Published in final edited form as:

Biochem Soc Trans. 2010 August ; 38(4): 1110–1115. doi:10.1042/BST0381110.

The function of the NineTeen Complex (NTC) in regulating spliceosome conformations and fidelity during pre-mRNA splicing

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Abstract

The NineTeen Complex (NTC) of proteins associates with the spliceosome during pre-messenger RNA splicing and is essential for both steps of intron removal. The NTC and other NTC associated proteins are recruited to the spliceosome where they participate in regulating the formation and progression of essential spliceosome conformations required for the two steps of splicing. It is now clear that the NTC is an integral component of active spliceosomes from yeast to humans and provides essential support for the spliceosomal snRNPs. Here we discuss the identification and characterisation of the yeast NTC and review recent work in yeast that supports the essential role for this complex in the regulation and fidelity of splicing.

Keywords

NineTeen Complex (NTC); Prp19; pre-mRNA splicing; spliceosome

Introduction

Pre-mRNA (pre-messenger RNA) splicing is the essential process by which introns are removed from pre-mRNA and the exons precisely ligated producing mature mRNAs ready for translation into protein. Splicing occurs by two sequential transesterification reactions, requiring accurate recognition of conserved RNA sequences within introns by a large RNP (ribonucleoprotein) machine called the spliceosome [1]. The spliceosome is composed of five snRNP (small nuclear RNP) particles and numerous protein splicing factors. Each snRNP contains a U-rich snRNA (small nuclear RNA), U1, U2, U4, U5 or U6, and a unique set of proteins. There is a cycle of snRNP assembly and disassembly with intron containing pre-mRNAs that results in the removal of introns (Figure 1). The U1 snRNP first recognises the 5' splice site with the U1 snRNA base-pairing with a conserved 5' splice site sequence within the intron. The U2 snRNP then binds the conserved branch site sequence to form complex A. The U2 snRNA forms a helix with the branch site sequence and bulges out a conserved intron adenosine required for the first transesterification reaction. A preformed tri-snRNP composed of the U4, U5 and U6 snRNPs then joins to form complex B. Within

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the tri-snRNP the U4 and U6 snRNAs are extensively base-paired. To form the active spliceosome (B^{ACT}) the U4/U6 base-pairing interaction is unwound allowing U6 to form two mutually exclusive interactions with the 5' splice site sequence and U2 snRNA, resulting in U1 and U4 destabilisation. These RNA/RNA interactions serve to position the bulged branch site adenosine to attack the 5' splice site for the first transesterification reaction, producing 5' exon and intron-3' exon intermediates. Several rearrangements then occur within the active spliceosome to form complex C for the second transesterification reaction, where the two exons are ligated removing the intron. In addition to the snRNPs, protein splicing factors play key roles in the splicing process [1]. For example, several RNA-dependent ATPases and one GTPase promote the rearrangement of the RNA/RNA and RNA/protein interactions that occur during spliceosome assembly, activation and disassembly [2,3] (Figure 1). Another set of splicing factors, that form a complex called the NTC (NineTeen Complex) in yeast, join the spliceosome before, or during, unwinding of U4 from U6 and stay associated with the spliceosome during the two steps of splicing (Figure 1). In recent years it has become evident that the NTC plays an important role in regulating spliceosome conformations and fidelity. This review will primarily focus on the NTC in the yeast *Saccharomyces cerevisiae*, however, the NTC is evolutionarily conserved from yeast to humans.

Prp19 and the composition of the NTC

The NTC is named after the splicing factor Prp19, which was first identified in 1993 as a splicing factor in the yeast *S. cerevisiae* [4]. Prp19 is essential for splicing but is not a constituent of any of the individual spliceosomal snRNPs [5,6]. Association of Prp19 with itself to form tetramers provides the basis for the hypothesis that Prp19 provides a scaffold for NTC organisation [7]. Prp19 contains a U-box domain which exhibits E3 ubiquitin ligase activity *in vitro* [8], however, a target for this activity in the spliceosome is still lacking. Prp19 purified from yeast is associated with a complex of at least ten proteins [9] (Table 1). When the NTC protein Cef1 was used for TAP (Tandem Affinity Purification) a much larger complex was identified containing 27 proteins [10]. Previously unidentified *S. cerevisiae* proteins in the Cef1-TAP complex were named Cwc (Complexed with Cef1) proteins. However, recent mass spectrometry analysis of different spliceosome complexes suggest that a bona fide yeast NTC consists of eight core proteins (Prp19, Cef1, Syf1, Syf2, Syf3, Snt309, Isy1, and Ntc20) [11] (Table 1). The other NTC associated proteins appear to have a more dynamic association with the NTC and spliceosome. Perhaps the most striking finding of the mass spectrometry analysis was the incorporation of the NTC into the spliceosome B complex, before the release of U1 and U4 [11], because previous immunoprecipitation experiments suggested NTC spliceosome recruitment after B complex formation and upon release of U4 [9,12]. The abundance of NTC proteins is greatly increased in activated spliceosomes suggesting that the NTC may be important for the transition of the spliceosome from the B to B^{ACT} complex [11]. Therefore, NTC association with the spliceosome appears to be dynamic and this may reflect the essential roles the NTC proteins play in splicing.

