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REPORT

A limited number of pseudouridine residues in the human atac spliceosomal UsnRNAs as compared to human major spliceosomal UsnRNAs

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ABSTRACT

Two forms of spliceosomes were found in higher eukaryotes. The major form contains the U1, U2, U4, U5, and U6 snRNAs; the minor form contains the U11, U12, U4atac, U5, and U6atac snRNAs. Assembly and function of the major form are based on a complex dynamic of UsnRNA–UsnRNA and UsnRNA–pre-mRNA interactions, and the involved UsnRNA segments are highly posttranscriptionally modified in plants and vertebrates. To further characterize the minor form of spliceosomes, we looked for the Ψ residues in HeLa cells' U11, U12, U4atac, and U6atac snRNAs, using chemical approaches. Four Ψ residues were detected in total for these four atac UsnRNAs, compared to 20 in their counterparts of the major spliceosomes. The two Ψ residues detected in U12 are also found in U2 snRNA. One of them belongs to the branch-site-recognition sequence. It forms one of the base pairs that bulge out the A residue, responsible for the nucleophilic attack. Conservation of this strategic Ψ residue probably reflects a functional role. Another Ψ residue was detected in a U4atac snRNA segment involved in formation of helix II with U6atac. The fourth one was detected in the additional stem-loop structure present at the 3′ end of U6atac snRNA. Differences in Ψ content of the atac and major UsnRNAs of human cells may participate in the differentiation of the two splicing systems. Based on secondary structure similarity, U2 and U12 snRNAs on the one hand and U4 and U4atac snRNAs on the other hand may share common Ψ synthases.

Keywords: atac spliceosome; HeLa cells; posttranscriptional modifications; splicing; U12-dependent intron

INTRODUCTION

For a very long time after their discovery, the introns, from the nuclear genes that encode proteins, were considered to be of a unique type, with 5′-GT and AG-3′ dinucleotides at the borders (for review, see Moore et al., 1993). They were thought to be all eliminated by the same processing machinery, the spliceosome, composed of 5 UsnRNAs (U1, U2, U4, U5, and U6) and a huge number of proteins (for review, see Moore et al., 1993). The increasing number of available genomic and cDNA sequences over the last few years has revealed the existence of introns that do not fulfill the GT-AG rule (Jackson, 1991; for review, see Wu & Krainer, 1999), and they were found to be spliced by a minor form of spliceosomes containing four specific UsnRNAs (U11, U12, U4atac, and U6atac) and U5 snRNA (Tarn & Steitz, 1996a, 1996b).

UsnRNAs were found to play a crucial role in the assembly and function of the major form of spliceosomes and, in the present stage of knowledge, UsnRNAs from the minor form of spliceosomes are supposed to play very similar roles (for review, see Wu & Krainer, 1999). Briefly, conventional GT-AG introns show the sequence GTRAGT at their 5′ end, and YNYTRAC at their branch site (BS). These two sequences are essential for the early steps of assembly of the major form of spliceosomes: U1 snRNA interacts with the 5′ sequence and U2 snRNA with the BS sequence (for review, see Nilsen, 1998). Similarly, the AT-AC introns of class I (for review, see Sharp & Burge, 1997) and some very recently discovered GT-AG introns (Dietrich et al., 1997) have the (G/A)TATCCTY

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Abbreviations: Ψ: pseudouridine; BS: branch site; CMCT, *N*-cyclohexyl-*N′*-(2-morpholinoethyl)-carbodiimid metho-*p*-toluolsulfonat; RT: reverse transcription.

sequence at their 5′ end and the TCCTTRAY sequence at their BS. The former sequence is recognized by U11 snRNA (Kolossova & Padgett, 1997; Yu & Steitz, 1997), the later one by U12 snRNA (Hall & Padgett, 1996; Tarn & Steitz, 1996b). After binding of U1 and U2 snRNAs, assembly of the major spliceosomes is completed by the association of the (U4/U6, U5) tri-snRNP particle and protein factors (for review, see Krämer, 1996). Similarly, a (U4atac/U6atac, U5) tri-snRNP is expected to join the minor form of pre-spliceosomes. At this stage of assembly, occurrence of conformational changes was demonstrated for the major spliceosomes (for review, see Nilsen, 1998): the U4/U6 interaction is disrupted and replaced by a U2/U6 interaction, U1 snRNA is discarded, and U6 snRNA interacts with the 5′ extremity of the intron. The resulting structure that is formed by the pre-mRNA, U2, and U6 snRNAs is supposed to play a direct role in catalysis. The 5′-terminal loop of U5 snRNA plays an important role for exon alignment.

