final concentration of 2mM along with ATP did not reproducibly enhance the amount of free U4 snRNP generated (Figure 9A). Moreover, the amount of U4/U6 di-snRNP present in the elution fractions was found to be higher than the ATP only elution fractions. Alternatively, increasing the ATP concentration in the elution mix to 10mM resulted in a significant amount of U4/U6 leaching off of the column (Figure 9B). In addition, a band that migrates faster than U4/U6, but slower than U4 is also enhanced in the 10mM ATP elution. While the identity of this band has not been confirmed, it is also present when probing for U6, indicating that this may be a di-snRNP with a truncated U4 or U6 snRNA. Varying other assay conditions including tRNA concentration, wash volume and length of washes, and incubation time with the resin did not improve U4 snRNP release compared to the conditions described above (Section 2.1; data not shown). I found that for this particular assay, 2mM of ATP in the elution mix gives the best enrichment of U4 snRNP, with the smallest amount of U4/U6 di-snRNP species.

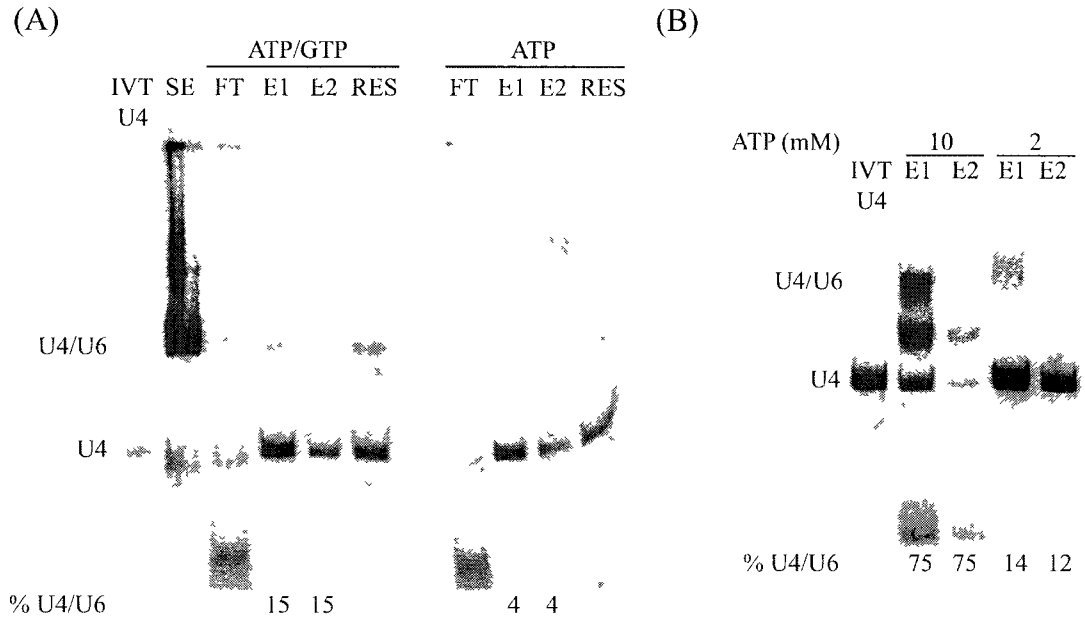


Figure 9. Brr2 release with 2mM ATP gives the best enrichment for free U4 snRNP. A) Northern blot of a Brr2 release with both 2mM GTP and 2mM ATP, or with 2mM of ATP only. B) Northern blot of a Brr2 release with 10mM or 2mM of ATP. (SE – splicing extract, FT – flowthrough, E1 and E2 – elutions, RES – resin).

Chapter Three – Structure Probing of Brr2 Released U4 snRNP

To gain insight into the mechanism of di-snRNP formation, the U4 snRNA secondary structure in the free snRNP must first be determined. Single stranded regions of RNA can be differentiated from double stranded regions with the use of chemical modifiers that modify nucleotide bases at Watson-Crick base pair positions (Moazed et al. 1986). 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-ρ-toluene sulfonate (CMCT) can be used to alkylate N3 of uridine, and dimethylsulfate (DMS) can be used to methylate N1 of adenine, and N3 of cytosine. The modifiers cannot react with bases that are hydrogen bonded at these positions; therefore double stranded RNA is protected from chemical modifiers. To verify the results of chemical modification, ribonucleases that cleave RNA in double or single stranded regions can be used. Double strand specific ribonuclease V_1 has been shown to cleave the RNA backbone of nucleotides hydrogen-bonded in Watson-Crick or wobble base-pairing, including G-U wobble base pairs (Lockard and Kumar 1981), and ribonuclease A has been shown to cleave phosphate bonds 3' of single-stranded pyrimidines (Reviewed in Raines 1998). This chapter summarizes the structure probing experiments with DMS, CMCT, RNase V_1, and RNase A in a model of free U4 snRNA.

3.1 Materials and Methods

Chemical Modification

The chemical modifiers used were dimethylsulfate (DMS; Sigma) and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-ρ-toluene sulfonate (CMCT; Sigma). Fifty microliters totaling approximately 7.5fmol of Brr2 released U4 snRNP was used for structure probing experiments. Modification with DMS was carried out in a total volume of 200μL

with HNM buffer (80mM K-HEPES pH 7.9, 20mM $MgCl_2$, 0.3M NaCl) in the presence of 10μg of yeast tRNA. The reaction was allowed to proceed in the presence of 2 to 100μL of 10.6M DMS (neat) for 50 minutes on ice, and stopped with 50μL of DMS stop buffer (1M Tris-HCl pH 7.5, 0.1M EDTA, 1M ß-mercaptoethanol) and 650μL of 100% ethanol. The RNA was pelleted and washed with 70% ethanol, resuspended in 500μL of resuspension buffer (0.3M NaOAc, 0.5% SDS, 6mM EDTA), and extracted before primer extension analysis. Modification with CMCT was carried out in a total volume of 200μL with BKM buffer (50mM borate/KOH pH 8.0, 50mM KCl, 10mM $MgCl_2$) in the presence of 5μg of yeast or *E. coli* tRNA at pH 8.0. The amount of CMCT added to the reaction ranged from 20μL to 50μL of 0.4M CMCT, and the reaction was carried out for 5 minutes at room temperature. The reaction was stopped with 800μL of 100% ethanol, and the RNA was precipitated and washed with 70% ethanol, then resuspended in 500μL of resuspension buffer for RNA extraction.

Ribonuclease Reactions

The enzymes used were Ribonuclease V_1 (RNase V_1; Ambion) and Ribonuclease A (RNase A; Sigma). Fifty microliters totaling approximately 7.5fmol of Brr2 released U4 snRNP in the presence of 5μg of tRNA was used for enzymatic probing experiments. Both RNase A and RNase V_1 reactions were carried out in a total volume of 200μL TNM buffer (30mM Tris-HCl pH 7.5, 300mM NaCl, 20mM $MgCl_2$). RNase V_1 reactions were carried out in the presence of 1μL of 0.1U/μL RNase V_1 at room temperature for 3 to 15 minutes. RNase A reactions were carried out in the presence of 3.4μL of 5 x 10^{-4}g/L RNase A (≥ 70 Kunitz units/mg protein) at room temperature for 3 to 15 minutes. The reactions were stopped by the addition of 150μL of 0.3M NaOAc, and 650μL of 100% ethanol. The RNA

was precipitated, washed with 70% ethanol, resuspended in 500μL of 0.3M NaOAc and phenol extracted before primer extension analysis.

Primer Extension

Phenol extracted RNA samples were resuspended in 8μL of water, and brought to a total volume of 12μL with 1μL of 10mM dNTPs, 1μL of 10mg/mL tRNA, and 2μL of labeled primer. Samples were incubated at 65°C for 6 minutes and subsequently cooled on ice for 2 minutes. Eight microliters of RT mix (0.5μL of AffinityScript Reverse Transcriptase (Stratagene), 0.5μL of SUPERase-In, 2μL of 10X AffinityScript buffer, 1μL of 0.1M DTT, and 4μL of ddH_2O) was added to the sample, and the sample was incubated at 37°C – 52°C for at least 1 hour (dependent on the Tm of the labeled primer). Reactions were stopped with 150μL of TE (10mM Tris-HCl pH 7.5, 1mM EDTA), 20μL of 3M NaOAc, 0.5μL of (20mg/mL) glycogen, and 500μL of 100% ethanol. Samples were precipitated and washed with 70% ethanol, resuspended in 5μL of 2X formamide loading buffer (deionized formamide, 50mM EDTA, 2X TBE, xylene cyanol, bromophenol blue), and heated to 95°C for three minutes before they were run on an 8%/7M denaturing sequencing gel. Dideoxy sequencing lanes contained 25 fmol of IVT U4 (T7U4 with two extra G nucleotides at the 5' end), and were treated identical to sample reactions except that the RT mix contained 1μL of 20mM ddNTP per lane.

Oligonucleotides Used for Primer Extension of U4 snRNA

675: 5' – AAAGGTATTCCAAAAATTC – 3' (U4 nucleotides 160 – 142)

14b: 5' – AGGTATTCCAAAAATTCCCTAC – 3' (U4 nucleotides 158 – 137)

SSU4: 5' – ACCATGAGGAGACGGTCTGG – 3' (U4 nucleotides 100 – 81)

CM6: 5' – TCAACCAGCAAAAACACAATCTCG – 3' (U4 nucleotides 66 – 43)

CM2: 5' – CTCGGACGAATCCTCAC – 3' (U4 nucleotides 46 – 30)

3.2 Results and Discussion

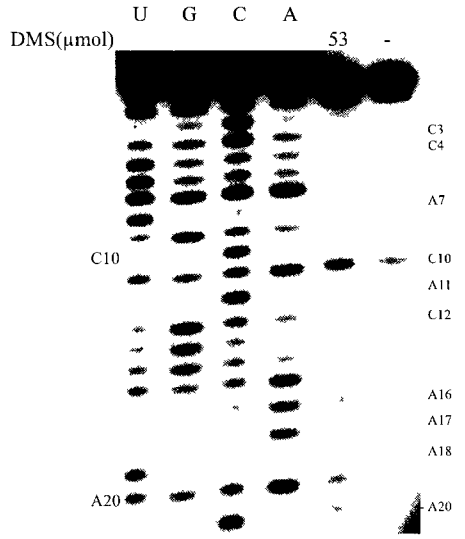
Chemical modification and enzymatic cleavage were optimized such that on average only one modification or cleavage should be present per snRNA molecule. This was accomplished by titration of the probes to a suitable amount, and with the addition of tRNA as another substrate for modification or cleavage. DMS structure probing was performed on ice because a low temperature was thought to minimize conformational changes. However structure probing of the Brr2 released U4 with CMCT did not result in any modifications when the reaction was performed on ice. In addition, the ribonucleases are not optimal below 4°C (Ambion; Sigma), thus the structure probing experiments with CMCT, RNase V_1, and RNase A were carried out at room temperature.