Regulation by the NTC of spliceosome conformations and fidelity

During spliceosome activation and catalysis the spliceosome adopts specific conformations that promote the two steps of splicing. The two state model of the active spliceosome states that spliceosome conformations that promote either the first or second step of splicing are in kinetic competition [13]. During spliceosome activation the NTC promotes RNA/RNA interactions that form the catalytic core for the first step of splicing [12,14]. For example, the NTC specifies the U6 base-pairing interaction with the 5' splice site and an interaction of the 3' end of U6 with the intron. Further, the Lsm proteins are dissociated from the 3' end of U6 in an NTC dependent manner [12]. The NTC also specifies an interaction of the U5 snRNA with the 5' exon of the pre-mRNA during spliceosome activation and, in the absence of the NTC, both the U5 and U6 snRNA associations with the active spliceosome are destabilised [12,14]. Therefore, the NTC is essential for specifying snRNA interactions with the pre-mRNA during splicing.

As well as base-pairing with the 5' splice site, U6 also forms helix I with the U2 snRNA, which is proposed to be part of the catalytic sites for both steps of splicing [15]. The ATPase Prp16 has long been proposed to bring about a conformational change after the first step of splicing that is required for the second step of splicing [16]. Recent work suggests that Prp16 promotes unwinding of U2/U6 helix I after the first step of splicing to allow substrate repositioning for the second catalytic reaction [17]. Helix I is then reformed for the second step conformation of the spliceosome. Deletion of the non-essential NTC protein Isy1 (Ntc30) suppresses a mutation in Prp16, suggesting that the NTC may also stabilise the U6 snRNA interaction with U2 for the first step of splicing [18]. The NTC may also be required to stabilise helix I in the second step of splicing as the NTC is associated with the spliceosome throughout the splicing cycle.

Suboptimal pre-mRNA substrates are rejected if they fail to accurately complete the first step of splicing before ATP hydrolysis by Prp16 [16]. This forms the mechanism by which the spliceosome can maintain fidelity by kinetic proofreading [19]. Mutation of the ATPase domain of Prp16 stabilises, and therefore slows exit from, the first step conformation of the spliceosome. This reduces the fidelity of branch site selection in the first transesterification reaction. The role of the NTC in the formation and stabilisation of RNA/RNA interactions in the spliceosome suggests its function may also be required to maintain splicing fidelity. In fact, deletion of Isy1 reduces the fidelity of 3' splice selection and also restores fidelity of branch site selection when Prp16 is mutated [18].

How the NTC specifies RNA interactions is poorly understood, however, interactions of two proteins place the NTC at the centre of the active spliceosome. The non-essential NTC protein Cwc21 binds directly to the C-terminus of the GTPase Snu114 and the N-terminus of Prp8, two proteins at the heart of the spliceosome [20]. Cwc21 also has a strong genetic interaction with Isy1 so may have a similar function in maintaining the fidelity of splicing [20,21]. Another NTC associated protein, Cwc2/Ntc40, crosslinks directly to U6 in first and second step spliceosomes thus may be involved in specifying/stabilising U6 interactions with the 5' splice site and/or U2 snRNA in the active spliceosome [22]. Cwc2 is the only essential NTC associated protein with motifs capable of binding RNA, an RRM (RNA

Recognition Motif) and zinc finger. Interestingly, Cwc2 also interacts with Isy1 by yeast two-hybrid analysis [23], providing a possible link between Isy1, the NTC, and the U6 snRNA [22]. It is likely that the NTC stabilises conformations and helps maintain splicing fidelity through these direct links with the RNA and proteins at the catalytic core of the spliceosome.

