

# Direct Probing of RNA Structure and RNA-Protein Interactions in Purified HeLa Cell's and Yeast Spliceosomal U4/U6.U5 Tri-snRNP Particles

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The U4/U6.U5 tri-snRNP is a key component of spliceosomes. By using chemical reagents and RNases, we performed the first extensive experimental analysis of the structure and accessibility of U4 and U6 snRNAs in tri-snRNPs. These were purified from HeLa cell nuclear extract and *Saccharomyces cerevisiae* cellular extract. U5 accessibility was also investigated. For both species, data demonstrate the formation of the U4/U6 Y-shaped structure. In the human tri-snRNP and U4/U6 snRNP, U6 forms the long range interaction, that was previously proposed to be responsible for dissociation of the deproteinized U4/U6 duplex. In both yeast and human tri-snRNPs, U5 is more protected than U4 and U6, suggesting that the U5 snRNP-specific protein complex and other components of the tri-snRNP wrapped the 5' stem-loop of U5. Loop I of U5 is partially accessible, and chemical modifications of loop I were identical in yeast and human tri-snRNPs. This reflects a strong conservation of the interactions of proteins with the functional loop I. Only some parts of the U4/U6 Y-shaped motif (the 5' stem-loop of U4 and helix II) are protected. Due to difference of protein composition of yeast and human tri-snRNP, the U6 segment linking the 5' stem-loop to the Y-shaped structure and the U4 central single-stranded segment are more accessible in the yeast than in the human tri-snRNP, especially, the phylogenetically conserved ACAGAG sequence of U6. Data are discussed taking into account knowledge on RNA and protein components of yeast and human snRNPs and their involvement in spliceosome assembly.

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## Introduction

Spliceosomes that catalyze intron removal in pre-messenger RNAs are formed by sequential assembly on selected pre-mRNA regions of modu-

lar elements (snRNPs U1, U2 and U4/U6.U5) and numerous non-snRNP proteins.<sup>1–4</sup> Whereas, the U1 and U2 snRNPs are associated as individual particles, the U4/U6 snRNP interacts with the U5 snRNP and additional proteins to form a stable 25 S tri-snRNP (U4/U6.U5 tri-snRNP), which is integrated in the pre-spliceosomal complex. Genetic and phylogenetic evidences revealed that U4 and U6 snRNAs interact by formation of two intermolecular helices in the U4/U6 snRNP.<sup>5</sup> However, up to now, no direct experimental study of the resulting Y-shaped RNA duplex has been performed.

Based largely on analysis of splicing complexes in non-denaturing gels, a sequential order of addition of the snRNPs has been defined: first, recognition of the 5' splice junction by the U1 snRNP,

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Abbreviations used: snRNA, small nuclear RNA; RNP, ribonuclear protein; DMS, dimethyl sulfate; CMCT, cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate.

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then interaction of the branch-site sequence with the U2 snRNP, and finally, association of the U4/U6.U5 tri-snRNP.<sup>2,3</sup> However, several recent data suggest that the assembly process is more complex than previously proposed.<sup>6,7</sup> Both spliceosome assembly and catalysis involve a dynamic network of snRNA/snRNA and snRNA/premRNA interactions.<sup>3,8,9</sup> In particular, integration of the U4/U6.U5 tri-snRNP into the pre-spliceosomal complex initiates a complete remodeling of the RNA/RNA interactions in this complex: the U4/U6 interaction is disrupted, allowing U6 snRNA to interact with the 5' region of U2 snRNA,<sup>10–13</sup> the initial interaction of U1 snRNA with the 5' splice site is also disrupted, and a short base-pair interaction is formed between the highly conserved ACAGAG sequence of U6 snRNA and the 5' extremity of the intron.<sup>14–21</sup> Establishment of this U6/5' splice site interaction is thought to be a prerequisite for U4/U6 dissociation<sup>22</sup> and U5 snRNA is likely involved in this process by formation of a U5/5' splice site interaction early in assembly.<sup>3,9,23</sup> Prior to the first step of the reaction, U5 snRNA interacts with the 3' extremity of the upstream exon. After this first step, it interacts with the two exon extremities, that have to be ligated.<sup>6,16,18,24–26</sup> This cascade of remodeling of the RNA/RNA interactions, that is initiated upon tri-snRNP integration, is highly conserved from yeast to vertebrates. This strongly suggests that UsnRNA structures and RNA/protein interactions in the tri-snRNP have been selected for a correct achievement of all these RNA-strand exchanges. In addition, tri-snRNP proteins also play a crucial role in these exchanges, as demonstrated for some of them.<sup>27</sup> Hence, a precise understanding of the tri-snRNP architecture is required for a complete elucidation of the mechanisms responsible for the structural transitions leading to active splicing complexes. The protein content of the tri-snRNP particle is now well established for the human and yeast systems.<sup>28–31</sup> About 30 distinct proteins were identified in the purified human tri-snRNP, many of which are essential for splicing.<sup>32</sup> Homologues of many human tri-snRNP proteins were found in the purified yeast tri-snRNP.<sup>30,31</sup> Some of these highly conserved proteins, with specific catalytic activities (ATPase, GTPase, proline isomerase) have been the subject of deep analyses.<sup>3,33,34</sup> However, little is known on the protein/protein and RNA/protein interactions that bridge the U4/U6 and U5 snRNP components within the tri-snRNP,<sup>35</sup> and knowledge on the binding sites of proteins on U4/U6 and U5 snRNAs remains limited also. Essentially, the yeast Prp6 and its human homologue 102 kDa may bridge the U4/U6 and the U5 components of the tri-snRNP; the yeast Prp3 and Prp4 proteins (human homologues 60 and 90 kDa proteins) associate with the yeast 5'-terminal stem-loop structure of U4 snRNA,<sup>36–38</sup> the human 15.5 kDa protein and its yeast homologue Snu13p bind the 5'-terminal loop of U4 snRNA,<sup>39,40</sup> and the Lsm proteins bind the 3' end of U6 snRNA.<sup>41,42</sup> Cross-

linking experiments showed that the human 220 kDa protein and its yeast homologue Prp8p cover large parts of the 5'-stem-loop structure of U5 snRNA.<sup>43,44</sup> It is also well established that the common set of Sm proteins binds the Sm site of U4 and U5 snRNAs.<sup>45,46</sup> At least, three sets of tri-snRNP proteins (Sm, Lsm and some of the proteins cited above) were found to form multi-protein complexes.<sup>35,47–50</sup>

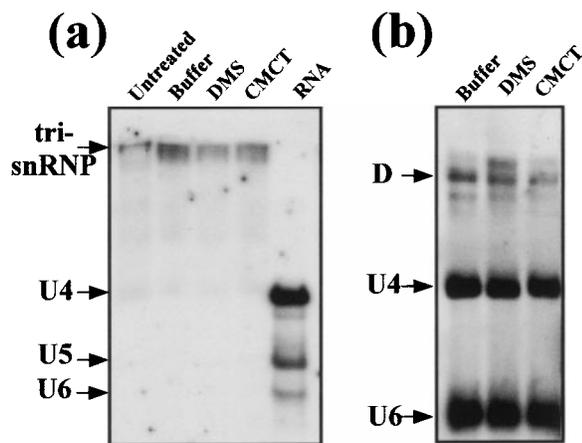
To obtain more information on UsnRNA/protein and RNA/RNA interactions in the yeast and human U4/U6.U5 tri-snRNPs, we performed footprinting experiments on UsnRNAs in these particles. Here, we describe a comparative study of the U4, U5 and U6 snRNA structures and their accessibility in tri-snRNP particles that were purified at low salt concentration from HeLa nuclear extract and *Saccharomyces cerevisiae* cellular extract.<sup>28,30</sup> This study is based on the use of several chemical reagents and RNases under conditions that were selected to preserve the tri-snRNP structure. For a better identification of the UsnRNA segments protected by proteins, analyses were performed under the same conditions on naked yeast and human UsnRNAs, the human U4/U6 snRNA duplex and the human U4/U6 10 S snRNP purified at high salt concentration by MonoQ chromatography.<sup>51</sup> Protection was found to be stronger in the human tri-snRNP than in the yeast tri-snRNP. The possible identity of components that afforded the protection is discussed, taking into account present knowledge on UsnRNAs and tri-snRNP proteins.<sup>30,31,34,78</sup>

## Results

### Strategy used in this study

To identify the segments of UsnRNAs that are protected by proteins in the tri-snRNPs, we had to use conditions for RNA probing that were as gentle as possible to maintain the structural integrity of human and yeast tri-snRNP particles. We had previously developed conditions to probe the U3 snoRNA structure in the yeast U3 snoRNP.<sup>52</sup> Starting from these established conditions, we modified the concentrations of reagents and enzymes, as well as times of incubation, in order to limit tri-snRNP dissociation. The integrity of the human and yeast 25 S tri-snRNPs was tested by gel electrophoresis and glycerol-gradient fractionation. Examples of fractionation by electrophoresis under non-denaturing conditions of the human 25 S tri-snRNPs modified by CMCT or DMS are shown in Figure 1(a).

To identify the segments of UsnRNAs that are protected by the proteins in the tri-snRNPs, we had to compare the accessibility in the particles to that in naked UsnRNAs and the U4/U6 snRNA duplex. To this end, we used a non-denaturing phenol extraction procedure that allowed us to keep a small part of the human U4/U6 RNA duplex in an intact form (Figure 1(b)). After phenol



**Figure 1.** Test of the integrity of the purified human tri-snRNPs and U4/U6 snRNA duplexes after chemical modification (a) The integrity of HeLa cell 25 S tri-snRNP modified by DMS or CMCT or incubated in the CMCT buffer (buffer), was tested by electrophoresis in a native 6% polyacrylamide gel followed by Northern blot analysis. RNA recovered by phenol extraction of the HeLa cell tri-snRNP was fractionated in parallel after heat denaturation (lane marked RNA). Positions of free U4, U5 and U6 snRNAs are shown. A mixture of the radiolabeled oligonucleotides U4-337, U6-411 and U5-409 was used as the probe. (b) The U4/U6 RNA duplexes, prepared as described in Materials and Methods, untreated (buffer) or treated with DMS or CMCT, were purified by 8.5% polyacrylamide gel electrophoresis under non-denaturing conditions and were detected by Northern blot analysis with the radiolabeled oligonucleotides U6-411 and U4-337. Positions of the RNA duplex (D), and free U4 and U6 snRNAs are indicated.

extraction, RNA was chemically modified or digested with enzymes, using the same conditions used for the tri-snRNPs, and was fractionated by electrophoresis in a non-denaturing polyacrylamide gel (Figure 1(b)). The fractionated modified or digested U4/U6 RNA duplex, free U4 snRNA and free U6 snRNA could thus be analyzed independently by primer extension analysis. As only the Sm proteins are present in the human U4/U6 10 S snRNP that are purified at high salt concentration<sup>32</sup> (Table 1), a comparative analysis of U4 and U6 snRNA accessibility in the 10 S particle and in the tri-snRNP was also expected to be useful in the identification of UsnRNA segments protected by components of the 25 S tri-snRNP. The accessibility of U4 and U6 snRNAs in 10 S U4/U6 snRNP was thus studied under the same conditions as used for the tri-snRNP. Experiments were not performed on the purified human 20 S U5 snRNP, as data on this particle were already available.<sup>53,54</sup> Only phenol extracted human U5 snRNA was analyzed for comparison. As the main goal of the study on yeast tri-snRNP was to compare the accessibility of U4, U5 and U6 snRNAs in the yeast and the HeLa cell's tri-snRNPs, we tested