Given the recent discovery of the minor form of spliceosomes, only a limited amount of experimental evidence for rearrangements prior to catalysis was obtained (Tarn & Steitz, 1996a; Yu & Steitz, 1997). However, based on potential base-pair interactions between U6atac and U12 snRNAs on the one hand and U6atac snRNA and the pre-mRNA on the other hand, a model of interaction of these three RNAs, mimicking the interactions of U2 and U6 snRNAs with the pre-mRNA, was proposed. This model is in accord with recent cross-linking and genetic data (Tarn & Steitz, 1996a; Yu & Steitz, 1997; Incorvaia & Padgett, 1998; Shukla & Padgett, 1999).

According to the proposed UsnRNA–pre-mRNA interactions, the major form of spliceosomes splices all the major conventional GT-AG introns and also the AT-AC introns of class II that contain sequences recognized by U1, U2, and U6 snRNAs. The minor form of spliceosomes splices the AT-AC introns of class I, and also a few GT-AG introns that contain sequences recognized by U11, U12, and U6atac snRNAs (for review, see Wu & Krainer, 1999).

In plants and vertebrates, the UsnRNAs involved in the formation of the major spliceosomes carry numerous posttranscriptional modifications, primarily pseudouridines (Ψ) and 2′-*O*-methylated residues. These modifications are concentrated in the segments involved in intermolecular interactions (for review, see Massenet et al., 1998). Based on the high phylogenetic conservation of some of these modifications, they are expected to play important roles in spliceosome assembly and/or function (Szkukalek et al., 1995; Massenet et al., 1999), such as stabilization of bimolecular interactions, recognition by protein factors, and stabilization of RNA three-dimensional structure, as already proposed for posttranscriptional modifications of other types of RNA (for review, see Agris, 1996; Auffinger & Westhof, 1998; Davis, 1998). Experimental proofs of the importance of posttranscriptional modifications in the formation of active spliceosomes were obtained for the vertebrate U2 snRNA (Ségault et al., 1995; Yu et al., 1998).

Our recent mapping of Ψ residues in the *Saccharomyces cerevisiae* spliceosomal UsnRNAs revealed a low number of Ψ residues in this organism as compared to vertebrates (Massenet et al., 1999). However, the detected Ψ residues were located in UsnRNA segments of extremely high functional importance. There are two possible explanations for the observed difference: either multicellular organisms produce a larger number of RNA-modification enzymes, leading to a higher level of posttranscriptional modification of the UsnRNAs, or, from a functional point of view, the *S. cerevisiae* splicing machinery is less dependent upon UsnRNA posttranscriptional modifications as compared to the plant and the vertebrate major splicing machineries. We wanted to test whether the vertebrate UsnRNAs from the minor spliceosomes were as highly posttranscriptionally modified as vertebrate UsnRNAs from the major spliceosomes. As a first step, we mapped Ψ residues in the U11, U12, U4atac, and U6atac snRNAs from HeLa cells. The data are presented in this report. They are discussed in terms of the possible role of Ψ residues in splicing and in connection with spliceosome evolution.

RESULTS AND DISCUSSION

Mapping of Ψ residues in the U11 and U12 snRNAs could be achieved on HeLa cell RNA extracts using two complementary approaches based on (1) the alkaline-resistant modification of Ψ residues by CMCT (Bakin & Ofengand, 1993), and (2) the absence of modification of Ψ residues by hydrazine (Peattie, 1979). Due to the low abundance of the U4atac and U6atac snRNAs, only the CMCT analysis could be achieved successfully for these two RNAs, and this with the use of specific conditions for CMCT modification (see Materials and Methods). In this case, control experiments with U4atac and U6atac snRNAs produced by in vitro transcription were performed. In addition, for U6atac snRNA, a preliminary step of affinity selection, with a complementary biotinylated oligonucleotide retained on streptavidin-agarose beads, was required to get a clear CMCT analysis. Presence of alkaline-resistant CMCT modifications and absence of hydrazine modification were detected by primer-extension analysis. Because of the difficulty of detecting CMCT modifications in U4atac snRNA, three different primers had to be used for reverse-transcriptase (RT) analysis. Representative examples of the analysis of atac UsnRNAs by the CMCT method are illustrated in Figures 1 and 2. To save space, only the control experiment with the U4atac in vitro transcript is shown (Fig. 2A). For the same reason,