The NTC and the first catalytic reaction

Recent research reveals that the NTC is a dynamic complex with roles in spliceosome activation, disassembly and recycling. The incorporation of the NTC and release of U1 and U4 mark the change from the inactive to the active spliceosome (from B to B^{ACT} complex) [12]. However, after NTC mediated activation, several other proteins are also required for the first catalytic reaction to proceed. One of these proteins is the DEAH-box RNA helicase Prp2, which acts to restructure the spliceosome from the activated spliceosome to the pre-catalytic state (B^{ACT} to B*) [24] (Figure 1). After Prp2 remodelling, at least two other proteins Yju2 (Cwc16) and the heat resistant factor Cwc25 are required to complete the first catalytic step in an ATP independent manner [25-27] (Figure 1). *In vitro* splicing assays using purified spliceosomes stalled in the pre-catalytic state, can be complemented with recombinant or native proteins to identify the necessary factors required for the transition to catalysis. These studies have revealed that the essential NTC associated protein Yju2 is required for spliceosome activation [26]. Its function and interaction with the spliceosome appears to be distinct from the NTC. Unlike the NTC, which remains associated through both steps of splicing, Yju2 only associates with the spliceosome upon activation and appears to dissociate after this step [26]. Similar studies have identified Cwc25 as another essential NTC associated protein required for catalysis [25,27]. Similar to Yju2, complementation analysis revealed that Cwc25 allows stalled pre-catalytic spliceosomes to proceed through splicing. Cwc25 is a heat resistant protein, and is believed to be the heat resistant factor (HP-X) responsible for the transition to splicing described in previous complementation analyses [24,25,27]. While complementation of the pre-catalytic spliceosomes with recombinant Cwc25 allows splicing [27], a separate complementation analysis found that addition of recombinant Cwc25 could only restore catalytic activity to a marginal level [25]. This indicates that other factors in addition to Cwc25 may be necessary for catalysis. Furthermore, addition of affinity purified Cwc25-HA improved catalytic activity, indicating that this additional factor may co-purify with Cwc25 [25]. These analyses reveal that essential conformational changes are performed by NTC associated proteins which are not helicases or necessarily require ATP hydrolysis.

Role of NTC in spliceosome biogenesis and recycling

Spliceosome disassembly is catalysed by the NTR (NineTeen complex-Related) proteins, which are Ntr1, Ntr2 and Prp43 [28]. The non-essential NTC associated protein Cwc23 has also been implicated in spliceosome disassembly through its interaction with Ntr1 [29,30]. Cwc23 is one of 13 J proteins found in *S. cerevisiae*. A partial loss of function Cwc23 mutant displays an increase in unspliced pre-mRNA and an increase in the lariat intron, a phenotype common in mutants which affect spliceosome disassembly [30,31]. Furthermore, deletion of the Cwc23 J domain is synthetically lethal with mutations that compromise the

interaction between Ntr1 and Prp43 [30]. These results imply that the J domain is important for the function of the NTR complex however, as the J domain is itself dispensable, its function may be important under different environmental conditions [30].

Reduced expression or deletion of the NTC proteins Prp19, Ntc90, Ntc77, Ntc20, Ntc25 or Ntc30 results in the accumulation of free U4 snRNP [32]. This accumulation of free U4 may be the consequence of reduced spliceosome recycling due to the defective NTC. Deletion of Ntc25 also results in reduced U4/U6 biogenesis, which in turn affects spliceosome recycling [32]. Reduced expression of Cwc2 results in the reduction of U1, U5 and U6 snRNAs and, unlike other NTC proteins, also results in the reduction of free U4 [22]. Although a mechanism is unclear, the evidence suggests that the NTC is required for the disassembly of the spliceosome and its subsequent recycling. Deletion or depletion of NTC proteins may stall the spliceosome at a specific stage where it is then subject to a discard pathway resulting in decreased spliceosome recycling.

The NTC in humans

The role of the NTC in splicing is evolutionarily conserved from yeast to humans. In humans the NTC is found associated with the spliceosome and is called the CDC5L-SNEV^{Prp19-Pso4} complex or human Prp19/CDC5L complex [33-36]. At the core of the human Prp19/CDC5L complex are hPrp19(SNEV^{Prp19-Pso4}), CDC5L, PRL1(PLRG1), SPF27(BCAS2), AD-002 and Hsp73. Some, but not all, of these core proteins have counterparts in yeast (Table 1). Additionally, as with the yeast NTC, the human Prp19/CDC5L complex appears to associate early with spliceosomes and is remodelled during splicing with its composition and associated factors changing [37-41]. Finally, proteins of the human Prp19/CDC5L complex have been implicated in many non-splicing functions including cellular senescence, DNA damage response, nucleotide excision repair, lipid biogenesis and antibody diversification [42-50].