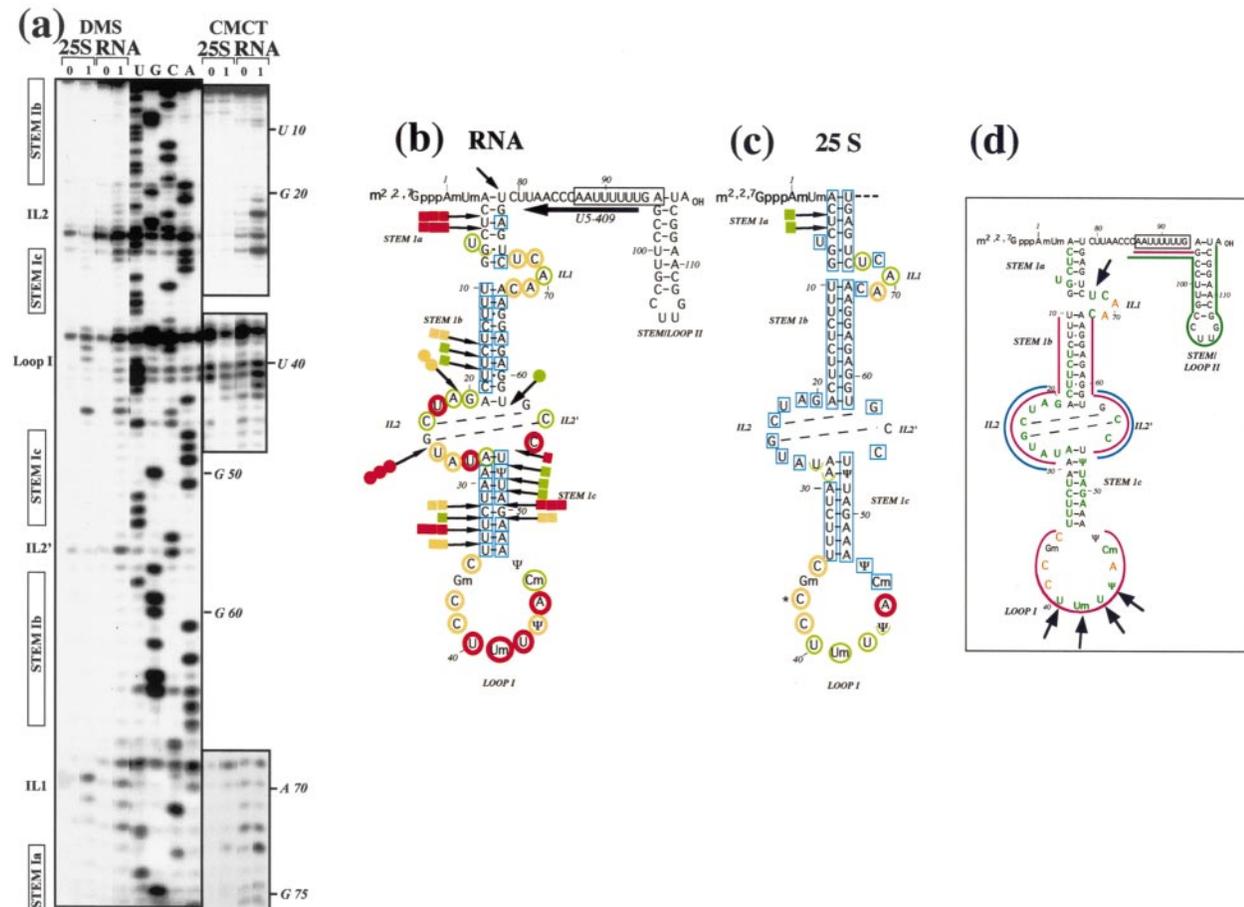
these accessibilities on the purified 25 S tri-snRNP and compared them to those in free U4, U5 and U6 snRNAs. Each probing experiment was repeated between two and five times, depending on the difficulty to get a very clear and reproducible picture of the accessibilities.

### A high protection of U5 snRNA in the human 25 S tri-snRNP

Representative examples of primer extension analyses of U5 snRNA modified by DMS or CMCT, in intact tri-snRNP or after phenol extraction, are shown in Figure 2(a). Results obtained from different experiments are summarized in Figure 2(b) and (c). Data for both chemical and enzymatic probing of free human U5 snRNA (Figure 2(b)) were in good agreement with the previously proposed secondary structure of U5 snRNA,<sup>55</sup> except for loop IL1. This turned out to be a bulged loop protruding out of the 3' strand of the helix, instead of an internal loop as previously proposed. Residues of the internal loops IL2 and IL2' were highly accessible in free RNA, except for the C<sub>23</sub> G<sub>24</sub> and C<sub>56</sub> G<sub>57</sub> dinucleotides, that probably formed base-pair interactions. Residues of terminal loop I were also highly accessible in free RNA (except  $\Psi$  46). Presence of stems 1a, 1b and 1c was confirmed by numerous RNase V<sub>1</sub> cleavages and the absence of chemical modifications. In the HeLa cell 25 S tri-snRNP (Figure 2(c)), U5 snRNA was poorly accessible. Only parts of the terminal loop 1 and bulge loop IL1 remained accessible. Protection of the internal loop IL2-IL2' and of stems 1b and 1c was total. As discussed later, these data are in accordance with the present knowledge of human U5 snRNA-protein interactions.<sup>44</sup>

### Only limited portions of U4 snRNA are protected in the human 25 S tri-snRNP

U4 and U6 snRNAs that had been chemically modified and enzymatically digested in: (i) the phenol-extracted U4/U6 RNA duplex (band D in Figure 1(b)); (ii) the 10 S U4/U6 snRNP; and (iii) the 25 S tri-snRNP, were probed by primer-extension analysis. Representative examples of fractionation of the cDNAs obtained are illustrated in Figure 3(a) and (b). Experiments were repeated several times, and all the data obtained are summarized schematically in Figure 4. A very clear analysis of the U4 snRNA portion 1 to 61, that is involved in helix I and II formation, was obtained with primer U4-337 (gels (a1), (a2) and (a3) in Figure 3(a)). The data bring strong evidence for the presence of the Y-shaped structure involving U4 and U6 snRNAs: (i) RNase V<sub>1</sub> cleavages are restricted to the U4 snRNA segments that are implicated in formation of the 5' stem and the intermolecular helices I and II (gel (a3) in Figure 3(a), and schemes in Figure 4(b) and (c)); and (ii) in both the U4/U6 RNA duplex and the 10 S particle, all the residues expected to be single-



**Figure 2.** Chemical and enzymatic probing of free human U5 snRNA and human U5 snRNA in the purified U4/U6.U5 tri-snRNP. Fractionation of cDNAs obtained after chemical probing of the RNAs are shown (a). Purified HeLa cell tri-snRNP (25 S) and phenol-extracted U5 snRNA (RNA) were modified by DMS or CMCT under the conditions described in Materials and Methods. Lanes marked 0 are control experiments performed in the absence of chemical reagent. Modified nucleotides were detected by extension of primer U5-409 with reverse transcriptase. Lanes U, G, C, A correspond to sequencing ladders made with the same oligonucleotide. Positions of nucleotides and of helices and loops in U5 snRNA are indicated on the right and left sides of the panel, respectively. (b) and (c) Schematic representation of the data obtained upon chemical (a) and enzymatic probing of free human U5 snRNA (b) and human U5 snRNA in the 25 S tri-snRNP particle (c). The secondary structure of U5 snRNA shown is according to Krol *et al.*<sup>55</sup>, with the modification of the internal loop IL1 described here (see Results). Nucleotides modified by DMS or CMCT are circled; colors indicate the level of modification (green, orange and red for weak, medium and strong modification respectively, very weak modifications are represented by a green half-circle). Nucleotides boxed in blue were not modified by chemical reagents. Nucleotides which are not boxed correspond to pauses in the reverse transcription or to sites of primer hybridization. RNase V<sub>1</sub> or T<sub>1</sub> cleavages are indicated by arrows linked to squares or circles, respectively. Colors and numbers of squares and circles are indicative of cleavage yields; the same color symbolism is used as for chemical modifications. Stem and loop numbering shown are according to Krol *et al.*<sup>55</sup> The site of hybridization of the U5-409 primer is shown. (d) The probing data on the tri-snRNP and the naked U5 RNA are compared: green, nucleotides protected in the tri-snRNP; orange nucleotides having identical reactivity in naked RNA and the tri-snRNP. Nucleotides crosslinked to the 220 kDa protein<sup>44</sup> are indicated by black arrows. U5 snRNA sequences important for binding of the 220 kDa protein and Sm proteins<sup>66,82</sup> are underlined in pink and green, respectively. The U5 snRNA sequences important for binding of the 116 kDa protein,<sup>67</sup> are underlined in blue.

stranded in the Y-shaped structure were modified by DMS or CMCT (gels (a1) and (a2) in Figure 3(a) and schemes in Figure 4(a) and (b)). The limited modification by DMS of residues C41 and C42 in the 5'-terminal loop (gels (a1) and (a2) in Figure 3(a) and schemes in Figure 4(a), (b) and (c)), suggests that two G·C pairs were present, which is in agreement with the 3-D structure established for the complex formed between the 5'-terminal stem-

loop structure of U4 snRNA and the 15.5 kDa protein.<sup>40</sup> On the basis of the analyses of the 25 S particles (gels (a2) and (a3) in Figure 3 and scheme in Figure 4(c)), only limited segments of the U4 snRNA portion 10 to 61 were protected in the tri-snRNP.

Some strong stops of reverse transcriptase, that occurred even in the absence of chemical or enzymatic treatment, disturbed the analysis by primer

U4-537 of the central U4 snRNA portion (positions 60 to 101; gel (a4) in Figure 3). However, the results were of sufficient quality to show that the U4 snRNA residues 64-84, which were expected to be single-stranded, were not equally accessible to chemical reagents in the 10 S particle (Figure 4(b)). Furthermore, comparative analysis of 25 S and 10 S particles reveals that only limited portions of the U4 snRNA segment 64-84 show additional protection in the tri-snRNP. Finally, the part of the central stem structure, that was analyzed with primer U4-537 (positions 110-126), was poorly protected in both the 10 S and the 25 S particles (see the strong RNase V<sub>1</sub> cleavages in the terminal stem; gel (a4) in Figure 3(a)).