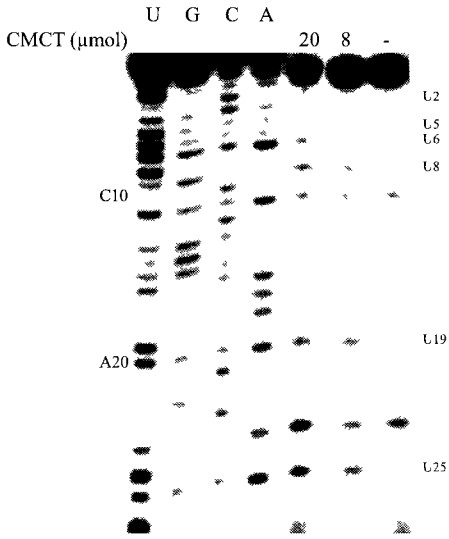
Structure probing of U4 with DMS, CMCT, RNase V_1, and RNase A shows strong support for the three stem loops (3' stem loop, central stem loop, and the 5' kink-turn stem loop) that were previously proposed for yeast U4 snRNA (Myslinski and Branlant 1991, Mougin et al. 2002). In addition, the modification pattern indicates the presence of a short stem loop at the extreme 5' end of U4 snRNA (Figure 10). This stem loop, which I will refer to as the 5' kissing stem loop of U4, is thought to consist of four base pairs encompassing nucleotides G15 – C12 and U2 – U5, with 6 nucleotides forming an apical loop. Stem nucleotides C4 and C12 are protected from modification, C3 is very weakly modified, and U5 is weakly modified. In addition, a fifth base pair may transiently form between the very weakly modified A11 and U6. Loop nucleotides C10 and U8 are strongly modified, demonstrating the availability of these nucleotides for an initial interaction with U6. This is

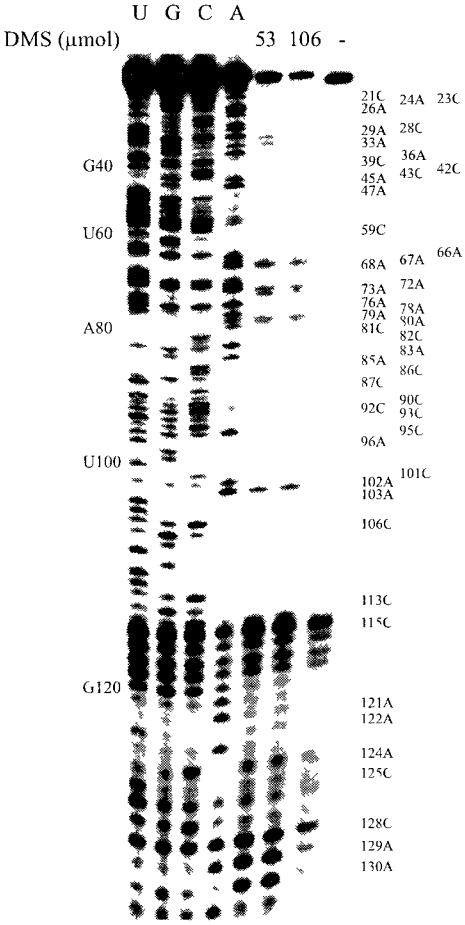
further supported by RNase A cleavage 3' of loop nucleotides U6, U8, and C10, indicating that this region is indeed single stranded (Figure 11).

Enzymatic cleavage with RNase V_1 supports the presence of the 5' kissing stem loop with moderate cleavages on the 5' side of the stem, and weak cleavages on the 3' side. The 5' kissing stem is a short stem structure, but the minimum substrate size for RNase V_1 is thought to be between 4 and 6 nucleotides (Lowman and Draper 1985), so cleavage in this region is credible. RNase V_1 is thought to recognize a substrate that is in an approximately helical conformation, and it does not require that the nucleotide bases be hydrogen bonded in a helix (Lowman and Draper 1985). Therefore stabilization of a helical arrangement near this stem, like the potential base pair A11 – U6, might further contribute to the cleavage site for RNase V_1. Notably the observed V_1 cuts in the 3' and central regions of U4 (3' of positions A122, U112, U111, U109, U100, U97, U63, and U49) were also found in the free phenol/chloroform extracted U4 RNA by Mougin et al. 2002. The fact that I also observe these cuts indicate a similarity in structure of the 3' and central regions of U4 regardless of the presence of proteins, and serve to validate the V_1 cleavages I have observed at the 5' end.

A)


U G C A
DMS(μmol) 53 -
C3
C4
A7
C10
C10
A11
C12
A16
A17
A18
A20
A20


U G C A
CMCT (μmol) 20 8 -
U2
U5
U6
U8
C10
U19
A20
U25


U G C A
DMS (μmol) 53 106 -
21C
26A
24A
23C
29A
28C
33A
39C
36A
G40
45A
43C
42C
47A
59C
U60
68A
67A
66A
73A
72A
76A
79A
A80
81C
82C
83A
85A
86C
87C
90C
92C
93C
95C
96A
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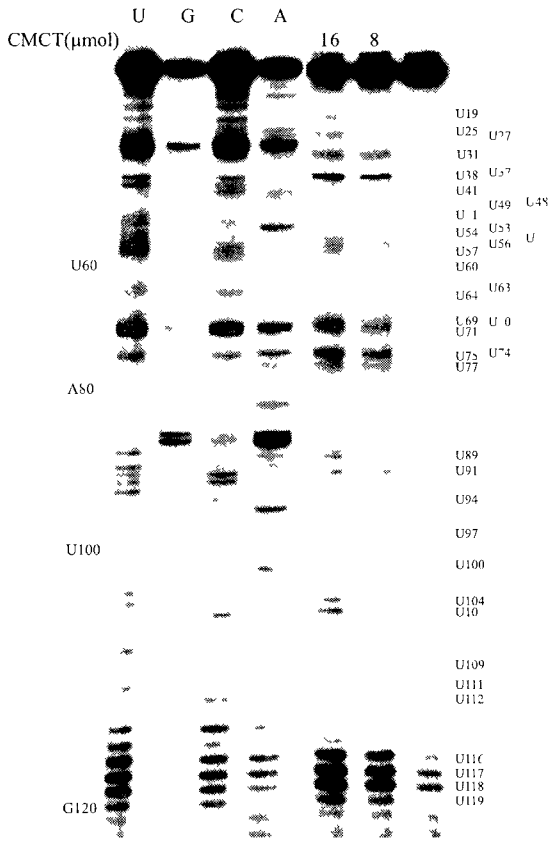
121A
122A
124A
125C
128C
129A
130A


U G C A
CMCT(μmol) 16 8
U19
U25
U31
U38
U41
U49
U54
U57
U60
U64
U69
U71
U75
U77
U60
A80
U89
U91
U94
U97
U100
U100
U104
U109
U111
U112
U117
U118
U119
G120

B)

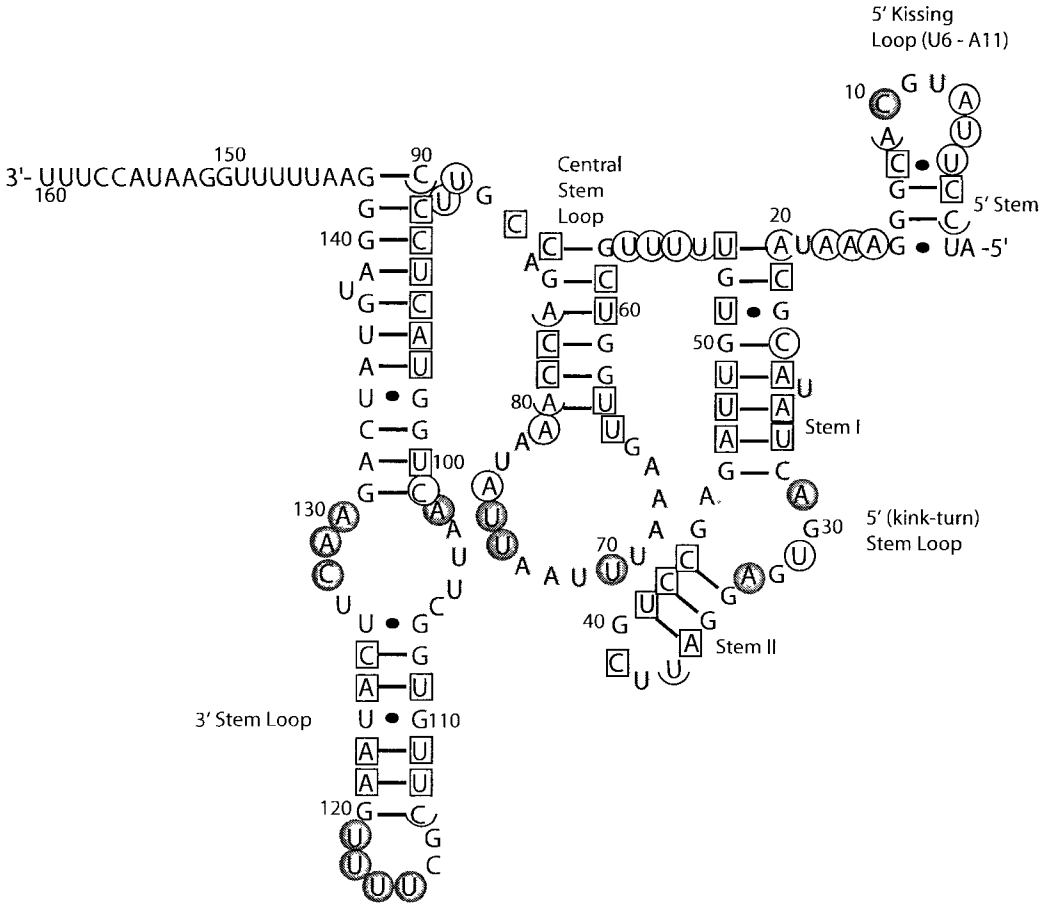
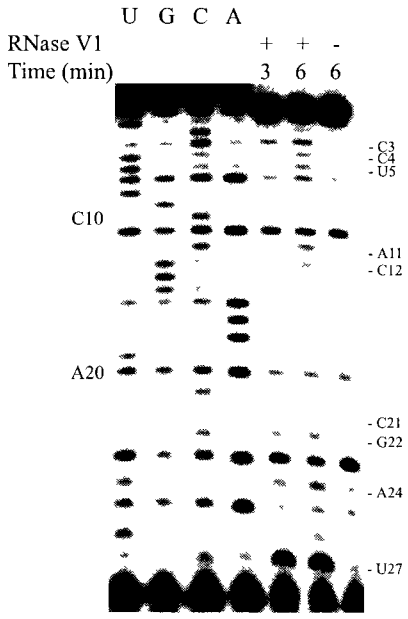
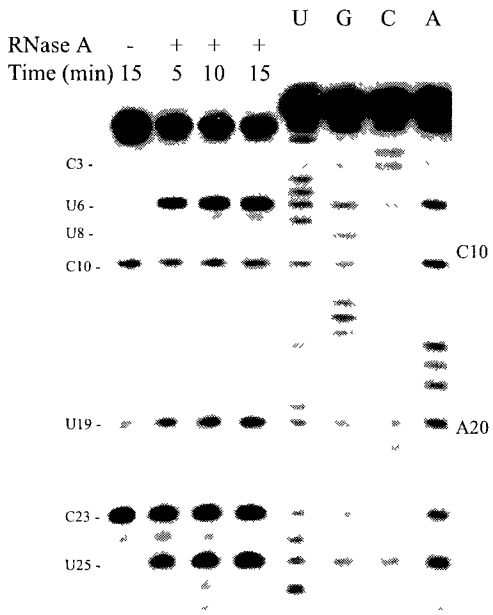
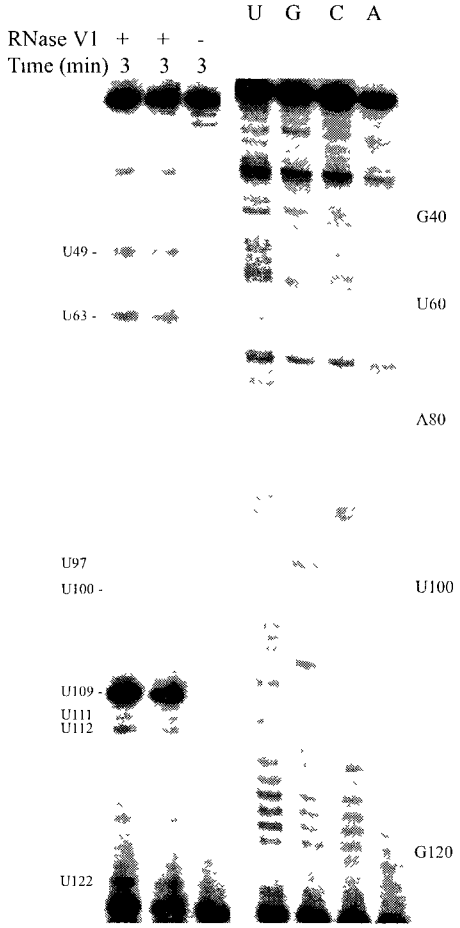