Summary/future directions

It is now clear that the NTC is an integral component of the active spliceosome. Mass spectrometry analysis of spliceosome complexes has revealed that the NTC core associates early with the spliceosome and additional NTC associated proteins are added as splicing progresses. Recent work has revealed that this dynamic association of the NTC with the spliceosome is involved in setting up spliceosome conformations required for splicing and maintaining the fidelity of splicing. The next step is to determine the exact mechanisms by which the NTC carries out these roles. For example, how do the proteins of the NTC interact with the spliceosome proteins, snRNAs and/or pre-mRNA to regulate spliceosome conformations and fidelity? It is known that the spliceosomal ATPases and GTPase are critically involved in facilitating structural transitions required for splicing. Is the NTC regulated directly or indirectly by the spliceosomal ATPases or GTPase? Finally, the question still remains as to whether there are any targets in the spliceosome for the E3 ubiquitin ligase activity of Prp19 and, if there are, whether their modification has any functional significance to splicing. Now that we have a fully characterised NTC in yeast the

door is open for future work into the mechanisms of how the NTC regulates spliceosome conformations and fidelity.

Acknowledgments

This work was supported by funding from the Biotechnology and Biological Sciences Research Council and the Wellcome Trust.

Abbreviations

pre-mRNA	pre-messenger RNA
RNP	ribonucleoprotein
snRNP	small nuclear RNP
snRNA	small nuclear RNA
NTC	NineTeen Complex
Cwc	complexed with Cef1
TAP	tandem affinity purification
RRM	RNA recognition motif
NTR	NineTeen complex Related

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Figure 1.

Complexes formed during spliceosome assembly and activation. Intron containing pre-mRNAs with 5'SS (5' splice site), BS (branch site) and 3'SS (3' splice site) sequences are recognised by the spliceosomal snRNPs during spliceosome assembly and activation. The U1 and U2 snRNPs interact with the 5'SS and BS, respectively, to form complex A. The ATPase Prp28 then allows the U4/U6.U5 tri-snRNP to assemble with the pre-mRNA along with the NTC proteins to form complex B. The GTPase Snu114 and the ATPase Brr2 induce U4/U6 unwinding with subsequent destabilisation of the U1 and U4 snRNPs to form the complex B^{ACT}. The ATPase Prp2 then remodels the spliceosome to the complex B^{*} which is the catalytically active spliceosome. The action of Cwc25 and Yju2 then induce step 1 of splicing and formation of complex C which contains the step 1 intermediates. Finally the ATPases Prp16 and Prp22 induce step 2 of splicing and formation of the post-splicing complex which contains the spliced mRNA and the removed intron. This post-splicing complex is then disassembled and the snRNPs and NTC proteins are recycled for another round of splicing.

Table 1NTC and NTC associated proteins in *Saccharomyces cerevisiae*

	Yeast gene names	Known domains	Essential	Human protein
Core NTC proteins	PRP19	U Box, WD40	Yes	hPRP19
	CEF1/NTC85	c-Myb DNA binding	Yes	CDC5L
	CLF1/SYF3/NTC77	TPR, HAT	Yes	CRNKL1
	SYF1/NTC90	ROS, HAT, TPR	Yes	hSYF1/XAB2
	SYF2/NTC31	-	No	GCIP p29
	ISY1/NTC30/UTR3	-	No	KIAA1160
	SNT309/NTC25	-	No	SPF27
	NTC20	-	No	-
NTC associated proteins	CWC2/NTC40	Zinc finger, RRM	Yes	RBM22
	PRP46/NTC50/CWC1	WD40	Yes	PRL1
	Bud31/CWC14	-	No	G10
	CWC15	-	No	AD-002/HSPC148
	Yju2/CWC16	-	Yes	CCDC130
	CWC21	-	No	Sm300
	CWC22	-	Yes	KIAA1604
	CWC23	J domain	Yes	-
	CWC24	Zinc finger, RING domain	Yes	RNF113A
	CWC25	-	Yes	CCDC49
	Bud13/CWC26	-	No	MGC13125
	CWC27	PPIase cyclophilin-type	No	NY-CO-10
	PRP17/CDC40/SLU4/SLT15	WD40	No	hPRP17
	PRP22	DEAH Box	Yes	hPRP22
	PRP45/FUN20	SKIP homology	Yes	SKIP1
	SLU7/SLT17	Zinc finger/knuckle	Yes	hSLU7
	ECM2/SLT11	Zinc finger, RRM	No	RBM22
	SPP2	G patch	Yes	GPKOW/T54