### The ACAGAG U6 snRNA sequence is protected in the human 25 S tri-snRNP

Analysis of U6 snRNA with primers U6-411 and U6-550 was also obscured at some positions by strong stops of reverse transcriptase that may be due to the numerous post-transcriptional modifications of human U6 snRNA.<sup>56</sup> However, the data obtained also bring strong experimental support for the existence of the Y-shaped interaction involving U6 and U4 snRNAs. This is demonstrated: (i) by detection of RNase V<sub>1</sub> cleavage in the segments involved in the formation of helices I or II (Figure 3(b2), (b3) and (b4) and schemes in Figure 4(a), (b) and (c)); and (ii) by modification by DMS or CMCT of all the U6 snRNA residues that are expected to be single-stranded in the Y-shaped structure (Figure 3(b1) and (b3) and schemes in Figure 4(a), (b) and (c)). Interestingly, the observed sensitivity to DMS and RNase T<sub>1</sub> in the RNA duplex of the U6 snRNA AmGmG trinucleotide (70-72) (Figures 3 (b1) and 4(a)) reflects some instability of the terminal part of helix II in the duplex. Such instability was not detected in the 10 S and 25 S particles.

Chemical and enzymatic analyses of the 5'-terminal region of U6 snRNA in the 10 S particle is in agreement with formation of the 5' stem-loop structure (Figure 3(b3) and scheme in Figure 4(b)). On the basis of the analysis of 25 S particles, protection of this area of U6 snRNA is limited in the tri-snRNP (Figure 3(b3) and scheme in Figure 4(c)). A highly complex pattern of accessibility was observed for the U6 snRNA segment 20-50, which links the U6 5'-terminal stem-loop structure to the Y-shaped structure. Interestingly, this U6 snRNA segment was protected in the 25 S tri-snRNP as compared with the 10 S snRNP (Figure 3 (b3) and schemes in Figure 4(b) and (c)). In particular, whereas the highly conserved ACm<sup>6</sup>AGAGAm sequence was accessible in the 10 S particle, it was buried in the 25 S tri-snRNP.

Another interesting observation is the presence of RNase V<sub>1</sub> cleavage 3' to positions 35 and 36 in the RNA duplex (Figure 3(b2)) and in the 10 S and 25 S particles (Figure 3(b5)). These cleavages might be explained by interaction of the U6 snRNA

segments 27-38 and 94-106, as previously proposed for the U4/U6 snRNA duplex<sup>57</sup> (Figure 5(c)). An alternative explanation was a base-pair interaction between the U6 snRNA segment 30-36 and the U4 snRNA segment 72-79. To choose between these two possibilities, the 10 S and 25 S particles were digested with RNase V<sub>1</sub> after incubation in the presence of oligonucleotide U6-411, which is complementary to the U6 snRNA segment 79-102, or oligonucleotide U4-337, which is complementary to the U4 snRNA segment 65-82. As illustrated in Figure 3(b5), cleavage at positions 35, 36 and 38 of U6 snRNA were abolished by pre-incubation of the 10 S and 25 S particles with oligonucleotide U6-411, whereas they were preserved after pre-incubation with oligonucleotide U4-337. This was a strong indication that U6 snRNA is able to form an internal long-range interaction (Figure 5(c)) in the purified 10 S snRNP and in the tri-snRNP. Results obtained upon direct probing of free U6 snRNA (Figure 5(a) and (b)), strongly suggest that a similar internal U6 base-pair interaction takes place in free U6 snRNA (Figure 5(c)).

### Structure probing of the yeast 25 S tri-snRNP

#### U5 snRNA

Purified yeast 25 S tri-snRNP was subjected to chemical and enzymatic probing under the same conditions used for analysis of the human tri-snRNP. Figure 6 shows representative examples of primer extension analyses of yeast U5 snRNA after modification with CMCT, DMS or kethoxal (Figure 6(a), (c) and (d)) or cleavage by RNase V<sub>1</sub> or T<sub>2</sub> (Figures (b) and (d)) in the 25 S tri-snRNP. The naked yeast U5 snRNA was treated under the same conditions as the tri-snRNP and analyzed in parallel. Results obtained from three distinct experiments are summarized in Figure 6(e) for free yeast U5 snRNA and (f) for U5 snRNA in the 25 S particle.

Most of the observed base modifications and phosphodiester bond cleavage in free U5 snRNA were in agreement with the secondary structure previously proposed for yeast U5 snRNA,<sup>58</sup> except for two areas. In these two areas (one extremity of stem 1a and its bordering region, and segment 146-154) base modifications were detected together with RNase V<sub>1</sub> cleavage, which suggests the occurrence of alternative conformations in solution (Figure 6(e)). Stem 1a may be slightly extended in some molecules and segment 146-154 may be alternatively single-stranded or base-paired with a downstream sequence. In the purified yeast 25 S tri-snRNP (Figure 6(f)), U5 snRNA was not extensively protected as much as in the purified human 25 S tri-snRNP (Figure 2(c)). For instance, large parts of the additional stem-loop structure 1.1 of yeast U5 snRNA, most of the residues of loop IL2' and almost the entire IL1 loop remained accessible in the yeast tri-snRNP. In contrast, loop IL2 was highly protected, as well as stem 1b, stem 1a and

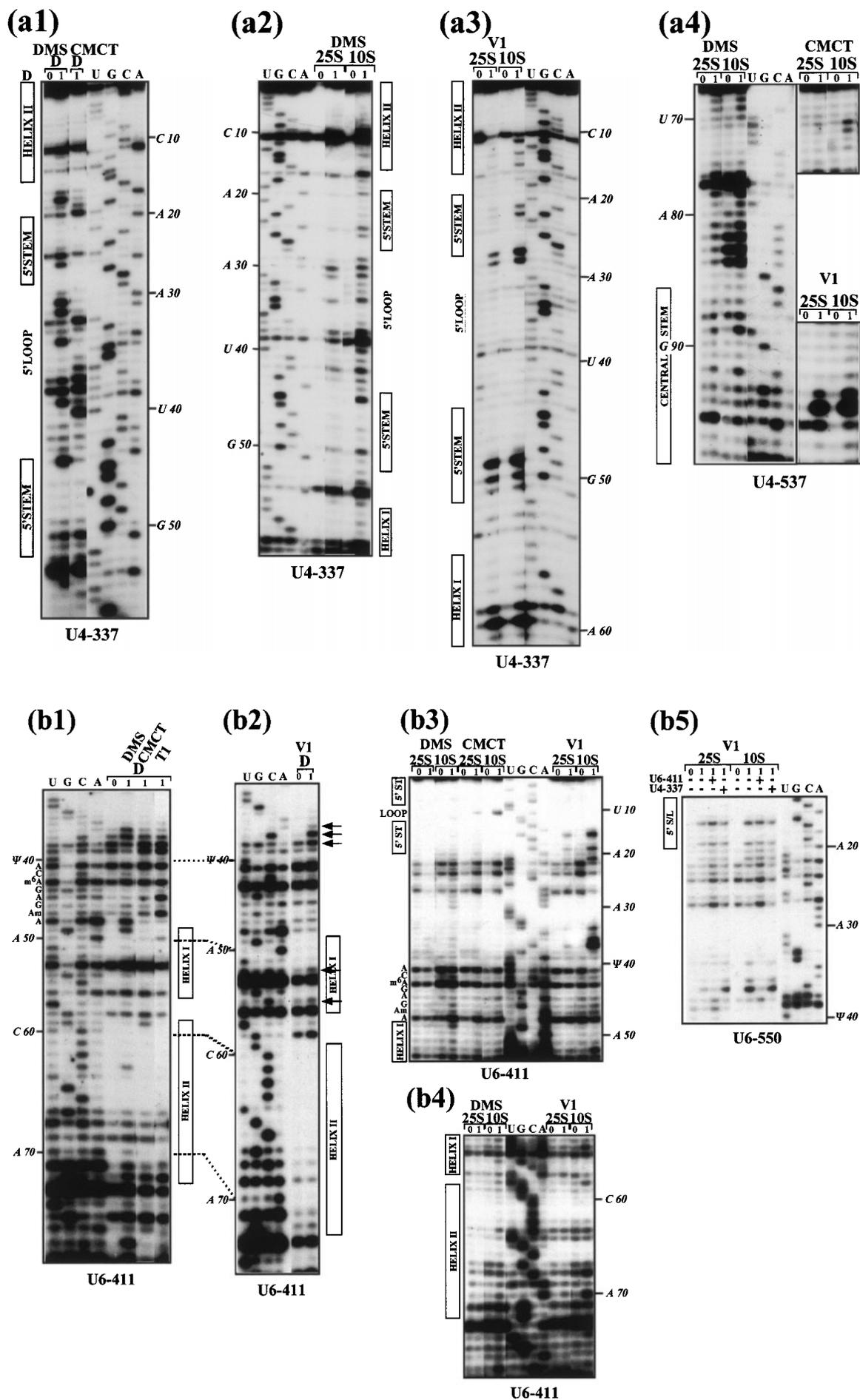


Figure 3 (legend opposite)

the sequence downstream from stem 1a. A common property of the human and yeast tri-snRNP was the partial accessibility of the U5 5'-terminal loop, that aligns the exons in the course of splicing.

### U4/U6 snRNA interaction

Free *S. cerevisiae* U4 and U6 snRNAs and U4 and U6 snRNAs in the purified yeast U4/U6.U5 tri-snRNP were probed with chemical reagents and enzymes using conditions selected to preserve tri-snRNP integrity. Representative examples of primer extension analyses are shown in Figure 7(a), (b) and (c) and results obtained from three distinct experiments are represented schematically in Figure 7(d), (e) and (f) on secondary structure models proposed for free yeast U6 snRNA,<sup>59</sup> free U4 snRNA,<sup>60</sup> and on the model proposed for the yeast U4/U6 snRNA duplex.<sup>5</sup> As shown by a high level of chemical modifications and by the co-localization of these modifications with RNase V<sub>1</sub> cleavage, yeast U6 snRNA structure was heterogeneous in solution. Only the 5'-terminal 25 nt folded into a defined structure (the 5'-terminal stem-loop structure) (Figure 7(d)), that was also found in the 25 S particle (Figure 7(f)). The accessibility observed for the remaining part of free U6 snRNA did not fit any of the secondary structures proposed for yeast U6 snRNA as well the first structure proposed,<sup>59</sup> as the recently proposed telestem structure.<sup>61</sup>

Analysis of free U4 snRNA confirmed the presence of the 5' stem-loop structure (residues 20-53) (Figure 7(e) and (f)). The low level of modification by kethoxal of G residues 34 and 35, and the absence of modification of C residues 43 and 44 by DMS were in agreement with formation of two G·C base-pairs within the terminal loop, generating the loop-helix-loop structure found in human U4 snRNA.<sup>40</sup> Chemical modifications and enzymatic cleavage clearly demonstrated formation of the 3' stem-loop structure in free U4 snRNA and the tri-snRNP (Figure 7 (e) and (f)). In free U4 snRNA, the data confirmed the formation of the central stem-loop structure (Figure 7(e)). However, as indicated by limited modification by chemical reagents, all the helical parts of U4 snRNA which are rearranged in the U4/U6 RNA duplex are characterized by a low stability (Figure 7 (e) and (f)).