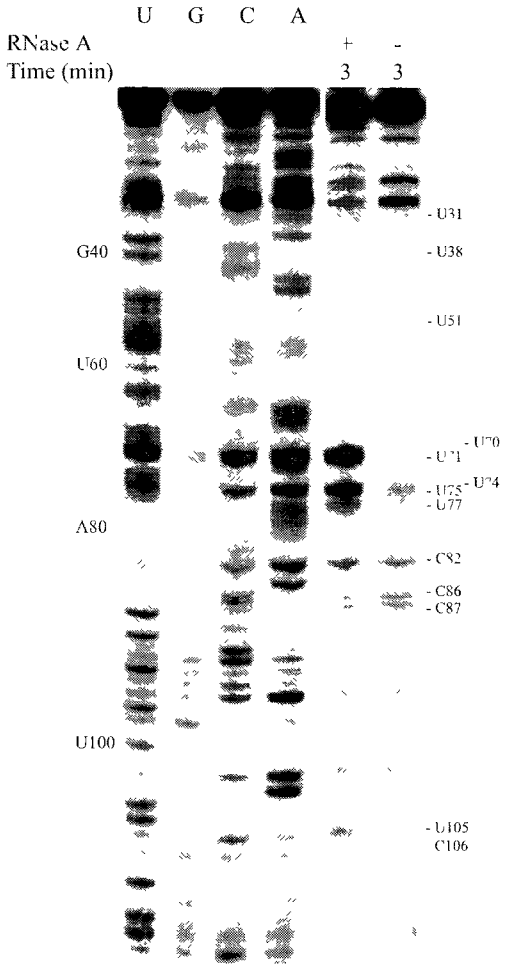
Figure 10. Chemical structure probing of Brr2 released U4 snRNPs with DMS and CMCT supports the novel 5' stem loop. A) Chemical modification of accessible nucleotide bases in the free U4 snRNP with DMS and CMCT. Primer extension reactions were carried out with radiolabeled CM2, 14b, or 675 oligonucleotides. The position where the reverse transcriptase was inhibited by a modification is shown on the right. Lane U, G, C, and A are dideoxy sequencing lanes made with the corresponding oligonucleotide. The amount of modifier used is given above the lane, where (–) indicates the omission of the modifier. DMS experiments were carried out on ice for 50 minutes, and CMCT experiments were performed at room temperature for five minutes. B) Secondary structure model of the free U4 snRNP with the modification pattern mapped on it. Boxes indicate protected nucleotides, semi circles indicate very weak modifications, circles indicate weak modifications, grey circles indicate medium modifications, and dark grey circles indicate strong modifications. Nucleotides at the 3' end of the molecule are where oligonucleotides 14b and 675 bind. Nucleotides lacking boxes or circles are nucleotides for which there is no information, or positions of strong reverse transcriptase stops. The model is based on probing experiments that were repeated two to more than five times, depending on the difficulty in reproducing modifications, and the region of U4. The 5' region was probed at least three times.

A)


U G C A
RNase V1 + + -
Time (min) 3 6 6
- C3
- C4
- U5
C10
- A11
- C12
A20
- C21
- G22
- A24
- U27


U G C A
RNase A - + + +
Time (min) 15 5 10 15
C3 -
U6 -
U8 -
C10 -
C10
U19 -
A20
C23 -
U25 -


U G C A
RNase V1 + + -
Time (min) 3 3 3
G40
U49 -
U63 -
U60
A80
U97
U100 -
U100
U109 -
U111
U112
G120
U122


U G C A
RNase A + -
Time (min) 3 3
- U31
G40
- U38
- U51
U60
- U70
- U71
- U74
- U75
- U77
A80
- C82
- C86
- C87
U100
- U105
C106

B)

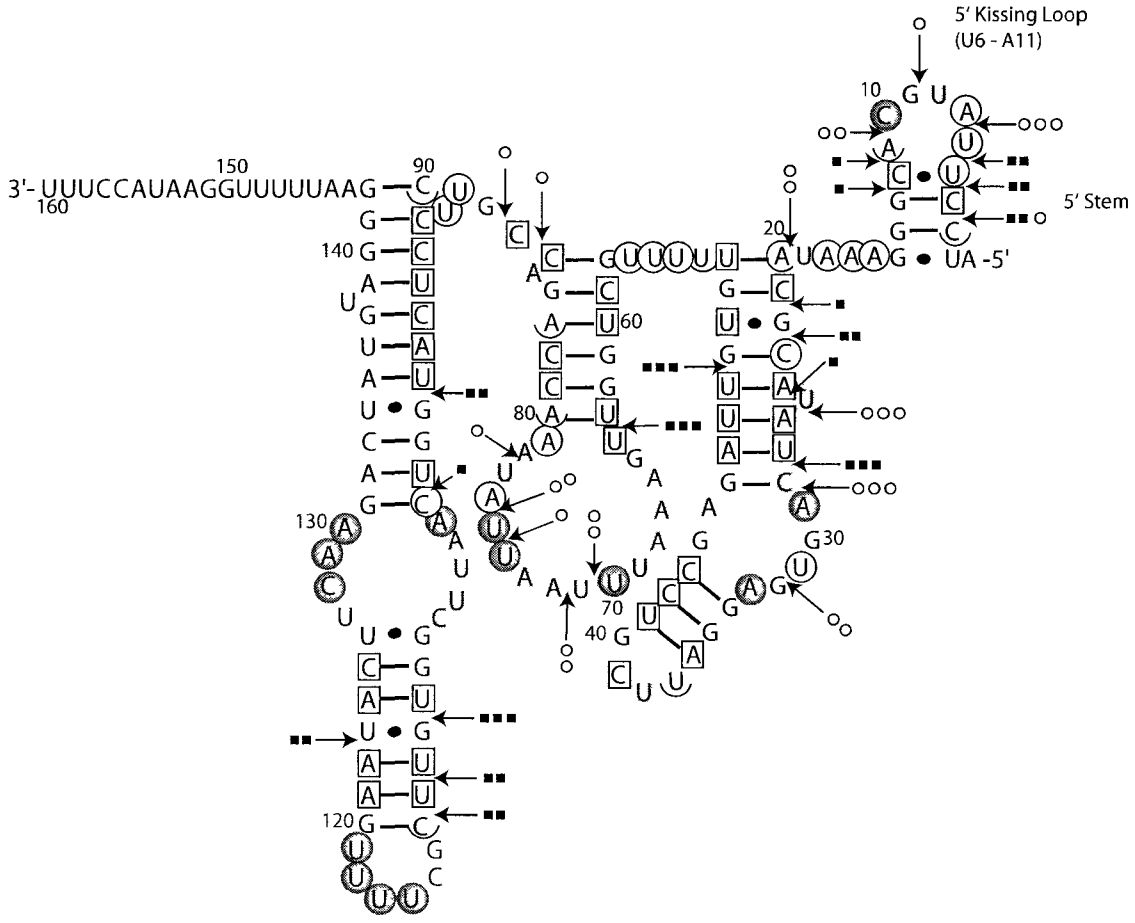


Figure 11. Enzymatic probing of Brr2 released U4 snRNPs with RNase V_1 and RNase A corroborates the novel 5' stem loop. A) Enzymatic cleavage of free U4 snRNP with RNase V_1 and RNase A. Primer extension reactions were carried out with radiolabeled CM2, 14b, or 675 oligonucleotides. The position where the reverse transcriptase stopped due to cleavage is indicated. Lane U, G, C, and A are dideoxy sequencing lanes made with the corresponding oligonucleotide. The presence of the ribonuclease is given above each lane, where (–) indicates the omission of the ribonuclease. RNase V_1 experiments were carried out at room temperature for 3 or 6 minutes with 0.1U of RNase V_1, and RNase A experiments were performed at room temperature for 3 – 15 minutes with 1.2×10^{-4} Kunitz units. B) Secondary structure model of the free U4 snRNP with the modification and ribonuclease cleavage pattern mapped on it. Chemical modifications are labeled as in Figure 10. Arrows linked to boxes indicate sites of RNase V_1 cleavage, and arrows with open circles indicate sites of RNase A cleavage. The strength of cleavage is indicated by the number of squares or circles, where 1 represents a weak cut in the RNA, 2 a moderate cut, and 3 a strong cut. Nucleotides at the 3' end of the molecule are where oligonucleotides 14b and 675 bind. Nucleotides lacking boxes or circles are nucleotides for which there is no information, or positions of strong reverse transcriptase stops. The model is based on probing experiments that were repeated two to more than five times, depending on the

difficulty in reproducing cleavages, and the region of U4. The 5' region was probed at least three times.

In the 5' kissing loop there was one position in the RNA backbone, 3' of C3, that was found to be cleaved by both RNase A and RNase V_1. RNase A cleaved this position only weakly, while RNase V_1 cut this position more readily. In addition, C3 was found to be very weakly modified on some occasions. Together this may indicate that C3 and the surrounding backbone may be involved in helix breathing, whereby it is partially exposed to RNase A and DMS for a small amount of time during the structure probing experiments. This is not surprising if indeed the 5' kissing loop is the nucleation site of di-snRNA formation. It may experience some conformational flexibility to enable it to form intermolecular base pairs when U6 is in close proximity. Although the 5' kissing loop is short, the Tm of this stem loop (nucleotides 1 – 15, not including the C – U pair) is thought to be approximately 48°C (Integrated DNA Technologies), which would suggest that this structure should be relatively intact at room temperature.

The 5' kink turn stem loop was found to be present in the Brr2 released U4 snRNP (Figure 10 and 11). There is evidence that stem I and II of the kink-turn stem loop are stabilized in the presence of human proteins 15.5K (yeast Snu13), and 61K (Prp31), and that without proteins the two G – C base pairs of stem II become unstable. The structure of yeast Snu13 was found to have a nearly identical conformation as that of the human 15.5K bound with the kink-turn stem of human U4 snRNA (Oruganti et al. 2005), suggesting that Snu13 may bind to yeast U4 in a similar manner to that of 15.5K. Nucleotides A36, U41, C42, and C43 are protected from chemical modification, form stem II of the kink turn stem loop, and may indicate the presence of Snu13 and Prp31.