In the tri-snRNP, chemical modifications were in perfect agreement with the Y-shaped structure. Only residues of U6 snRNA that are expected to be single-stranded, and a few residues located in helical regions of low stability (GU or AU pairs), were modified (Figure 7(f)). As the free yeast U4/U6 snRNA duplex was not studied, only the accessibility of the U4 and U6 snRNA segments that have identical secondary structure in the free RNAs and the tri-snRNP can be compared accurately. This comparison revealed: (i) a low level of protection of the 3'-terminal stem-loop structure of U4 snRNA (modifications and cleavages were identical in the free RNA and the tri-snRNP; Figure 7(e) and (f)); (ii) limited protection of the U4 snRNA 5' stem-loop structure (the bulged A<sub>45</sub>, and residues 29-33, 38-40, 44 in the terminal loop-helix-loop structure; Figure 7(f), and inset). Examination of the parts of U4 and U6 snRNAs that have different conformations in free RNAs and the duplex, reveals that all the single-stranded residues linking the three helices of the Y-shaped structure were modified in low yield, suggesting some protection (Figure 7(f)). The U4 snRNA segment 65 to 90 was poorly protected compared to its counterpart in the human tri-snRNP. Accessibility of the U6 snRNA segment 26-54 was lower than that of the U4 snRNA segment 65-90. However, the ACAGAG sequence was subjected to mild modification, whereas it was completely buried in the human tri-snRNP.

## Discussion

The present work is the first complete experimental analysis of the structure and accessibility of UsnRNAs in purified U4/U6.U5 tri-snRNPs. Both in the human and the yeast particles, U5 snRNA is more extensively protected than U4 and U6 snRNAs, indicating that U5 snRNA is wrapped within a large protein complex, whereas large parts of U4 and U6 snRNAs are located on the surface of the particle. In spite of these common properties, we observed significant differences of accessibility of UsnRNAs in the two particles. This may be explained by a slightly different protein content (Table 1). Indeed, except for Spp381p and Prp18p, most of the yeast proteins have a counterpart in the human tri-snRNP. However, the yeast counterparts of the human U5-specific proteins

**Figure 3.** Chemical and enzymatic probing of human U4 and U6 snRNAs in the purified U4/U6 RNA duplex, U4/U6 snRNP and U4/U6.U5 tri-snRNP. (a) Primer extension analyses of U4 snRNA probed in the U4/U6 RNA duplex (D) (a1), the tri-snRNP (25 S) ((a2) to (a4)) and the U4/U6 snRNP (10 S) ((a2) to (a4)). (b) Primer extension analysis of U6 snRNA probed in the U4/U6 RNA duplex (D) ((b1) and (b2)), the tri-snRNP (25 S) ((b3) to (b5)) and the U4/U6 snRNP (10 S) ((b3) to (b5)). Chemical reagents (DMS, CMCT) and enzymes (V1 for RNase V<sub>1</sub> and T1 for RNase T<sub>1</sub>) used for RNA probing are indicated at the top of the lanes, together with the identity of the material analyzed (D, 10 S or 25 S). Control experiments in the DMS, CMCT or RNase V<sub>1</sub> buffer are marked 0. Lanes U, G, C, A correspond to sequencing ladders. The oligonucleotides used for primer extension analysis are given below each panel. In (b5), addition of oligonucleotide U6-411 or U4-337 prior to the digestion with RNase V<sub>1</sub> is indicated. Positions of nucleotides in U4 and U6 snRNAs and helices and loops in the U4/U6 RNA duplex are indicated on the left and right sides of the panels, respectively. Position of the ACm<sup>6</sup>AGAGAmA sequence is shown.

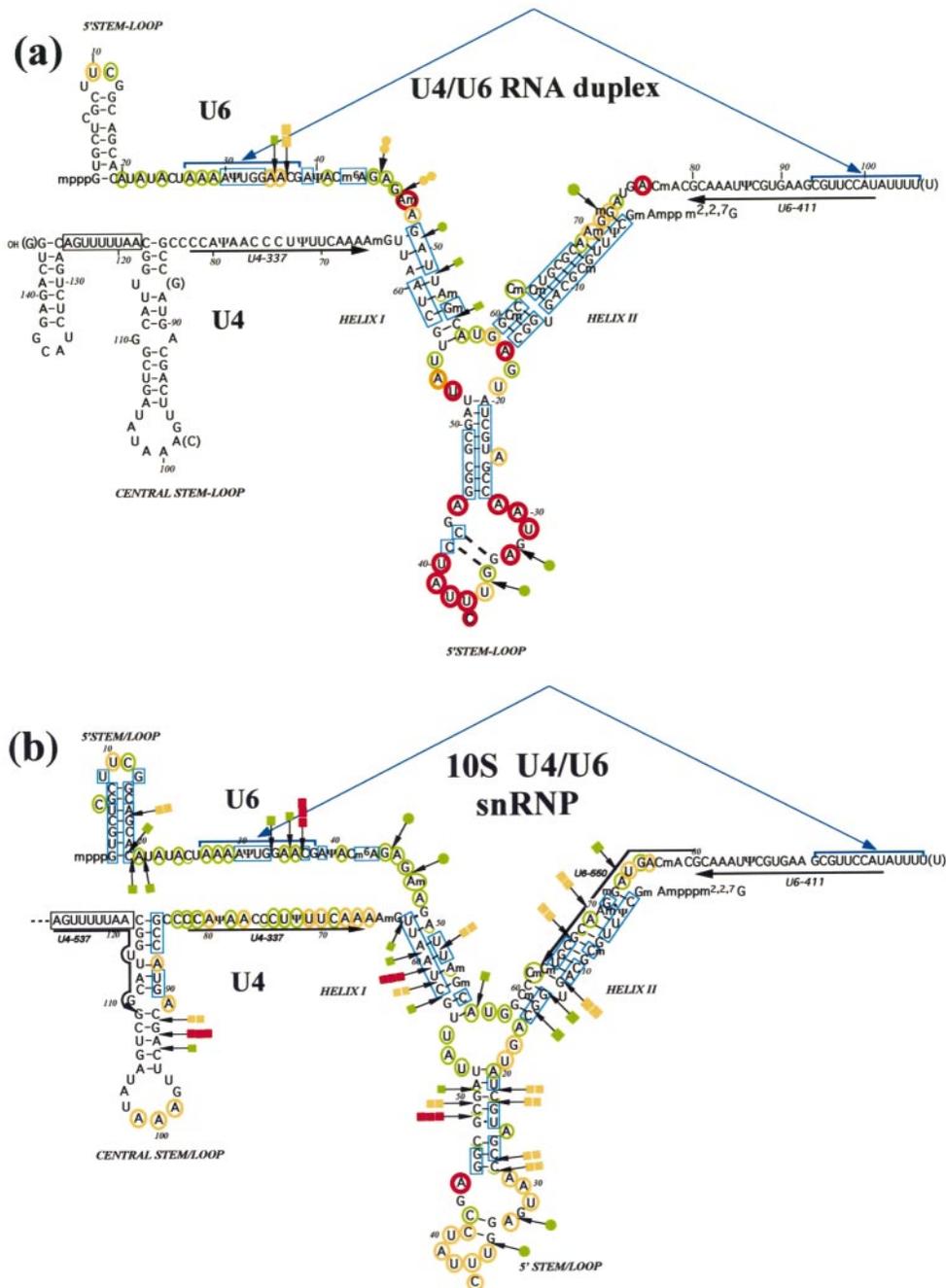


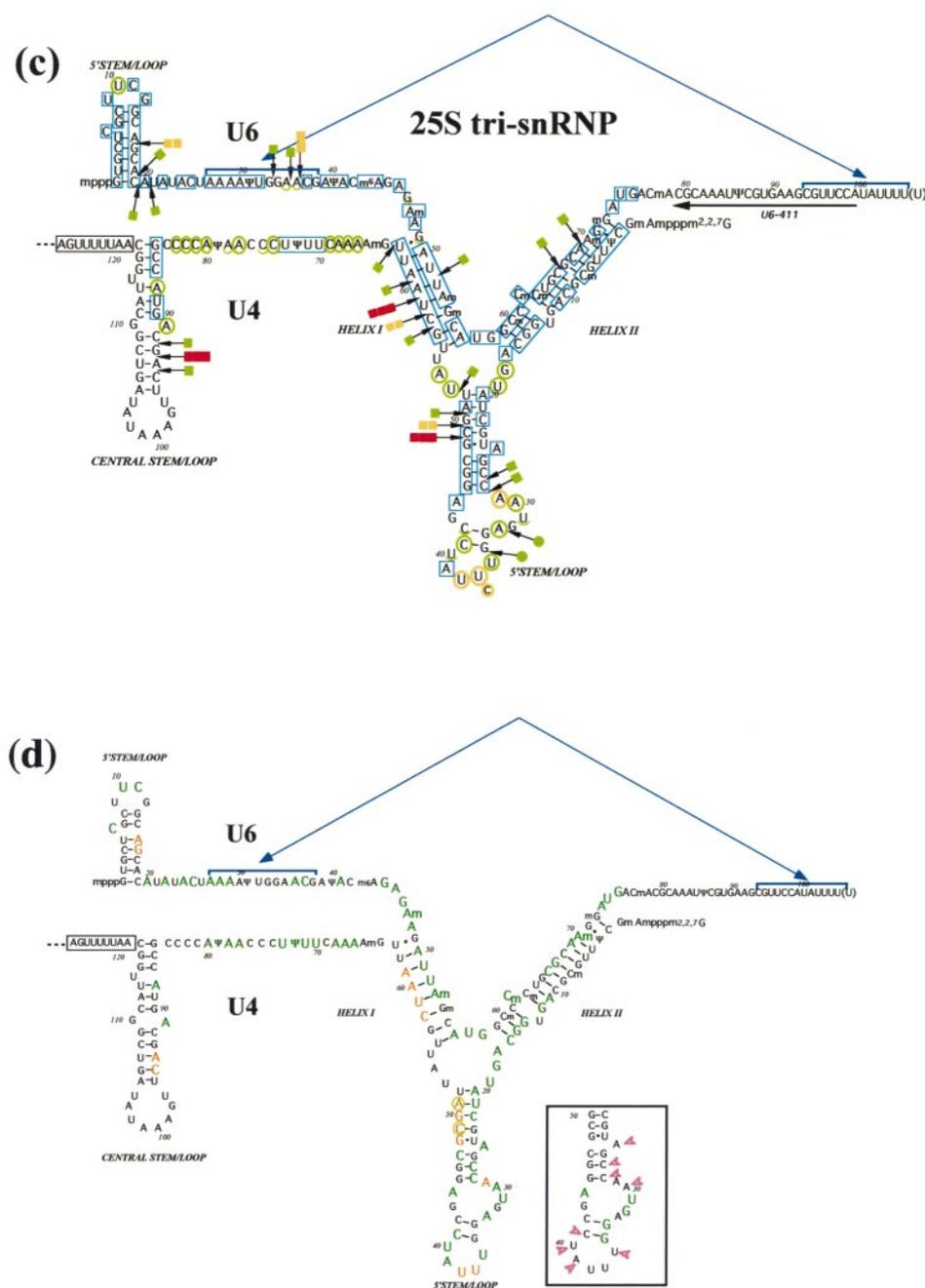
Figure 4 (legend opposite)

40 kDa and 100 kDa<sup>62</sup>, of the human tri-snRNP-specific proteins 65 kDa (SadIp<sup>63</sup>) and 27 kDa and of the human U4/U6 snRNP-specific protein 20 kDa (cyclophilin H<sup>49,64</sup>) were not detected until to now in the purified yeast tri-snRNP.<sup>30,31</sup>

#### Protection of the U5 snRNA 5' stem-loop in the tri-snRNP is due mainly to interactions with U5-specific proteins

In the human tri-snRNP, protection of loops IL2 and IL2' and of stems 1b and 1c of U5 snRNA is stronger than previously observed in the 20 S U5

snRNP.<sup>54</sup> This observation is in agreement with some previous analyses on human tri-snRNP,<sup>53,65</sup> although these analyses were not as precise as the one presented here. On the basis of crosslinking data, loop IL1 and the terminal loop I of U5 snRNA were proposed to contact the U5 snRNP 220 kDa protein.<sup>44</sup> Interestingly, the four cross-linked residues in loop I form a stretch of protected U and Ψ residues bordered by chemically modified A and C residues in the human tri-snRNP (Figure 2(c)). In accordance with the protection of stem 1b and loop I in the tri-snRNP, these two U5 snRNA elements were proposed to be required for



**Figure 4.** Schematic representation of probing data on the secondary structure model for human U4/U6 snRNA interaction. The U4/U6 RNA secondary structure model used was established by Brow & Guthrie.<sup>5</sup> Data obtained upon analysis of the U4/U6 RNA duplex, the U4/U6 snRNP (10 S) and the tri-snRNP (25 S) in several distinct experiments are represented schematically in (a), (b) and (c), respectively. Symbols for representation of chemical modifications and enzymatic cleavages are as for Figure 2. The primer (U6-411 and U4-337) hybridization sites are indicated. The interaction between the U6 snRNA segments 27 to 38 and 94 to 106 (overlined in blue), evidenced by RNase V<sub>1</sub> digestions that disappeared in the presence of oligonucleotides U6-411, but not in the presence of oligonucleotide U4-337 (Figure 3 (b5)) is shown. (d) The difference of accessibility to chemical reagents and enzymes of U4 and U6 snRNAs in the 25 S tri-snRNP as compared with the 10 S snRNP. Residues protected in the tri-snRNP are given in green. Residues with identical reactivity in both particles are in orange. For the 5' stem of U4, where very mild modification with chemical reagents were observed in 10 S but not in 25 S snRNP, whereas strong cleavages by RNase V<sub>1</sub> found in 10 S persist in 25 S, nucleotides are shown in orange, circled in green. In the inset, residues of the U4 snRNA 5' stem-loop, which were protected by the recombinant 15.5 kDa protein in the U4 snRNA-15.5 kDa protein complex, are shown in green (S. Nottrott, unpublished results) and nucleotides protected in the tri-snRNP but not in the 15.5 kDa/U4 snRNA complex are indicated by pink arrows.

**Table 1.** Comparison of the protein composition of the purified yeast and human U4/U6.U5 tri-snRNP

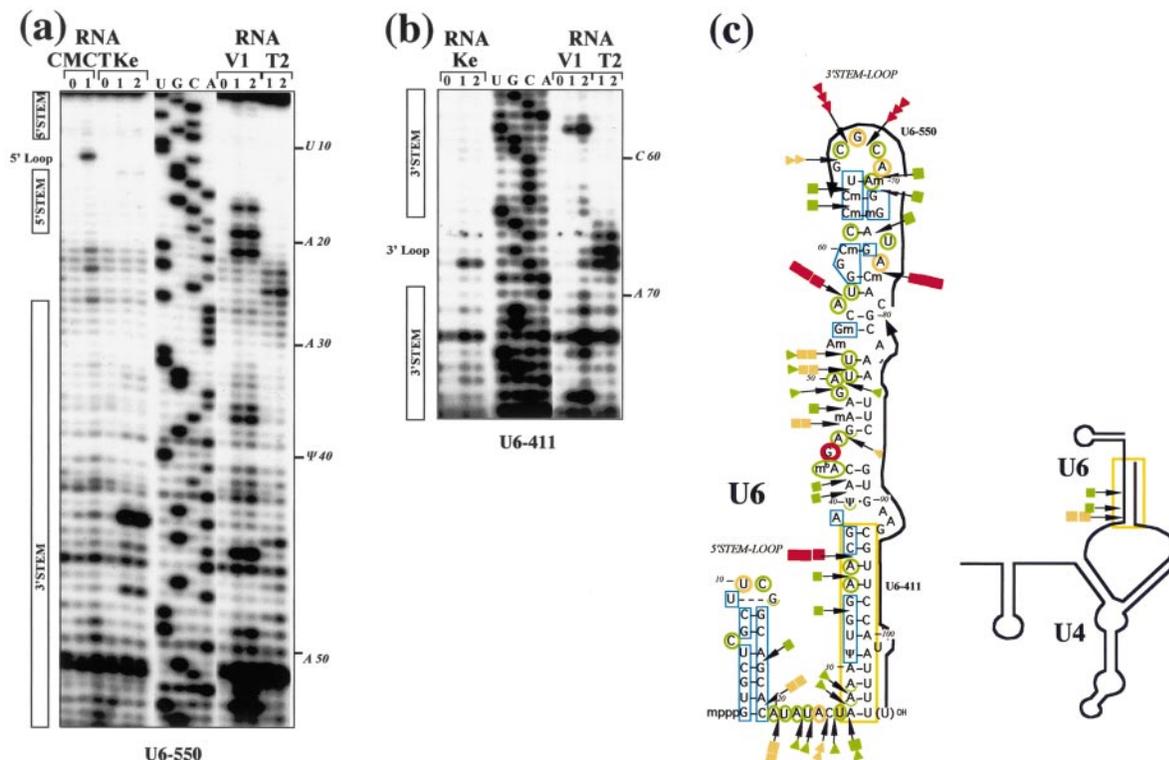
Human proteins	20 S U5 snRNP	10 S U4/U6 snRNP	25 S U4/U6.U5 snRNP	Yeast homologues	U5 snRNP	25 S U4/U6.U5 snRNP
220 kDa	+	-	+	Prp8p	+	+
200 kDa	+	-	+	Brr2p	+	+
116 kDa	+	-	+	Snu114p	+	+
100 kDa	+	-	+	Prp28p	+	-
52 kDa	+	-	-	Snu40p	+	-
40 kDa	+	-	+	-	-	-
15 kDa	+	-	+	Dib1p (Snu16p)	+	+
102 kDa	+	-	+	Prp6p	-	+
110 kDa (SART1)	+	-	+	Snu66p	-	+
61 kDa	-	-	+	Prp31p	-	+
90 kDa	-	-	+	Prp3p	-	+
60 kDa	-	-	+	Prp4p	-	+
-	-	-	-	Prp38p	-	+
-	-	-	-	Snu23p	-	+
15,5 kDa	-	-	+	Snu13p	-	+
-	-	-	-	Spp381p	-	+
-	-	-	-	Prp18p	-	+
20 kDa (cyclophilin H)	-	-	+	-	-	-
65 kDa	-	-	+	(Sad1p)	-	-
27 kDa	-	-	+	-	-	-
SmB/B'	+	+	+	SmB	+	+
SmD1	+	+	+	SmD1	+	+
SmD2	+	+	+	SmD2	+	+
SmD3	+	+	+	SmD3	+	+
SmE	+	+	+	SmE	+	+
SmF	+	+	+	SmF	+	+
SmG	+	+	+	SmG	+	+
Lsm4	-	-	+	Lsm(Uss1)	-	+
Lsm8	-	-	+	Lsm8	-	+
Lsm6	-	-	+	Lsm6	-	+
Lsm2	-	-	+	Lsm2	-	+
Lsm5	-	-	+	Lsm5	-	+
Lsm7	-	-	+	Lsm7	-	+
Lsm3	-	-	+	-	-	-

The human proteins listed in the left column were found in the purified U5 snRNP or the 10 S U4/U6 purified snRNP, and/or the 25 S U4/U6.U5 purified tri-snRNP,<sup>28,32</sup> as indicated by (+) or (-) in columns 2 to 4. Their counterparts in yeast are given in the fourth column. The detection of these yeast proteins in purified yeast U5 snRNP,<sup>78</sup> purified 25 S U4/U6.U5 tri-snRNP<sup>30,31</sup> are indicated by (+) in the two last columns, respectively. A (-) indicates the absence of a protein counterpart either in yeast or human purified particles.

binding of the 220 kDa protein.<sup>66</sup> Also consistent with the strong protection of loop IL2 and IL2' in the tri-snRNP, deletion of loop IL2 was found to prevent the reconstitution of an active U5 snRNP, since specific binding sites for some of the U5-specific proteins were lost, especially that for the 116 kDa protein.<sup>67</sup> The U5-specific proteins 116, 40 and 200 kDa form a complex that interacts with the 220 kDa protein.<sup>50</sup> The 102 kDa U5-specific protein associates with this complex and links the U5 and U4/U6 parts of the tri-snRNP.<sup>35</sup> On the basis of our protection data, this multi-protein complex is probably anchored on stems 1b and 1c, and the internal loops IL2 and IL2'. Within this complex, the 220 kDa protein is probably highly extended contacting loop IL1 on one side, and the terminal loop I on the other. In naked U5 snRNA, loops IL2 and IL2' are linked by two base-pair interactions; such peculiar structuration may participate in the recognition of the U5-specific protein complex.