Nucleotides U37 and C39 in the apical loop of the 5' kink turn stem loop were found to be very weakly modified and protected respectively. In the crystal structure of human Prp31 with 15.5K and nucleotides 20 – 52 of human U4 snRNA, Prp31 was found to contact the major groove of stem II and the capping pentaloop (Lui et al. 2007). Upon binding of Prp31 the pentaloop becomes stabilized and protected from hydroxyl radicals (Schultz et al. 2006, Lui et al 2007), and UV cross-linking of purified native 25S human tri-snRNP particles indicated that 61K contacts the loop nucleotides U36, U37, and U38 of human U4 snRNA (Nottrott et al. 2002). Therefore it is possible that protection of C39 and U37 in Brr2 released U4 may be due to binding of the yeast protein Prp31. Alternatively, tertiary RNA contacts may protect these nucleotides from modification.

Given the protection of nucleotides A36, U37, C39 and U41 – C43, it is possible that both Snu13 and Prp31 bind the Brr2 released U4 snRNP. However, there are a number of hydrogen bonds present with these proteins bound that have not been observed in the Brr2 released U4. In the crystal structure of full length 15.5K bound to nucleotides 26 – 47 of human U4 snRNA, the 15.5K protein was shown to stabilize the formation of two G – A sheared base pairs at the top of stem II (Vidovic et al. 2000). These hydrogen bonds do not involve N1 of adenine so DMS structure probing gives no information about these possible base pairs. Furthermore 15.5K binding to a fragment of U4 snRNA is characterized by a base pair between N1 of A44 (A45 in yeast) and the 2'-OH group of A29, which is not seen to occur in the Brr2 released U4 (Figure 10). Finally, N3 of U31 is thought to form a hydrogen bond with the main chain oxygen of Glu-61, yet I found this position to be weakly modified (Figure 10).

The difference in protection may reflect the difference between yeast and human RNP interactions. Alternatively it is possible that a small percentage of the U4 snRNA released via Brr2 is not complexed with any proteins, and this fraction of U4 species may be sufficient to produce the strong modification observed at A45, and the weak modification at U31. Although in humans the U4/U6 di-snRNP specific proteins are thought to dissociate concurrently with the U4 snRNP, they may not be bound to U4, or they may be destabilized from U4. Therefore the difference in protection may be due to a difference in protein composition following release of U4 from U6.

In humans 61K has a strong requirement for a two base pair long stem II of the kink-turn stem loop (Schultz et al. 2006). Addition of third base pair of either C – G or U – A, or extension of stem II to 5 or 7 base pairs significantly reduced binding of 61K to a binary complex consisting of the U4 kink-turn stem loop and 15.5K. However, yeast U4 snRNA has 3 base pairs in stem II of the kink-turn stem loop, implying that yeast Prp31 (and possibly Snu13) may bind in a slightly different manner to yeast U4 snRNA to accommodate this extra base pair.

In summary, the structure probing experiments indicated that there is a short stem loop present at the 5' end of the Brr2 released U4. The loop sequence is accessible to chemical modification, and available for intermolecular base pairing with U6 snRNA. It is unclear whether any snRNP specific proteins are associated with this U4 snRNP species. It is possible that when U4 is unwound from U6, no specific proteins associate with U4, or that U4 snRNP specific proteins are knocked off during the unwinding process. Although, data from our lab indicate that U4 snRNA from Brr2 release is probably associated with proteins, it may be only the Sm proteins that remain bound (Aukema and Rader, unpublished data).

For a complete understanding of the structure of the U4 snRNP, the location of the snRNP specific proteins associated with the molecule, if any, will need to be elucidated (see Chapter 6 for further discussion).

Chapter Four – Genetic Analysis of U4 at the C12 Position

The U4 5' kissing stem loop must undergo a conformational rearrangement during di-snRNP formation. Hyperstabilization of the stem loop may inhibit this rearrangement and lead to a di-snRNP or spliceosome assembly defect. In an attempt to test this hypothesis *in vivo* I used site-directed mutagenesis to mutate the C12 position of U4 snRNA. C12 is the only nucleotide in the short 5' stem loop that is not base paired in U4/U6 di-snRNA, but bulged out of the helix opposite U6 C69. Therefore, altering this position to an A should not affect U4/U6 stem II stability, but it may stabilize the 5' kissing stem by changing a C · U base pair to an A – U base pair. The A – U base pair may in turn stabilize an interaction between U4 nucleotides U6 and A11 to form a fifth base pair at the top of the stem. Hyperstabilization could possibly inhibit the ability of the 5' kissing stem to unwind, and a decreased growth temperature may exacerbate this effect. Therefore, stabilization of the 5' stem could result in cold sensitive growth or lowered levels of di-snRNP formation. A decrease in di-snRNP can be detected by an increase in free U4 snRNP compared to free U4 snRNP in wild-type extracts, which exists in a minimal amount. If a cold sensitive growth phenotype or lower levels of di-snRNP formation occur due to hyperstabilization of the short stem, this would provide strong evidence for the existence of the 5' kissing stem loop *in vivo*.

4.1 Materials and Methods

Generation of mutant SNR14

Point mutations were introduced into *SNR14*, the gene encoding U4 snRNA, via site directed mutagenesis of pSR20 (pSE362 with a 552 bp EcoRV-EcoRI genomic fragment containing *S. cerevisiae SNR14*). PCR was carried out with 2.5 units of Pfu Turbo AD DNA polymerase (Stratagene) for 18 cycles of 95°C for 45 seconds, 55°C for 45 seconds, and

68°C for 10 minutes, followed by a 20 minute extension at 68°C, and storage overnight at 4°C. Twenty units of DpnI (NEB) was added directly to the PCR mix and incubated at 37°C for 6hrs to digest wild-type *SNR14* template plasmid. The mutagenized plasmid DNA was precipitated and transformed into $RBCl_2$ competent DH5α by standard protocol. Transformations were plated onto LB-carbenicillin (LB – carb) plates and incubated overnight at 37°C.

A single colony was grown overnight at 37°C, 200RPM, in LB-carb medium, and plasmid DNA was isolated with an EZ-10 spin column plasmid DNA kit (BioBasic Inc.) following the manufacturer's instructions. Plasmids were sent for sequencing at the UNBC Genetics Facility to check for a mutation. After identification of the mutation, 1μg of plasmid DNA was cut with 20 units of EcoRI (NEB) and 20 units XbaI (NEB) for 1hr and 20 minutes at 37°C, run on a 1% agarose gel and purified with an EZ-10 spin column DNA gel extraction kit (BioBasic Inc.) following manufacturer's instructions. Vector DNA (pSR20) was digested in the same manner for 30 minutes at 37°C, and isolated as above. Ligations between the vector and the insert containing the mutation were carried out with 5000 units of T4 DNA ligase (NEB) for 1.5 hours at room temperature, and heat inactivated for 10 minutes at 65°C. The ligated plasmids were ethanol precipitated, transformed into DH5α cells and plated onto LB-carb plates. All mutant plasmids and wild-type pSR20 were sequenced from the EcoRI to XbaI sites to ensure the presence of the desired mutation at C12, and the absence of mutations within the rest of the sequence between these sites.

PCR primer pairs:

U4 – C12A

FWD: CTCCATCCTTATGCA**A**GGGAAATACGCATATC
RVS: GATATGCGTATTTCCC**T**TGCATAAGGATGGAG

U4 – C12U

FWD: CTCCATCCTTATGCA**T**GGGAAATACGCATATC
RVS: GATATGCGTATTTCCC**A**TGCATAAGGATGGAG

U4 – C12G

FWD: CTCCATCCTTATGCA**G**GGGAAATACGCATATC
RVS: GATATGCGTATTTCCC**C**TGCATAAGGATGGAG

Yeast Transformations

HIS3 marked mutant and wild-type pSR20 plasmids were transformed into the yeast strain YSR169 2H4 (a strain with a chromosomal disruption of *SNR14*, covered on a *URA3* marked plasmid (Open Biosystems)) by standard methods. Briefly, YSR169 2H4 was patched onto a YPD plate and incubated at 30°C for two days. A large glob of yeast was resuspended in 400µL of TEL (10mM Tris-HCl pH 7.5, 3.33mM EDTA, 100mM LiOAc), and 100µL was aliquoted per transformation. Forty micrograms of sheared salmon sperm DNA, 2µL of plasmid (300-400ng), and 1mL of PEG-TEL (10mM Tris-HCl pH 7.5, 3.33mM EDTA, 100mM LiOAc, 40% PEG-4000) was added to the resuspended yeast cells, and incubated overnight at room temperature. The cells were heat shocked for 25 minutes at 42˚C, spun down for 8 seconds at 4000RPM, and as much PEG-TEL was removed as possible. The cells were resuspended in 200µL of ddH_2O and plated onto selective -his media. A single colony was selected and streaked onto fresh -his plates. This plate was replica plated onto -his, -ura, 5-FOA, and YPD. A single colony of the wild-type and each

mutant that grew on -his, 5-FOA, and YPD, but not on -ura was selected and replica plating was performed again to ensure that the strain had indeed lost the *URA3* marked plasmid.

Dot Dilutions to Examine Growth at 37°C, 30°C, and 16°C

A single colony of the wild-type and each mutant strain was grown overnight in 10mL of YPD at 30°C. One OD_{600}/mL of cells was spun down at 5000RPM for 10 seconds, resuspended in 250µL of ddH_2O, and placed into a sterile 96 well plate. Five total 5 fold dilutions were made with ddH_2O, and the dilutions were plated onto three YPD plates with a frogger. The plates were incubated at 37°C for 2 days, 30°C for 2 days, or 16°C for 5 days.

Growth Curves

A single colony of the wild-type and each of the mutant strains was grown overnight in 5mL of YPD at 30°C, 200RPM. Twenty-five milliliters of YPD was inoculated with overnight culture (OD_{600} between 0.4 and 2.5) so that the initial OD_{600} was equal to 0.05. Cells were grown in pre-equilibrated (37°C, 30°C, or 16°C) culture and the OD_{600} was recorded at regular time intervals. Data was plotted using Excel and an exponential line of best fit was used to determine the doubling time. The growth curve analysis for the wild-type and each mutant strain was done at least three times. The average doubling time and the standard deviation was calculated using Excel. A statistical f-test was employed to determine the type of unpaired t-test required, and an unpaired t-test was used to determine if any of the mutant doubling times were statistically slower than wild-type doubling time (Excel).

Preparation of Yeast Total RNA

The wild-type strain and each U4 mutant was grown overnight in YPD at 30°C, 200RPM, until the cultures reached an OD_{600} between 0.5 and 1. Between 1 and 3mL of

cells were spun down and resuspended in 200μL of YPD, and used to inoculate 2mL of pre-equilibrated YPD at 16°C, 30°C, or 37°C. The cells were heat shifted for three hours, after which 2mL of culture was spun down at 4000RPM for 10 seconds, washed twice with 1mL of cold ddH_2O, and resuspended in 300μL of cold RNA buffer (50mM Tris-HCl pH 7.5, 100mM NaCl, 10mM EDTA). Approximately 200μL of baked zirconium beads were added to the samples, which were then vortexed for 1 minute, put on ice for 5 minutes, and vortexed again for 1 minute. Three hundred microliters of cold RNA buffer, 60μL of 10% SDS, and 400μL of cold acid equilibrated phenol/chloroform (Ambion) was added to the samples, vortexed for 1 minute, and spun at high speed for 2 minutes at 4°C. This was repeated twice with cold acid equilibrated phenol/chloroform, and once with cold chloroform (Sigma). The aqueous layer was transferred to a new tube, and 40μL of 3M NaOAc and 1mL of 100% ethanol was added. The RNA was precipitated, washed, and resuspended in 30μL of 10mM Tris-HCl pH 7.5 and quantified with a NanoDrop ND-1000 Spectrophotometer. Five to 10μg of total RNA was run on a non-denaturing gel at 4°C, and the U4 species was analyzed by northern blot with labeled oligonucleotide 14b.

4.2 Results and Discussion

In an attempt to demonstrate the existence of the 5' kissing stem loop *in vivo*, a C12A mutation was introduced into *SNR14*. This mutation was designed to hyperstabilize the 5' kissing stem loop, and was predicted to result in a cold sensitive growth phenotype, or possibly a U4/U6 di-snRNP assembly defect. The C12U mutation was introduced as a control mutation because it was not predicted to affect the stability of U4/U6 stem II or the U4 kissing stem loop. Finally, the C12G mutation was made to complete the analysis of possible mutations at this position. This mutation would stabilize U4/U6 stem II by forming

a base pair between U4 G12 and U6 C69 (Figure 12). In addition, C12G may stabilize the 5' kissing stem by forming a G – U wobble base pair. There was no prediction for the growth phenotype of this mutation as it was unclear where the mutation would affect RNA stability, if at all.

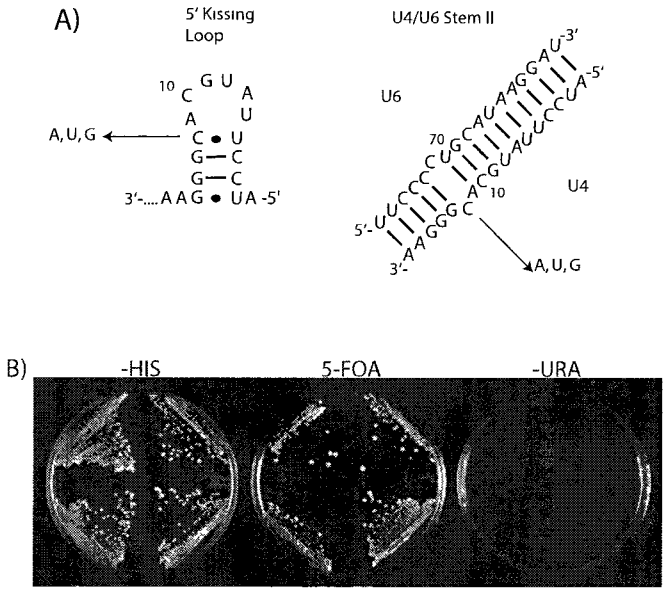


Figure 12. Yeast strains with *HIS3* marked plasmids containing wild-type or mutant *SNR14* grow on -his and 5-FOA, but not on –ura selective media. A) Schematic diagram showing the location of C12 in the free U4 snRNA, and in the base-paired U4/U6 di-snRNA. B) Yeast strains with *HIS3* marked wild-type or mutant plasmids plated on –his, 5-FOA, and –ura plates.

HIS3 marked plasmids containing wild-type and mutant *SNR14* (the gene encoding U4 snRNA) sequences were successfully introduced into YSR 169 2H4 yeast strain using the plasmid shuffle technique. The starting yeast strain, YSR169 2H4, contains a chromosomal disruption of *SNR14* that is covered with a *URA3* marked plasmid (Open Biosystems). Yeast transformation with the *HIS3* marked pSR20 plasmids results in a strain that contains wild-type *SNR14* on a *URA3* plasmid, and wild-type or mutant *SNR14* on a *HIS3* plasmid. Growth on plates lacking histidine (-his plates) causes selective pressure for the yeast to maintain the histidine encoding gene *HIS3* marked plasmids. Absence of selective pressure for the *URA3*

gene results in the loss of *URA3* marked wild-type plasmid. The resulting yeast strain will carry a copy of *SNR14* on the *HIS3* marked plasmid, and will grow on –his plates (Figure 12B). Loss of the uracil encoding *URA3* marked wild-type plasmid is indicated by the loss of growth on plates lacking uracil (-ura plates) (Figure 12B). Also, the ability of mutant strains to grow on media containing 5-FOA confirms the loss of the *URA3* wild-type plasmid, as in the presence of *URA3* 5-FOA is converted to fluorodeoxyuridine, a toxic chemical that inhibits yeast growth (Figure 12B).

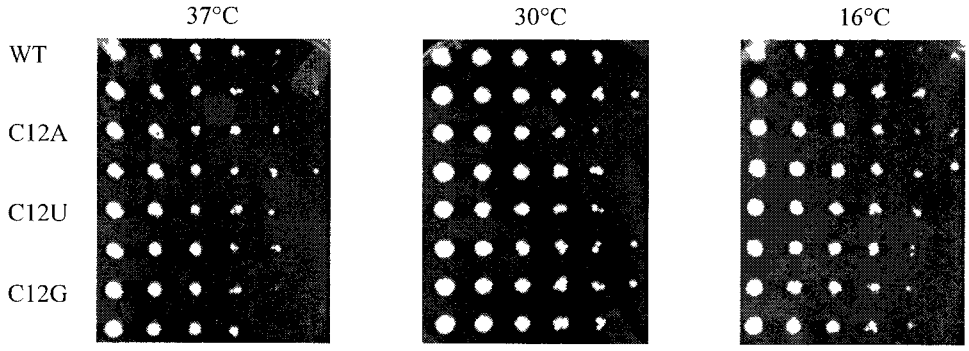


Figure 13. Wild-type and mutant growth phenotypes assayed by dot dilution.

Dot dilutions of the wild-type and mutant yeast strains were used to examine the growth phenotypes at 37°C, 30°C, and 16°C. Dot dilutions were performed in duplicate on a single plate, and carried out a total of 6 times for each strain. Overall, the size and the appearance of yeast colonies indicated that the mutants appeared to have wild-type growth at all temperatures tested (Figure 13). However, subtle differences in growth phenotypes are hard to detect by dot dilutions, so growth curve analysis was used to determine the doubling time for each strain (Table 1).

The doubling time for each mutant and the wild-type strain was calculated at least three times, and the average doubling time and standard deviation was determined using Excel. To determine if there was a statistical difference between the doubling times of a

mutant compared to the wild-type strain, an unpaired t-test was used. First, an f-test was utilized to find the probability that the variances in mutant doubling times and wild-type doubling times are not significantly different. The f-test results determine the type of t-test that should be used based on the equality or inequality of variances. If the p value was found to be less than 0.05, the variances were determined to be not equal. Only one f-test with C12 and WT data at 16°C indicated that the variances were not equal (Table 2). However, a conservative approach is to use the t-test that does not assume equality of variance (Excel) so both types of t-tests were performed to determine if the mutant doubling times were significantly different from the wild-type (Table 3).

For the t-test employed here, a p value less than 0.05 indicates that one should reject the null hypothesis of equal means. Table 3 shows that there is no statistical difference between the C12A and wild-type strain doubling times at any temperature tested. This indicates that C12A does not hyperstabilize the 5' stem loop, or that hyperstabilization of the stem with C12A does not affect the growth phenotype of yeast. In contrast, both C12U and C12G double slightly slower than wild-type at 37°C and 30°C, and C12U also doubled slower than wild-type at 16°C. Altering C12 to a guanine may stabilize both the U4 5' kissing stem loop and the U4/U6 di-snRNA, resulting in the slightly slower conversion of both species within the splicing cycle. Alternatively, the slower doubling time observed for both C12U and C12G may be caused by a slight disruption in an RNA-protein interaction at this site.

Table 1. Doubling time and standard deviation determined from growth curves of wild-type and mutant *SNR14* yeast strains grown at 37°C, 30°C, and 16°C.

Sample	Doubling Time 1	Doubling Time 2	Doubling Time 3	Doubling Time 4	Doubling Time 5	Average Doubling Time (hrs)	Standard Deviation (hrs)
WT 37°C	1.7174	1.7259	1.6885	1.8654	1.8805	1.7755	0.090155
A12 37°C	1.8713	1.8217	1.8284			1.8405	0.026912
U12 37°C	1.9275	1.8892	1.9249			1.9139	0.021401
G12 37°C	2.0056	1.9254	1.9586			1.9632	0.040297
WT 30°C	1.8188	1.8415	1.8523	1.785	1.7902	1.8176	0.029594
A12 30°C	1.8155	1.7178	1.7376			1.7570	0.051649
U12 30°C	1.8698	1.8938	1.8944			1.886	0.014033
G12 30°C	1.9074	1.9345	1.8795			1.9071	0.027501
WT 16°C	6.3533	6.5268	6.3533	6.2221	6.3825	6.3676	0.10857
A12 16°C	6.4599	6.3767	6.2389			6.3585	0.11162
U12 16°C	6.7165	6.7427	6.7889			6.7494	0.036658
G12 16°C	6.7823	7.1903	6.5639			6.8455	0.31795

Table 2. F-test results for each of the mutant strains compared to wild-type ($\alpha = 0.05$).

Sample	p Value	Equality of Variances
WT/A12 37°C	0.08349	Yes
WT/U12 37°C	0.05406	Yes
WT/G12 37°C	0.17340	Yes
WT/A12 30°C	0.16180	Yes
WT/U12 30°C	0.18793	Yes
WT/G12 30°C	0.50498	Yes
WT/A12 16°C	0.42802	Yes
WT/U12 16°C	0.10500	Yes
WT/G12 16°C	0.03576	No

Table 3. Un-paired t-test results for determination of doubling time differences between mutant and wild-type strains ($\alpha = 0.05$).

Samples	p Value (assume equal variance)	p Value (assume variance not equal)	Statistically different from WT
WT/A12 37°C	0.28203	0.19325	No
WT/U12 37°C	0.044222	0.021948	Yes
WT/G12 37°C	0.015836	0.0068695	Yes
WT/A12 30°C	0.074979	0.16086	No
WT/U12 30°C	0.010876	0.0047134	Yes
WT/G12 30°C	0.0056794	0.0076316	Yes
WT/A12 16°C	0.91318	0.91564	No
WT/U12 16°C	0.0012197	0.00080093	Yes
WT/G12 16°C	0.018363	0.12820	No

To see if U4/U6 di-snRNP formation was inhibited in any of the mutant strains, I isolated total RNA grown at permissive temperature and after heat shifting the cultures to non-permissive temperature for 3 hours. No U4 accumulation was detected for any of the mutants at 37°C, 30°C, or 16°C compared to the wild-type strain. This strongly suggests that the 5' kissing stem loop is not hyperstabilized in any of the mutants. The results also reveal that the slightly slower doubling times observed for C12U and C12G mutants are not due to a di-snRNP formation defect, although this does not rule out a di-snRNP dissociation defect.