Yeast U5 snRNA is longer than human U5 snRNA, explaining in part why yeast U5 snRNA is less protected than human U5 snRNA in the tri-

snRNP particle. In naked yeast U5 snRNA, loops IL1 and IL1' form a C·G and a G·C base-pair. This reinforces the idea that this loop pair is the counterpart of the IL2 and IL2' loop pair of human U5 snRNA,<sup>58</sup> which are also connected by two base-pair interactions. However, based on this correspondence, the low protection of loop IL1 in the yeast tri-snRNP was not expected, since loops IL2 and IL2' are highly protected in the human tri-snRNP. The additional loop of yeast U5 snRNA (loop IL2) is protected, suggesting that it is wrapped by the U5 snRNA-specific protein complex. Hence, due to differences in the U5 snRNA structure, the interaction between the U5-specific multi-protein complex and U5 snRNA differs slightly in yeast compared to the human particle. However, as found for the human particle, stems 1b and 1c are highly protected in yeast tri-snRNP, and protection of loop I is similar in the two tri-snRNPs (protection of the uridine-pseudo uridine stretch, and high sensitivity to DMS of the downstream nucleotide A100). In accordance with our observation of protection in almost all the loops of yeast U5 snRNA, the yeast Prp8 protein (the



**Figure 5.** Chemical and enzymatic probing of free human U6 snRNA. (a) and (b) Primer extension analysis of free human U6 snRNA modified with CMCT and kethoxal (Ke), or cleaved with RNases  $V_1$  or  $T_2$  ( $V_1$  and  $T_2$ ). Lanes marked 0 (no reagent); for kethoxal and for RNases  $V_1$  and  $T_2$ , lanes marked 1 and 2 correspond to two concentrations of the chemical reagent, or the enzyme (see Materials and Methods). The oligonucleotides U6-550 (a) or U6-411 (b) were used as primers. (c) Schematic representation of the results obtained on a revised secondary structure model for human U6 snRNA. Symbols for representation of chemical modifications and enzymatic cleavages are as for Figure 2; in addition, RNase  $T_2$  cleavage is indicated by arrows linked to triangles. The positions of primers U6-411 and U6-550 complementary sequences are indicated. The helix of the 3' stem-loop structure that can be formed in the U4/U6 snRNA duplex is boxed in a yellow rectangle, and its position in the U4/U6 snRNA duplex is represented in the scheme on the right.

counterpart of the human 220 kDa protein) was found to form covalent bonds with residues of loops IL2, IL1, loop I, IL1' and IL2',<sup>43</sup> and all the crosslinked residues were protected in the yeast tri-snRNP (Figure 6(f)). The strong conservation between the yeast and the human pattern of chemical modification in terminal loop I is in accordance with the high functional importance of the RNA-protein interaction involving this loop: early in assembly, loop I and protein 220 kDa/Prp8 together with other spliceosomal proteins are involved in the displacement of the U1/5' splice site interaction.<sup>6,23,68–71</sup> Further on in the course of splicing, loop I and protein 220 kDa/Prp8 align the two exon extremities.<sup>72–74</sup>

#### U4 and U6 snRNAs form the Y-shaped structure in tri-snRNPs and only specific segments of these RNAs are protected

Our experimental analysis of the U4/U6 snRNA interactions in the yeast and human tri-snRNPs and in the human U4/U6 snRNP is the first direct and complete demonstration of the Y-shaped structure formed between U4 and U6 snRNAs, and it

shows that this structure is identical in U4/U6 snRNP and tri-snRNP. The Y-shaped structure is largely preserved after deproteinization of U4/U6 snRNP or tri-snRNP. Only the base-pair interaction at one extremity of the heterologous helix is disrupted. Interestingly, the long-range interaction in U6 snRNA, whose formation destabilizes the naked human U4/U6 snRNA duplex,<sup>57</sup> can be formed in human U4/U6 snRNP and tri-snRNP without any destabilization effect. The only difference between the free human U4/U6 snRNA duplex and the 10 S U4/U6 snRNP, purified at high salt concentration, is supposed to be the presence of the Sm proteins in the U4/U6 snRNP. Under these conditions, the Lsm proteins are expected to be absent, or present in very low amounts.

This raises the question of how the Sm proteins bound to the Sm site at the 3' end of U4 snRNA can stabilize the U4/U6 snRNA interaction. The presence of the Lsm proteins was not investigated in the tri-snRNPs used in this study, since Lsm proteins were not discovered at the time they were prepared. As Lsm proteins interact with the 3' end

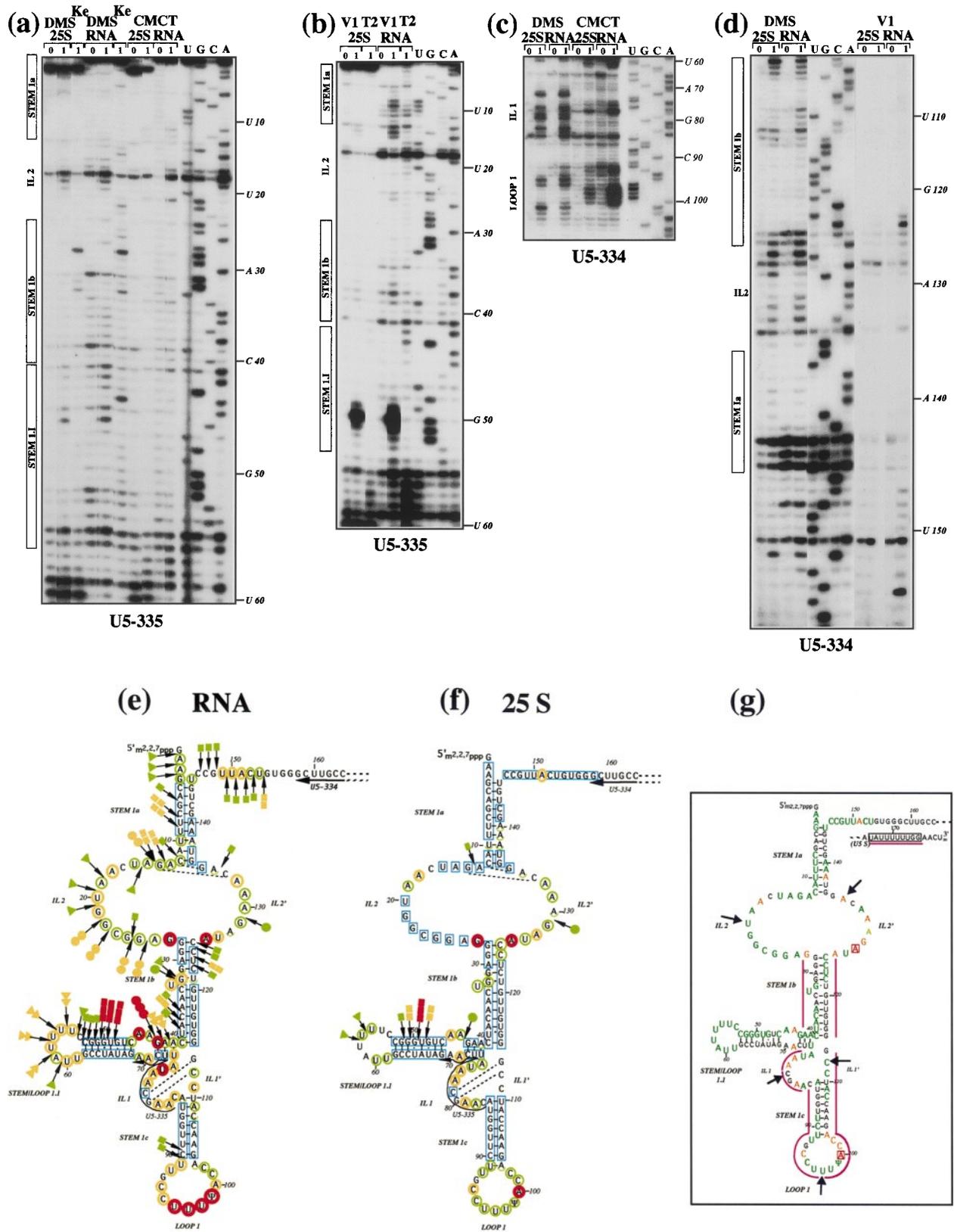


Figure 6 (legend opposite)

of U6 snRNA,<sup>41,42</sup> we cannot exclude the possibility that their binding abolishes formation of the long-range interaction in human U6 snRNA. Our observation of the U6 long-range interaction on purified tri-snRNP may reflect the loss of Lsm proteins in some of the tri-snRNPs particles during purification. The 3-D structure recently established by X-ray crystallography for a portion of the 5'-terminal loop of human U4 snRNA bound to the 15.5 kDa protein<sup>40</sup> demonstrated the formation of a loop-helix-loop structure with two canonical G·C base-pairs, and two tandem sheared G·A base-pairs. Our data revealed the formation of the two internal G·C pairs of the loop-helix-loop structure in the naked RNA (free U4 snRNA and the U4/U6 duplex). The Watson-Crick positions of adenosine residues in sheared A·G pairs are available for DMS modification, so that we cannot predict whether these sheared pairs are formed in naked RNA. The 3-D structure of the terminal loop was not solved by X-ray analysis. The unequal sensitivity to chemical reagents of residues in this terminal loop suggests a higher-order structure. Indeed, residue U<sub>37</sub> in yeast U4 snRNA and its counterpart U<sub>36</sub> in human U4 snRNA were poorly modified by CMCT while they are located in the loop (Figures 4(a) and 7(e)).

According to the established 3-D structures,<sup>40</sup> and to a footprint analysis of U4/15.5 kDa complex (S. Nottrott, unpublished results), binding of protein 15.5 kDa is expected to protect residues U<sub>31</sub>, G<sub>32</sub>, G<sub>34</sub>, G<sub>35</sub> and A<sub>44</sub> against the action of CMCT, kethoxal and DMS (green residues in the inset of Figure 4(d)). The additional protections observed in the human tri-snRNP (pink arrows in the inset of Figure 4(d) namely, protection against DMS of: the AUC (39-41) trinucleotide on the 3' side of the terminal loop, residue A<sub>30</sub> in the internal loop and the bulge A<sub>25</sub> residue of the 5' stem, protection against RNase V<sub>1</sub> cleavage of the C<sub>27</sub>-C<sub>28</sub> dinucleotide at the extremity of the stem (Figure 4(c) and (d)) and the protection against RNase V<sub>1</sub> and chemical reagents in the heterologous helices I and II and their linking segments (Figure 4(c)) may be generated by interaction of the Y-shaped motif with U4/U6-specific proteins.

The complex formed by the human proteins 20, 60 and 90 kDa and the human protein 61 kDa (the homologue of the *S. cerevisiae* Prp31p),<sup>34</sup> are known to interact with the 15.5 kDa protein.<sup>48,49</sup> Furthermore, the yeast Prp3p and Prp4p proteins (homologues of the human 60 and 90 kDa proteins) were found to bind the 5'-stem-loop structure of U4 snRNA in yeast.<sup>36-38</sup> Hence, proteins Prp3/60 kDa and Prp4/90 kDa may account for at least a part of the observed protection in the U4 snRNA 5' stem-loop. Another possibility to consider is the protection of some parts of the Y-shaped structure by U5-specific proteins. Interestingly, there is strong asymmetry in the protection of the U4 snRNA 5' stem-loop structure: the upper part is far more protected than the bottom part (Figure 4(c)). Noticeable is also the stronger protection of helix II, as compared with helix I (Figure 4(c)). Hence, components of the tri-snRNPs (U4/U6-specific proteins and the U5 part of the tri-snRNP) contact only the defined area of the Y-shaped structure of U4/U6, and protection is very similar in yeast and human tri-snRNPs.