In summary, the C12A mutation did not appear to hyperstabilize the U4 5' kissing stem loop as hypothesized. It is possible that the 5' kissing stem loop is not present *in vivo*, however the change from a C · U pair to an A – U pair may not be severe enough to hyperstabilize this stem loop. Although there was a statistical difference between the doubling time of C12U and wild-type at all temperatures tested, and between C12G and wild-type at 37°C and 30°C, the difference is very subtle as it is not readily detectable by dot

dilution Overall, the C12 position of U4 snRNA in yeast is very tolerant to mutation This is in agreement with a deletion experiment in yeast that showed deletion of C12 resulted in a viable strain when grown at 37°C, 30°C, and 16°C (Hu et al 1995) The deletion experiment does not refute the presence of a 5' kissing stem loop, as A11 may become the base pair partner for U5 in the ΔC12 strain

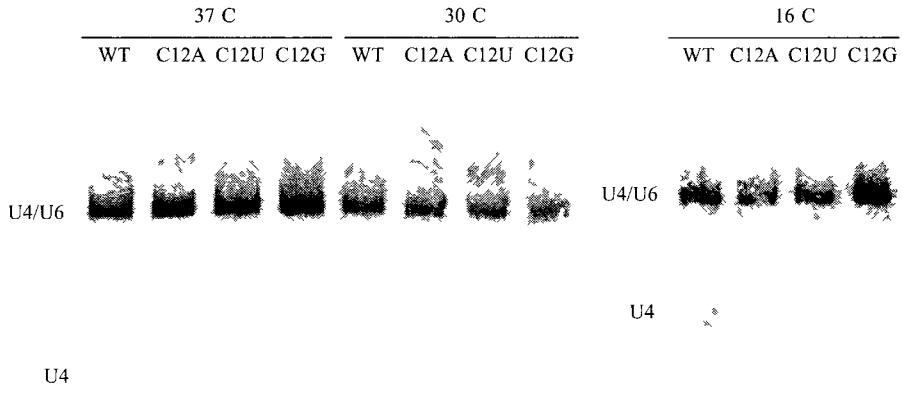


Figure 14 Northern blots of total RNA prepared from wild-type and mutant *SNR14* strains indicate a lack of U4 accumulation Blots were probed for U4 with labeled oligonucleotide 14b

Chapter Five – Modification/Interference Analysis with CMCT

Modification/interference assays are designed so that modification of important nucleotides prevents an RNA molecule from participating in a biochemical process. Segregation of the RNA molecules in a functional assay followed by mapping of the locations of modified residues allows functionally important regions to be determined (Merryman and Noller 1998). To gain insight into the mechanism of di-snRNP formation I used a modification/interference assay that establishes the positions in U4 that are necessary for base pair formation.

For the modification/interference assay the uridine bases of Brr2 released U4 were modified at Watson-Crick base pair positions with CMCT. Prp24, a protein known to catalyze U4/U6 formation *in vitro*, was added to the modified snRNPs to initiate their interaction, after which non-denaturing polyacrylamide gel electrophoresis was used to separate base-paired U4/U6 from free U4 and U6 (Raghunathan and Guthrie 1998, Rader and Guthrie 2002, Bell et al. 2002). Modified nucleotides were detected by primer extension as described above (Chapter 3). Chemical modification of a nucleotide that is critical to U4/U6 base pair formation results in the inability of that base to hydrogen bond to the corresponding nucleotide in U6, and interferes with di-snRNP formation. If the nucleotide is not critical, the modified U4 will be incorporated into the di-snRNP. Therefore, the pattern of modification in the U4/U6 di-snRNP, compared to the modification pattern of free U4 snRNP, identifies the nucleotides that are required for U4/U6 di-snRNP formation. This chapter presents the determination of functionally important uridines of U4 that are necessary for base pair formation.

5.1 Materials and Methods

Modification/Interference Assay

Fifty femptomoles of Brr2 released U4 snRNP was modified with 20µL of 0.4M CMCT in the presence of 5µg *E. coli* tRNA. CMCT reactions were performed in a total volume of 200µL borate buffer (50mM borate/KOH pH 8.0, 50mM KCl, 10mM $MgCl_2$) at pH 8.0, for 5 minutes at room temperature, and stopped with 20µL of 1M Tris-HCl pH 6.8. U4 and U6 snRNPs were annealed with 25pmol 10His-tagged Prp24 in the presence of 7µg BSA (NEB), 10U SUPERase-In (Ambion), and 1.5µg *E. coli* tRNA for 15 minutes at room temperature. The reaction was stopped by addition of 25µL Brow stop buffer (10mM EDTA, 0.5% SDS, 300mM NaOAc), and the RNA was immediately extracted with phenol/chloroform. Samples were run on a 6% non-denaturing gel at 300V for 45 minutes at 4°C to separate free U4 from base paired U4. U4 containing bands were eluted from the gel by crushing the gel slice with a disposable pestle (VWR), adding 200µL of 0.25mg/mL *E. coli* tRNA, heating for 10 minutes at 70°C, and spinning through an EZ-10 spin column (Bio Basic Inc.) for 6 minutes at 900RCF. Samples were primer extended for at least an hour at 45°C, run on an 8%/7M denaturing gel, and visualized by autoradiography.

10His-Prp24 Expression and Purification

Plasmid pSR175 containing an N-terminal 10His-tagged Prp24 was alkaline lysis mini-prepped, and transformed into competent Rosetta *E. coli* cells. For purification, 1L of cells were grown at 37°C to an $OD_{600} = 0.7$. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1mM and the cells were grown for an additional 3 hours. The cells were harvested at 8000RCF for 5 minutes at 4°C, washed with 40mL of wash buffer (20mM Tris-HCl pH 8.0, 500mM NaCl), spun at 3270RCF for 10 minutes at

4°C, and frozen and stored at -80°C. The frozen pellet was resuspended in 25mL of wash buffer with protease inhibitors (250µL of 10X Albert's protease inhibitor mix, and 250µL 10X [4-(2-Aminoethyl) benzenesulfonyl fluoride, hydrochloride] (Bio Basic Inc.)). Cells were disrupted by sonication at power 3 for 15 cycles of 10 second bursts with a 10 second pause between each burst (Fischer Scientific Sonic Dismembrator Model 100), and centrifuged at 72,000RCF for 20 minutes at 10°C. The supernatant was filtered and loaded onto a 1mL His Trap column (Amersham Biosciences). After the sample was loaded the column was washed with 13 column volumes of column wash buffer (500mM NaCl, 20mM Tris-HCl pH 8.0, 5mM ß-mercaptoethanol, 30mM imidazole, 10% glycerol). 10His-tagged Prp24 was eluted from the column with a gradient of 0 – 100% elution buffer (column wash buffer with 500mM imidazole) over 10 column volumes at 0.5mL/min, followed by 100% elution buffer for an additional 10 column volumes. The peak fractions (total 2mL) were dialyzed against buffer TNB (500mM NaCl, 20mM Tris-HCl pH 8, 5mM ß-mercaptoethanol, 30% glycerol) overnight. The pooled fractions were run alongside bovine serum albumin (BSA) on a 10% SDS polyacrylamide gel, and the concentration of Prp24 was determined by Bradford assay. The purified protein was stored at -80°C.

5.2 Results and Discussion

Due to the nature of the Brr2 preparation, Prp24 is not associated with the Brr2 released U6 snRNP particle. Purified recombinant Prp24 must be added to catalyze di-snRNP formation of Brr2 released snRNPs. 10His-tagged Prp24 was partially purified from crude *E. coli* lysate with a 1mL HisTrap column (Figure 15, lanes 2 – 5). Clearly some contaminants were still present after purification; however, the partially purified protein had the ability to anneal free U4 and U6 snRNPs to form the U4/U6 di-snRNP (Figure 16), so

further purification was deemed unnecessary. In total, 1mL of dialyzed protein at a concentration of 196μM was obtained after column purification.

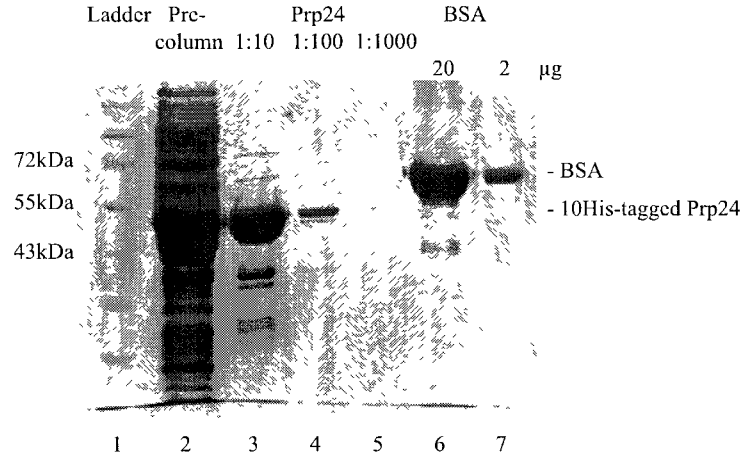


Figure 15. Purification of 10His-tagged Prp24. (Lane1 – ladder, 2 – crude *E. coli* lysate, 3 – 1:10 dilution of Prp24, 4 – 1:100 dilution of Prp24, 5 – 1:1000 dilution of Prp24, 6 – 20 μg of bovine serum albumin (BSA), 7 – 2 μg of BSA).

Brr2 released U4 and U6 snRNPs do not anneal in the absence of Prp24 over a period of 45 minutes at room temperature (Figure 16, lanes 2 – 5; data not shown), proving that Prp24 is indeed necessary for *in vitro* di-snRNP formation. The maximum amount of di-snRNP produced was 73% using 25pmol of Prp24 (Figure 16, lane 7), and did not increase with the addition of more Prp24, even up to 196pmol (Figure 16, lanes 6 – 8; data not shown). Therefore all subsequent experiments were carried out with 25pmol of Prp24. The addition of 150μL of CMCT buffer decreased the amount of di-snRNP formed (Figure 16, lanes 7 and 9; Figure 17, lane 8; data not shown), and more noticeably the addition of CMCT itself considerably inhibited the formation of the di-snRNP (Figure 17). Titration of CMCT in structure probing experiments (Chapter 3) showed that the addition of 20μL of CMCT (8μmoles) was near the minimum amount of CMCT that showed distinct modifications. Although the addition of 20μL of CMCT showed a decrease in the amount of di-snRNP

formed, this decrease is not as extreme compared to the addition of 50 or 40μL of CMCT. As a result, 20μL of CMCT was chosen for subsequent modification/interference experiments.

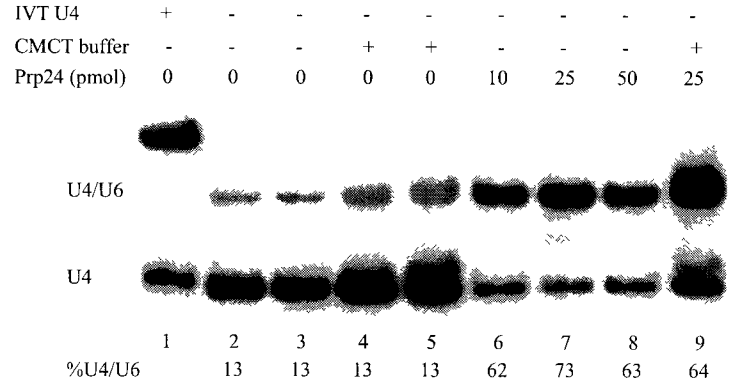


Figure 16. 10His-tagged Prp24 anneals Brr2 released U4 and U6 snRNPs. Approximately 7.5fmol of Brr2 released U4 snRNP was annealed to Brr2 released U6 snRNP with Prp24 for 15 minutes at room temperature. Reactions were allowed to proceed in the presence (Lanes 6 – 9) or absence (Lanes 2 – 5) of annealing factor Prp24, for 15 minutes at room temperature (Lanes 3, 5, 6 – 9), or stopped immediately with Brow stop buffer (Lanes 2 and 4). Annealing reactions were performed with an additional 150μL of CMCT buffer (Lanes 4, 5, 9) to determine how this buffer affects the annealing reaction.