The highly phylogenetically conserved ACA-GAG segment of U6 snRNA (positions 41-46 in human, and 47-52 in yeast) plays an essential role in catalysis by formation of a base-pair interaction with the 5' splice site.<sup>8,21,75</sup> Early in tri-snRNP assembly, protein Prp8 interacts directly or indirectly with the region upstream of the ACA-GAG sequence.<sup>7</sup> Later on, the interaction takes place with the ACAGAG sequence,<sup>74,76</sup> and an implication of U5 snRNA in promoting this interaction was also proposed.<sup>6,23,65,77</sup> Surprisingly, whereas the ACAGAG sequence was totally protected in human tri-snRNP (Figure 4(c)), it was partially accessible to chemical reagents in yeast tri-snRNP (Figure 7(f)). In addition, the accessibilities of the single-stranded segment of U6 that links the 5' stem-loop to helix I (26-54), and of the single-stranded segment of U4 that links the 3' stem-loop to helix I (65-90) were as strong in yeast tri-snRNP as they were in human 10 S U4/U6 snRNP. Interestingly, recently, these two U4 and U6 snRNA segments, which are more protected in human than in yeast tri-snRNP, were both found

**Figure 6.** Chemical and enzymatic probing of free yeast U5 snRNA and yeast U5 snRNA in the purified U4/U6.U5 tri-snRNP. (a) to (d) Primer extension analyses of yeast free U5 snRNA (lanes marked RNA) and U5 snRNA in the purified yeast 25 S tri-snRNP (lanes marked 25 S). The chemical reagents (DMS, kethoxal (Ke), CMCT) and RNases (V<sub>1</sub> and T<sub>2</sub> RNases) used are indicated above the lanes. Lanes marked 0 are control experiments. Oligonucleotides U5-335 or U5-334 were used as primers. Lanes U, G, C, A correspond to sequencing ladders. Positions of nucleotides and of helices and loops in U5 snRNA are indicated on the right and left sides of the panels, respectively. (e) and (f) Schematic representation of the data obtained by probing free yeast U5 snRNA (e) and the yeast tri-snRNP (f). The U5 snRNA secondary structure and stem and loop numbering was described by Frank *et al.*<sup>58</sup> The symbols used for representation of chemical modifications and enzyme cleavages are the same as for Figures 2 and 5. Sites of hybridization of the U5-334 and U5-335 primers are shown. (g) Schematic representation of U5 snRNA protection in the yeast tri-snRNP compared to free U5 snRNA. Nucleotides in green are protected in the tri-snRNP; nucleotides in orange have identical reactivity in naked RNA and the tri-snRNP; red boxed nucleotides have increased reactivity in the tri-snRNP. The sites of crosslinking of protein Prp8p on U5 snRNA<sup>43</sup> are indicated by black arrows. The U5 sequences important for U5 snRNA function<sup>58</sup> are indicated by pink lines.

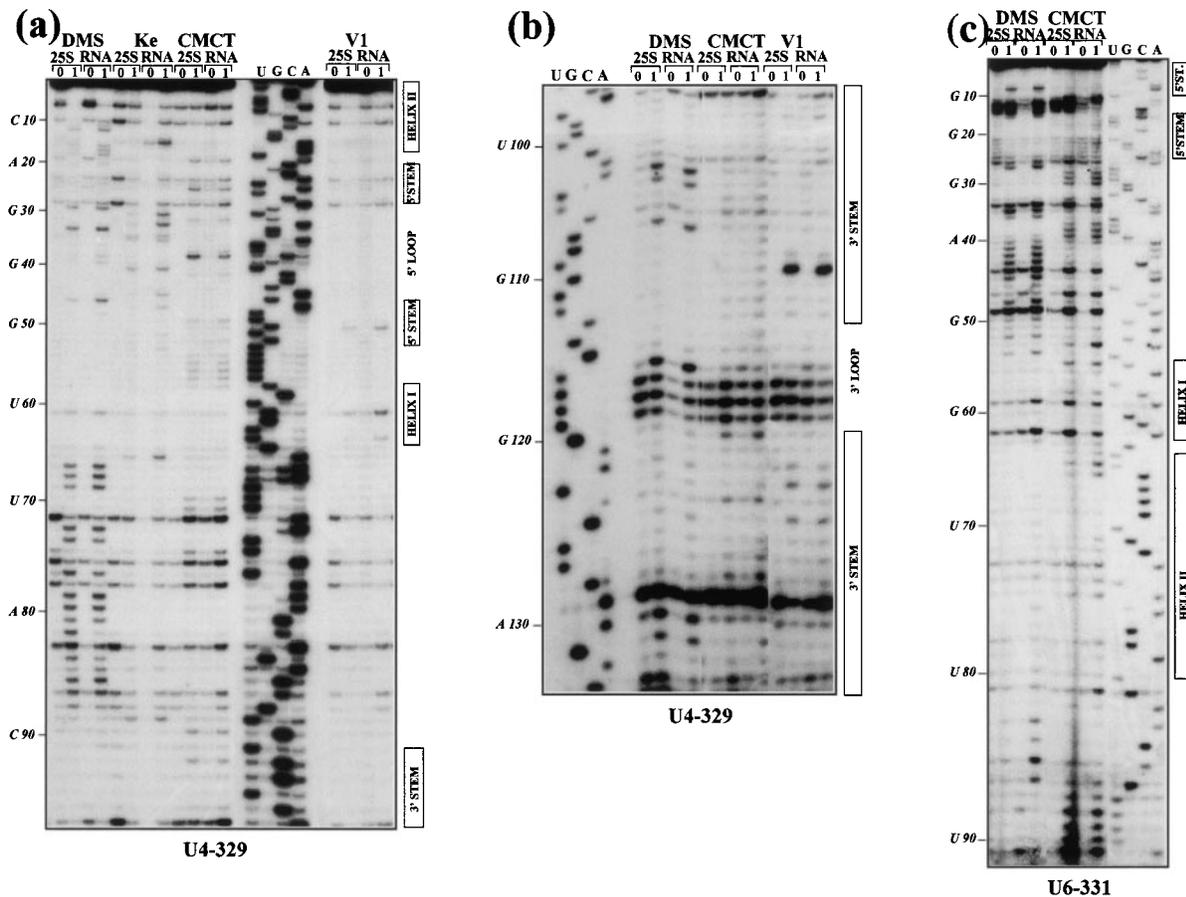


Figure 7 (legend opposite)

to interact with the 5' splice site, in an ATP-dependent fashion, at an early stage of the interaction between spliceosome and pre-mRNA: in a *trans*-splicing reaction, nucleotides U<sub>75</sub>, A<sub>78</sub> and C<sub>82</sub> in the U4 snRNA central single-stranded segment were crosslinked with photoactivable residues incorporated at position +1 or +2 in the intron; in U6 snRNA, residue C<sub>43</sub>, located upstream of the ACAGAG sequence, was also crosslinked.<sup>7</sup> The authors proposed that prior to interaction of the ACAGAG sequence with pre-mRNA, the A<sub>42</sub>C<sub>43</sub>A<sub>44</sub> sequence of U6 snRNA interacts with the UGU 5' splice site sequence, and that U4 snRNA help to align U6 snRNA with the 5' splice site. Proteins Prp8, Brr2 and Prp28 may be involved in this process. Whereas all the U4 and U6 snRNA residues, found to be crosslinked with the intron 5' extremity in the *trans*-splicing assays, were accessible to chemical probes in the purified yeast tri-snRNP, their counterparts in the human tri-snRNP were much less sensitive to chemical reagents, indicating an interaction with other tri-snRNP components. This suggests that some of the yeast proteins involved in the early step of tri-snRNP assembly are loosely bound to the yeast tri-snRNP and are lost during purification. As an

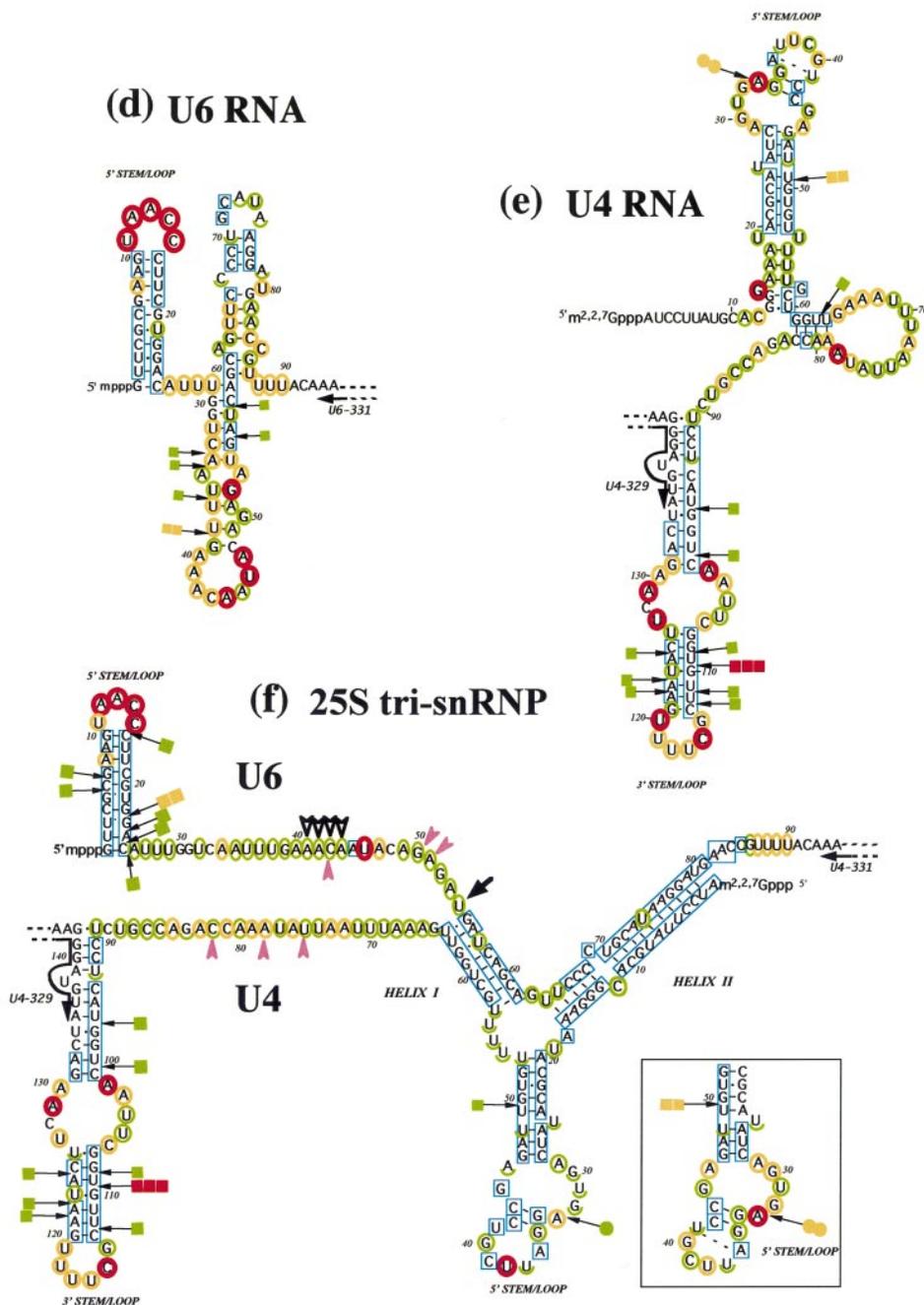
example, protein Prp28 involved in the U1-U6 switch, that was recently shown to be present in yeast U5 snRNP,<sup>78</sup> is not found in the purified yeast tri-snRNP, whereas its human counterpart, the 100 kDa U5-specific protein, is present in the purified tri-snRNP.<sup>28,30,31</sup> (Table 1).