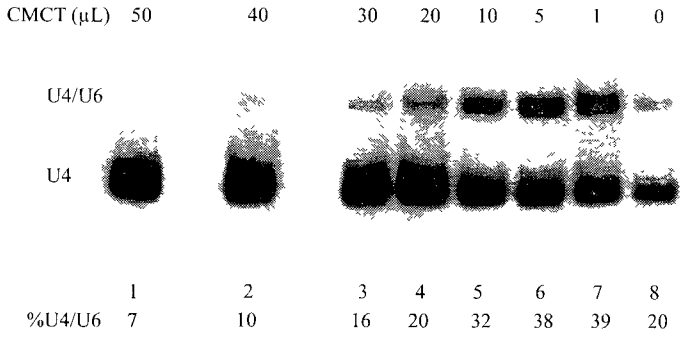


Figure 17. Addition of CMCT impairs di-snRNP formation. Approximately 7.5fmol of Brr2 released U4 snRNP was annealed to Brr2 released U6 snRNP with 25pmol of Prp24 for 15 minutes at room temperature in the presence of 150μL CMCT buffer, and 0 to 50μL of 0.4M CMCT.

The amount of U4 used for modification/interference was increased from 7.5fmol to 50fmol in hopes of increasing the amount of U4/U6 formed. The increase in starting

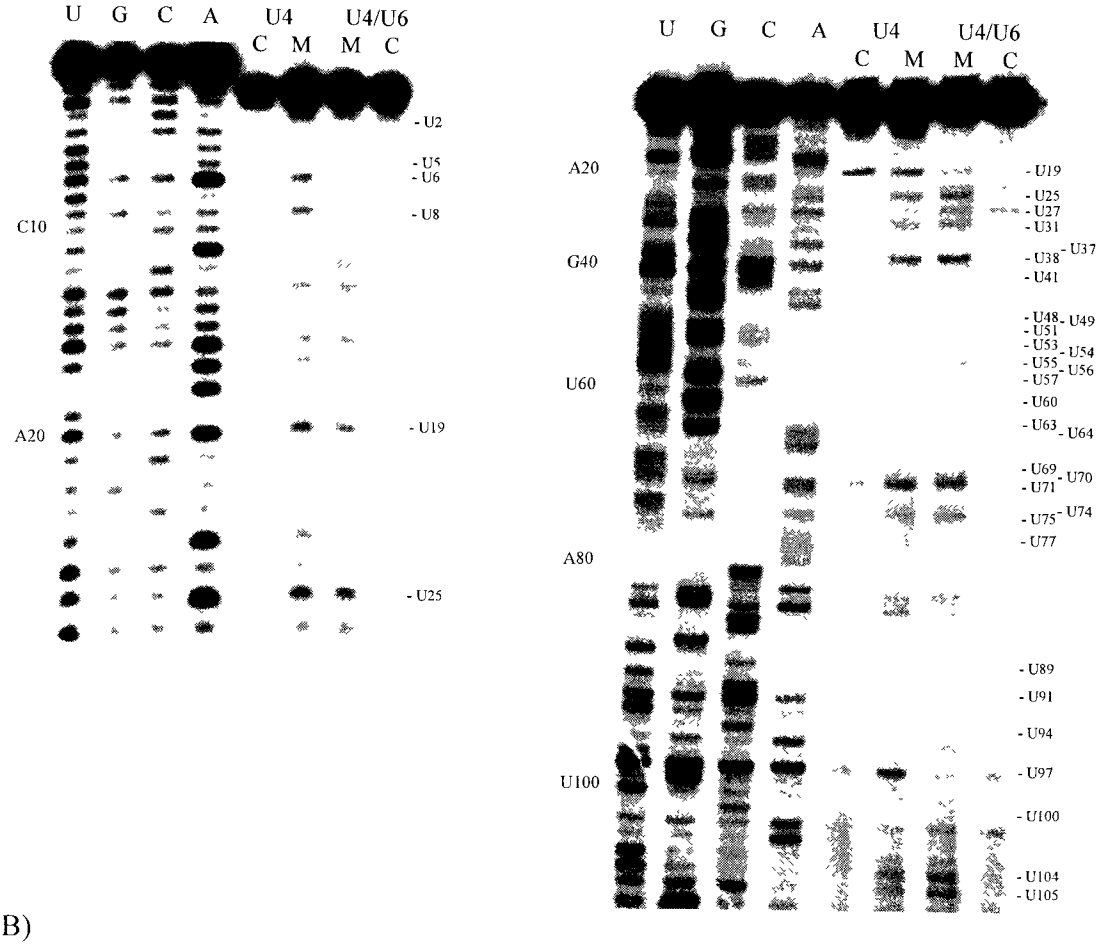
material consistently resulted in 50% U4/U6 di-snRNP formation with no CMCT added, and approximately 35% di-snRNP formation with 8μmoles of CMCT added (data not shown). The increase in U4 snRNP did not change the modification pattern observed, as all of the uridine residues that have the potential to be modified continue to be modified by this protocol except for U91, which was observed to be a weak modification during structure probing (Chapter 3).

To facilitate analysis and discussion of the degree of interference observed in these experiments, I developed an interference index whereby the strength of interference at a particular position can be reported as a numerical value. The interference index was calculated by first determining the intensity of the band in each lane by subtracting a background band and normalizing to the full length band for that lane. The interference index at each position indicated in Figure 18B was calculated by dividing the value of the U4 modified band subtracted by the U4 control band by the U4/U6 modified band subtracted by the U4/U6 control band. In principle, an interference index greater than 1 would indicate some interference in di-snRNP formation, and an interference index equal to 1 would indicate no interference in di-snRNP formation. However, some uridines in the central and 3' region of U4 had a calculated interference index as low as 0.49. While it is possible that some modifications alter the U4 structure in such a way as to allow it to be incorporated more readily into the di-snRNP compared to wild-type U4, it is unlikely that modification that should inhibit a base-pair interaction accomplishes this. It is more likely that +/- .5 indicates the amount of error associated with the calculation of the interference index (data not shown). Therefore, if the interference index was 1 +/- 0.5 the nucleotide was said to not

inhibit di-snRNP formation, and if the interference index was greater than 1.5 the nucleotide was said to inhibit di-snRNP formation.

Most of the modified uridines are readily incorporated into the U4/U6 di-snRNP (interference index = 1 +/- 0.5), except for uridines 5, 6 and 8 (Figure 18; Table 4; Table 5). The interference indices indicate that these modified uridines are underrepresented in the di-snRNP compared to the free U4 snRNP. Thus, modification at U5, U6, or U8 interferes with the ability of these residues to base pair with their corresponding partners in U6, and consequently inhibits the formation of the di-snRNP. The results support the kissing loop model, in which the loop region of the extreme 5' stem loop is critical for formation of the di-snRNP. Notably this is in agreement with literature regarding the necessity of stem II for di-snRNP formation in yeast, humans, and Xenopus. In humans, deletion of all nucleotides that form stem II, or half of these (nucleotides 1 – 8) resulted in complete abolishment of di-snRNP formation *in vitro* (Wersig and Bindereif 1990), and in Xenopus deletion of stem II was found to inhibit U4/U6 di-snRNP assembly (Vankan et al. 1990, Vankan et al. 1992). Notably in Xenopus, substitution of nucleotides 2 – 6 or nucleotides 12 – 16 of U4 (analogous to the stem nucleotides of the short 5' stem loop) resulted in an intermediate phenotype, but substitution of nucleotides 7 – 11 (analogous to loop nucleotides) inhibited formation of the di-snRNP (Vankan et al. 1990, Vankan et al. 1992). Therefore the loop nucleotides of the short stem loop are essential for di-snRNP formation.

A)


U G C A U4 U4/U6
C M M C
- U2
- U5
- U6
C10
- U8
A20
- U19
- U25
U G C A U4 U4/U6
C M M C
A20
- U19
- U25
- U27
- U31
G40
- U38
- U37
- U41
- U48
- U49
- U51
- U53
- U54
U60
- U55
- U56
- U57
- U60
- U63
- U64
- U69
- U71
- U70
- U75
- U74
A80
- U77
- U89
- U91
- U94
U100
- U97
- U100
- U104
- U105

B)

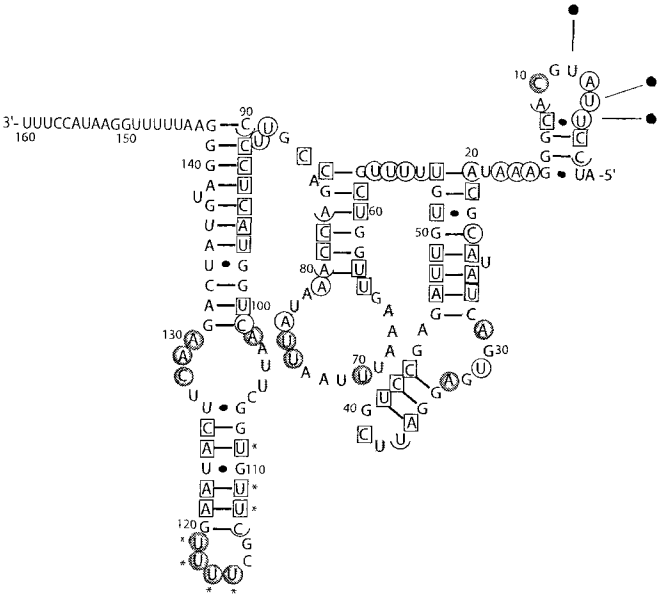

3'- UUUCCAUAAGGUUUUUAAG
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
UUUUUU
AUAAAG
UA -5'

Figure 18. Modification/interference analysis with CMCT reveals that modification of U5, U6, and U8 interferes with di-snRNP formation. A) Modification/interference analysis for the uridine nucleotides of U4. Primers used in this analysis were 14b and CM2. U, G, C, A are dideoxy sequencing lanes made with the corresponding oligonucleotide. Uridine residues are labeled on the right. (U4 C – unmodified U4 that was not incorporated into the di-snRNP, U4 M – modified U4 that was not incorporated into the di-snRNP, U4/U6 M – modified U4 that was incorporated into the di-snRNP, U4/U6 C – unmodified U4 that was incorporated into the di-snRNP). Modification/interference experiments were performed at least twice. B) Schematic diagram of the free U4 snRNP with the modification pattern determined from structure probing experiments mapped on it (Chapter 3). Boxes indicate protected nucleotides, semi circles indicate very weak modifications, circles indicate weak modifications, grey circles indicate medium modifications, and dark grey circles indicate strong modifications. Nucleotides at the 3' end of the molecule are where oligonucleotide 14b binds. Nucleotides lacking boxes or circles are nucleotides for which there is no information, or positions of strong reverse transcriptase stops. Nucleotides that interfere with di-snRNP formation are indicated with lines linked to closed circles. (*) Indicates uridines that were not assayed in this experiment.

Table 4. Interference Index used to determine if a modified nucleotide inhibits di-snRNP formation. The Interference Index was determined as follows: The intensity of the band in each lane was determined by subtracting a background band and normalizing to the full length band for that lane. The interference index at each position was calculated as (U4 modified – U4 control) / (U4/U6 modified – U4/U6 control). If the interference index is 1 +/- 0.5 the nucleotide does not inhibit di-snRNP formation; if the interference index is > 1.5 then the nucleotide inhibits di-snRNP formation. Modification/interference experiments with CMCT were completed twice for all uridine residues indicated in Figure 18, with similar interference indices being obtained. Additional experiments showed that modified residues outside of the 5' kissing loop did not interfere with di-snRNP formation (interference indices not shown).

Nucleotide	U4 control	U4 modified	U4/U6 modified	U4/U6 control	Interference Index	Inhibits di-snRNP formation
U5	.163	.407	.211	.137	3.30	Yes
U6	.490	.900	.363	.221	2.89	Yes
U8	.330	.802	.354	.231	3.84	Yes
U19	.679	1.04	.951	.372	.617	No
U25	.702	1.45	1.47	.527	.792	No

Table 5. Interference Index calculated from two independent experiments for U5, U6, U8, U19, and U25. The Interference Index was determined as above (Table 4).