In conclusion, although the purified yeast and human tri-snRNPs share strong common properties, the accessibilities of the U4 and U6 snRNA segments involved in the first steps of tri-snRNP association differ in these two particles.

## Materials and Methods

### Isolation of HeLa 25 S U4/U6.U5 tri-snRNPs, HeLa 10 S U4/U6 snRNPs and yeast 25 S tri-snRNP

Total snRNPs from HeLa cell nuclear extract were purified by immunoaffinity chromatography with anti-m3G antibody, essentially as described by Bach *et al.*,<sup>51</sup> except that the salt concentration was decreased from 420 mM to 150 mM KCl for the recovery of tri-snRNPs. U4/U6 10 S snRNPs were further purified by monoQ chromatography,<sup>51</sup> and 25 S tri-snRNPs by centrifugation on a 10%-30% (v/v) glycerol gradient as described.<sup>28</sup> Isolation of the yeast 25 S tri-snRNPs from



**Figure 7.** Chemical and enzymatic probing of free yeast U4 and U6 snRNAs and U4 and U6 snRNAs in the yeast U4/U6.U5 tri-snRNP. (a) and (b) Primer extension analyses of U4 snRNA after modification in the purified tri-snRNP (25 S) or as free RNA (RNA). Chemical reagents (DMS, Ke, CMCT) and the enzyme (RNase V<sub>1</sub>) used are indicated above the lanes. Lanes 0 are control experiments. Oligonucleotide U4-329 was used as the primer for reverse transcriptase analysis. (c) Primer extension analyses of U6 snRNA after modification in the purified 25 S tri-snRNP or as free RNA. Chemical reagents (DMS and CMCT) and the enzyme (RNase V<sub>1</sub>) used are indicated above the lanes. Oligonucleotide U6-411 was used as the primer for reverse transcriptase analysis. (d), (e) and (f) Schematic representation of the results of chemical and enzymatic probing of: the free U6 snRNA, on the U6 snRNA secondary structure proposed by Fortner *et al.*<sup>59</sup> (d), free U4 snRNA on the U4 snRNA secondary structure proposed by Krol *et al.*<sup>60</sup> (e), and the U4/U6 RNA duplex on the U4/U6 secondary structure proposed by Brow & Guthrie.<sup>5</sup> (f). Symbols used for representation of chemical modifications and enzymatic cleavages are as for Figure 2. The primers hybridization sites (U4-329 and U6-331) are indicated. (f) The binding site for protein Prp24 on U6 snRNP is shown by open arrows.<sup>83</sup> The site of crosslinking of protein Prp8 on U6 snRNA.<sup>77</sup> is shown by a black arrow. Crosslinks of U4 and U6 snRNA to the pre-mRNA 5' splice site are shown by pink arrows.<sup>7</sup> For an easier comparison, the probing pattern of the U4-5' stem-loop of free U4 snRNA is shown in the inset.

strain W303-Prp4-8xHIS was performed according to Gottschalk *et al.*<sup>30</sup>

### Human U4/U6 snRNA duplex extraction, gel purification and analysis

The U4/U6 snRNA duplex was extracted either from total HeLa cell snRNPs (10 to 200  $\mu$ g), or from purified HeLa cell 10 S U4/U6 snRNPs (2 to 20  $\mu$ g), using conditions for phenol extraction that preserve RNA-RNA interactions. The snRNPs, dissolved in 100  $\mu$ l of G buffer (50 mM KCl, 10 mM MgCl<sub>2</sub>, 50 mM Hepes-KOH (pH 7.9)) were phenol-extracted for ten minutes at 0°C. After extraction with chloroform in ice, RNA was recovered by ethanol precipitation.

For RNA structure probing, the extracted U4/U6 snRNA duplex was subjected to chemical modification and enzymatic digestion before fractionation on an 8.5% (w/v) polyacrylamide gel in TBE buffer. Electrophoresis was at 4°C for 12 hours at 8V/cm. The modified or hydrolyzed RNAs were loaded in parallel in two pockets of the gel using non-denaturing loading buffer (10% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanole blue, 50 mM KCl, 10 mM MgCl<sub>2</sub>, 50 mM Hepes-KOH (pH 7.9)). One lane was used for localization of the U4/U6 RNA duplex and free U4 and U6 snRNAs, by Northern blot analysis, using 5' end-labeled oligonucleotides specific for U4 or U6 snRNAs as the probes. Hybridization after electrotransfer to a Biotodyne B Pall membrane was carried out at 26°C in 6 $\times$  SSC buffer for four hours.<sup>79</sup> Positions of modified or cleaved U4/U6 RNA duplex and free U4 and U6 snRNAs fractionated in the second lane were deduced from the Northern blot data; these RNAs were then recovered by an overnight elution at 4°C in elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 0.1% (w/v) SDS, 0.1 mM EDTA (pH 8.0)), in the presence of phenol (1:10, v/v). RNA was precipitated with ethanol in the presence of glycogen (1 mg/ml) or carrier tRNAs (1 mg/ml).

### Extraction of UsnRNAs under denaturing conditions

Free UsnRNAs used for comparison in probing experiments were extracted from HeLa cell or yeast tri-snRNPs (10 to 200  $\mu$ g), by successive phenol and chloroform extractions. Ethanol precipitated UsnRNAs were dissolved in tri-snRNP buffer<sup>28,30</sup> to a final concentration similar to that of the tri-snRNPs.

### Chemical modifications and enzymatic cleavages

Chemical modifications and enzymatic digestions were performed on human and yeast 25 S tri-snRNPs, human 10 S snRNPs, and RNAs that were phenol-extracted from these particles, either under denaturing (U4, U6, U5) or non-denaturing (U4/U6) conditions. For each sample, modifications and cleavages were repeated two to five times. The following chemical probes were used: dimethylsulfate (DMS) that methylates G, A and C residues at positions N7-G, N1-A and N3-C, the carbo-diimide derivative CMCT that alkylates U and G residues at positions N3-U and N1-G; kethoxal that reacts with G residues at the N1 and N2 positions. Except for methylation by DMS at position N7-G, all these modifications cannot occur on base-paired residues, and they stop the extension of primers by reverse transcriptase. Two enzymatic probes for single-stranded regions were

used: RNase T<sub>1</sub> that cleaves after G residues, and RNase T<sub>2</sub> that cleaves after any residue. RNase V<sub>1</sub> was used to cleave specifically the double-stranded or structured regions. As well as for modification for enzymatic cleavages, UsnRNP was pre-incubated for ten minutes in the reaction buffer at the temperature used for the reaction (20°C, except for kethoxal reaction, which was performed at 0°C). Before chemical or enzymatic treatment, naked RNAs, were heated ten minutes at 65°C in the reaction buffer and then slowly cooled at 20°C for renaturation. Except for RNase T<sub>1</sub>, incubations were done in the presence of 1.25  $\mu$ g of yeast tRNA mixture to ensure random modification and cleavage. CMCT modifications were performed for six minutes in 50  $\mu$ l of 100 mM KCl, 2.5 mM MgCl<sub>2</sub>, 50 mM sodium borate (pH 8.0), at CMCT concentrations of 30 or 60 mM (lanes marked by CMCT and 1 or 2, respectively, in the Figures). DMS modifications were performed for six minutes in 50  $\mu$ l of 100 mM KCl, 2.5 mM MgCl<sub>2</sub>, 50 mM sodium cacodylate (pH 7.5), with 1  $\mu$ l of a DMS/ethanol solution (1:1, v/v). Kethoxal modifications were performed at 0°C for ten minutes at a kethoxal concentration of 5 or 10 mg/ml (lanes marked by Ke and 1 or 2, respectively, in the Figures). CMCT modifications were stopped by ethanol precipitation, and were followed by phenol and chloroform extractions in the case of snRNP analysis. DMS modification reactions were stopped by the addition of DMS stop buffer (1.5 M sodium acetate, 1 M  $\beta$ -mercaptoethanol, 0.1 mM EDTA, 1 M Tris-acetate, (pH 7.5), 0.2 of the reaction mixture volume), prior to phenol extraction. Kethoxal modifications were stopped by addition of 0.5 M potassium borate (pH 7.0) (0.25 of the reaction mixture volume), followed by ethanol precipitation and phenol chloroform extractions in the case of snRNP analysis. Before primer extensions, modified RNA pellets were dissolved in 25 mM potassium borate (pH 7.0) to stabilize the chemical adducts.<sup>80</sup> Incubation with RNases T<sub>1</sub>, T<sub>2</sub> and V<sub>1</sub> was for six minutes in 40  $\mu$ l of 100 mM KCl, 2.5 mM MgCl<sub>2</sub>, 50 mM Tris-HCl (pH 7.5). Two enzyme concentrations were used for RNases V<sub>1</sub> and T<sub>2</sub> ( $2 \times 10^{-3}$  and  $5 \times 10^{-3}$  unit/ $\mu$ l), (lanes marked by V<sub>1</sub> and 1 or 2 and by T<sub>1</sub> and 1 or 2, respectively, in the Figures). A unique RNase T<sub>1</sub> concentration was used ( $5 \times 10^{-3}$  unit/ $\mu$ l). RNase T<sub>1</sub> and T<sub>2</sub> digestions were stopped by the addition of 5  $\mu$ l of 100 mM EDTA before phenol/chloroform extractions. RNase T<sub>1</sub> digestions were stopped by the addition of an excess of commercial tRNA mixture (10  $\mu$ g), followed by rapid phenol extraction on ice. Modification and digestion products were ethanol-precipitated, washed with 70% (v/v) ethanol, and dissolved in MilliQ water before primer extension analysis. As described above, chemically modified and enzymatically cleaved U4/U6 snRNA duplex was purified in a non-denaturing polyacrylamide gel prior to primer extension analysis.

### Primer extension analysis

For primer extension analysis, 5'-labeled primers were annealed to chemically modified RNAs or digested RNAs and extended with the avian myeloblastosis virus reverse transcriptase (Life Sciences) and the four deoxynucleotide triphosphates, as described by Mougins *et al.*<sup>81</sup> For a given sample, primer extension analysis was repeated two to three times. Positions of chemical modifications and enzymatic cleavage were identified by reference to the sequencing ladder made with unmodified RNA and the 5' end-labeled primer. The UsnRNA segments complementary to oligonucleotides used for

primer extension were: in U5 snRNA, segment 80-95 (U5-409), in human U4 snRNA segments, 110-126 (U4-537) and 66-82 (U4-337), in human U6 snRNA, segments 62 to 79 (U6-550) and 80 to 103 (U6-411), in yeast U5 snRNA, segments 158-179 (U5-334) and 68 to 82 (U5-335), in yeast U4 snRNA, segment 135-160 (U4-329), in yeast U6 snRNA, segment 95-113 (U6-331).

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