Nucleotide	Interference Index for Gel #1	Interference Index for Gel #2	Average Interference Index	Inhibits di-snRNP formation
U5	3.30	3.69	3.50	Yes
U6	2.89	2.81	2.85	Yes
U8	3.84	3.19	3.52	Yes
U19	.617	.842	.730	No
U25	.792	1.40	1.10	No

All of the residues that compose Stem I of the U4/U6 di-snRNP are also found base-paired in the free U4 snRNP (Chapter 3), except for U57, which did not interfere with di-snRNP formation. In humans deletion of all nucleotides that form stem I in U4 snRNA inhibited but did not abolish di-snRNP formation (~ 50% compared to wild-type), as deletion of stem II nucleotides did (Wersig and Bindereif 1990). Moreover, *in vivo* mutational analysis in yeast showed that stem II nucleotides (1 – 15) were the most sensitive to mutational change, but stem I nucleotides were very tolerant (Hu et al. 1995). It seems that base pairing in stem I may be optimal for di-snRNP formation, but it does not appear to be crucial like stem II.

Interestingly modification of uridines 69, 70, 71, 74, and 75, that are predicted to be base paired to U6 in the putative stem III helix (Jakab et al. 1997) does not interfere with di-snRNP formation. While it is unclear whether stem III formation is important during spliceosome assembly, the U4 uridine residues of this stem do not appear to be critical for U4/U6 di-snRNP formation. It is possible that stem III may function in U4/U6 di-snRNP dissociation by helping to position U6 correctly with the 5' splice site.

It is not surprising that uridines of the 5' kink-turn stem loop do not interfere with base pair formation given that none of these nucleotides are predicted to be base-paired to U6 in the di-snRNP, and also because deletion experiments in yeast and humans have found this region of U4 snRNA to be dispensable for di-snRNP formation. Deletion of human U4 snRNA 5' kink-turn stem loop reproducibly enhanced U4/U6 formation, but resulted in a block at subsequent spliceosome assembly stages. This is supported by yeast deletion experiments showing that deletion of U4 snRNA nucleotides 19 – 52 still had the ability to base pair to U6, but could not subsequently associate with the U5 snRNP (Bordonne et al. 1990). This suggests that the 5' kink turn stem loop is necessary for spliceosome assembly at stages following di-snRNP formation.

No interference was detected from uridines in the 3' region of the molecule. Unfortunately a limitation of the assay is the inability to analyze nucleotides that are originally sequestered in the free snRNP, as many of the uridines in this region are. However, the few modified uridines 3' of the putative stem III interaction domain did not interfere with di-snRNP formation. The role of the 3' stem loop in di-snRNP formation appears to differ between species. The 3' portion of human U4 (nucleotides 91 – 145) including the Sm binding site has been found to be dispensable for both U4/U6 di-snRNP formation and subsequent spliceosome assembly and catalysis *in vitro* (Wersig and Bindereif 1990, Wersig and Bindereif 1992). Contrary to these results, a deletion mutant in yeast consisting of nucleotides 1 – 90 could not support di-snRNP formation, while U4 mutant 1 – 142 could, indicating a requirement for nucleotides 90 -142 (Hayduk and Rader 2010). The discrepancy observed between human and yeast may reflect differences in the splicing machineries of the organisms. It is possible that one or more splicing factors in humans for

which there is no homologue in yeast fulfill the functional requirements that the 3' region of yeast U4 satisfies in di-snRNP formation.

In conclusion, it is clear that stem II nucleotides are essential for di-snRNP interaction *in vitro*, and the loop nucleotides of the novel 5' stem loop appear to be the most significant. This supports a model of di-snRNP formation where loop nucleotides 6 – 11 of U4 snRNA and single stranded nucleotides 75 – 71 of U6 snRNA are the initial nucleation site of di-snRNP formation, and stem I nucleotides may contribute a smaller auxiliary function. However, it is not clear what steps of base-pair formation are monitored with the modification/interference experiment used here. While it is possible that the modifications inhibit the initial step of di-snRNP formation, they may also inhibit subsequent interactions with U6, or protein interactions that proceed through the Watson-Crick base pair positions of the RNA. Further experiments are needed to determine the temporal association of stem I, II, and III in the formation of the U4/U6 di-snRNP.

Chapter Six – Future Directions and Concluding Remarks

6.1 Future Directions

The structure probing experiments presented here demonstrate the presence of a short stem loop at the 5' end of Brr2 released U4 snRNP *in vitro*. To complete chemical secondary structure probing of all positions in this molecule the chemical modifier kethoxal, which modifies guanine bases, can be used. Protection of the 5' kissing stem nucleotides G13 – G15 would strongly support the presence of this short stem, and preliminary tests suggest that they are indeed protected from modification.

Modification/interference analysis of U4 with CMCT indicates that modification of 5' loop nucleotides U6 and U8, and closing stem nucleotide U5 inhibits di-snRNP formation. Modification of uridines throughout the remainder of the molecule does not interfere with di-snRNP formation, suggesting that the 5' kissing loop is important in di-snRNP formation, possibly as a means of initial di-snRNP nucleation. Modification/interference assays would be complete with the use of DMS and kethoxal modifiers. The use of DMS would provide information for the 5' kissing loop nucleotides A7, C10, and A11, and modification/interference assays with kethoxal would provide information for the 5' kissing loop nucleotide G9. The use of these modifiers would confirm that the 3' and central regions of U4 do not inhibit di-snRNP formation, as indicated by CMCT modification/interference experiments.

Although I have determined the secondary structure of a Brr2 released U4 snRNP, it remains unclear if this snRNP is structurally similar to a biogenesis U4 snRNP that has not been base-paired with U6, or if it is similar to U4 snRNP released from U6 in the spliceosome assembly pathway. The biogenesis U4 snRNP is thought to be a species that

has been transcribed and associates with the Sm and possibly U4 specific proteins, but has not base-paired to U6. The U4 snRNP species released from the spliceosome has been base-paired to U6, but is thought to be recycled in a way that it can undergo another association with U6, to be used for a future round of splicing. The literature suggests that Brr2 is the protein responsible for unwinding of the U4/U6 di-snRNP in the spliceosome, but it is unclear whether the released U4 snRNP undergoes compositional or conformational rearrangements in the recycling process prior to being re-annealed to U6 snRNP. Therefore, it is possible that the product of *in vivo* recycling is different from the product of the *in vitro* Brr2 release. An accumulated U4 snRNP, presumably a U4 snRNP species that has not base paired with U6, can be generated by specific mutations in Prp24 that inhibit di-snRNP formation. Structure probing of an accumulated U4 snRNP will provide insight into the possible conformations of U4 snRNA throughout the splicing cycle.

Crystal structure determination and cross-linking experiments indicate that 15.5K and hPrp31 (61K) make direct contact with the 5' kink-turn stem loop of human U4 snRNA (Vidovic et al. 2000, Nottrott et al. 2002, Lui et al. 2007; Chapter 3). Notably, these experiments were only carried out with a small portion of the 5' region of U4, so it is possible that these proteins induce a slightly different conformation in the presence of full length RNA. Also, it will be interesting to see if the yeast proteins Snu13 and Prp31 bind to yeast U4 snRNA in a similar manner as 15.5K and 61K are thought to bind to human U4 snRNA. A technique that may give some insight into the binding locations of these proteins in yeast is hydroxyl radical probing. If the U4 snRNA is bound by proteins (Snu13 or Prp31) then the RNA backbone will be protected from hydroxyl radical cleavage. However, if the RNA backbone is not bound by proteins it will be accessible to hydroxyl radicals. The

difference in hydroxyl radical cleavage pattern between proteinated and deproteinized U4 snRNP may indicate a protein binding site. Protection from hydroxyl radicals at and around the 5' kink turn stem loop of Brr2 released U4 would be a strong indication of the presence of Snu13 and Prp31. Alternatively, it may be possible to express recombinant Snu13 and Prp31 and incubate them with U4 snRNA both separately and in combination to determine the distinct footprint pattern obtained from each protein.

Existing literature suggests that Snu13 and Prp31 may bind to the free U4 snRNP, but an accurate determination of the protein complement of the free U4 snRNP remains to be elucidated. Mass spectrometry of a purified U4 snRNP would return protein candidates, and can be verified with further biochemical experiments, including immunoprecipitation with tagged candidate proteins. First, a U4 snRNP of sufficient purity and quantity must be obtained for mass spectrometry analysis. The Brr2 released U4 snRNP contains large amounts of free U6 snRNP, and small amounts of U4/U6 di-snRNP (Chapter 2). A second purification of the Brr2 released U4 may result in a free U4 sample of sufficient purity for mass spectrometry. This may be accomplished with an additional affinity capture and release using biotinylated 2'-O-methyl oligonucleotides, and streptavidin agarose (Aukema and Rader, unpublished results). The biotinylated 2'-O-methyl oligonucleotide would be designed to bind to the free U4 snRNP, but not to U4 that is base-paired with U6. Free U4 that is bound by the biotinylated oligonucleotide can then be separated from the other snRNP species by interaction with streptavidin agarose. This procedure has been employed to yield purified U4 snRNP, although the amounts obtained may be insufficient for mass spectrometry (Aukema and Rader, unpublished results). Alternatively, it may be possible to purify the Brr2 released U4 snRNP, or an accumulated U4 snRNP, using glycerol gradients

that fractionate snRNPs based on composition and size. An accurate determination of the protein complement of free U4 snRNP is necessary for understanding the role of snRNP specific proteins in U4/U6 di-snRNP formation.

There is still considerable debate about the structure of free U6 snRNP and whether free U6 contains a 3' intramolecular stem loop (ISL), a structural feature of U6 that is present in the catalytically active spliceosome. The structure of the Brr2 released U6 snRNP has yet to be determined, and it will be interesting to see if Brr2 released U6 is similar to a free U6 snRNP that does not contain a 3' ISL, or if it adopts a conformation comparable to U6 in the catalytic spliceosome. If Brr2 unwinds the di-snRNP in the spliceosome and allows U6 to fold into a conformation that facilitates assembly of the active spliceosome, free U6 from the Brr2 release may fold into a conformation that contains the 3' ISL. To address this question, the secondary structure of Brr2 released U6 snRNP should be probed with the chemical modifiers, and ribonucleases used here, and compared to structure probing experiments of free U6 snRNP (Fortner et al. 1994, Jandrositz and Guthrie 1995, Karaduman et al. 2006).

6.2 Concluding Remarks

In the field of RNA splicing, there are no examples of RNA interactions whose genesis has been determined. The U4/U6 di-snRNP formation is a significant structural rearrangement in splicing that takes place outside of the active spliceosome, and it is therefore an important model for the regulation of spliceosomal RNA conformational changes. By the use of structure probing methods with chemical modifiers DMS, and CMCT, and with enzymatic probes RNase V_1 and RNase A, I have provided evidence for the existence of a 5' kissing stem loop in Brr2 released U4 snRNP. Modification/interference experiments with CMCT show that modification of 5' kissing loop nucleotides U5, U6, and

U8 inhibits di-snRNP formation, yet modification of uridine bases in other regions of U4 do not inhibit di-snRNP formation. This suggests a model of di-snRNP initiation in which U4 kissing loop nucleotides 6 – 11 initiate contact with U6 single-stranded nucleotides 75 – 71 to provide the site for di-snRNP nucleation. The experiments presented in this thesis suggest a preliminary model for the initiation mechanism of di-snRNP formation that provides a foundation for future mechanistic analysis.

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