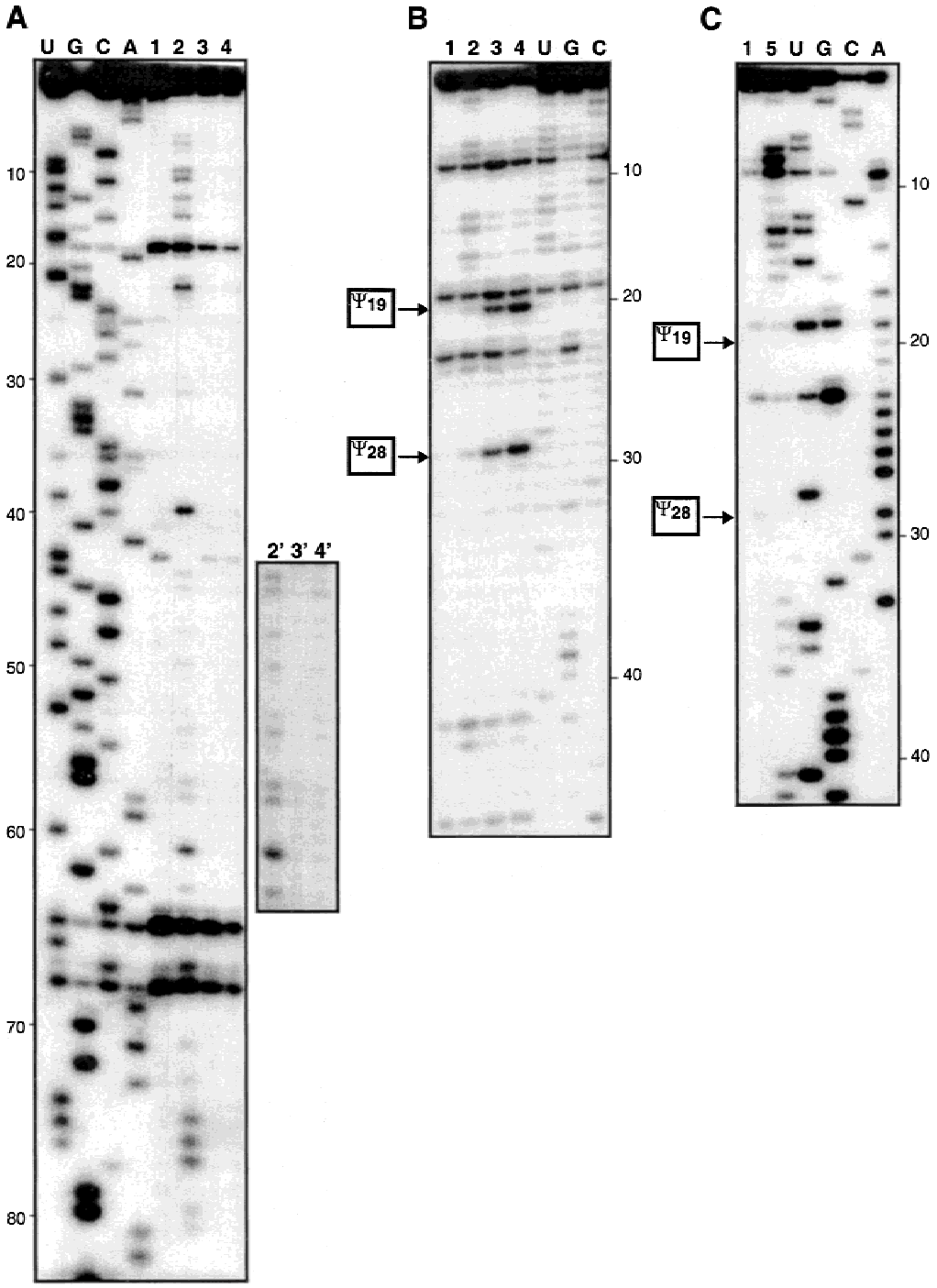

FIGURE 1. **A**: Absence of Ψ residue in the HeLa cell's U11 snRNA. Ten micrograms of total nuclear RNA were modified by CMCT for 1, 10, and 20 min (lanes 2, 3 and 4, respectively), with (lanes 3 and 4) or without (lane 2) alkaline treatment at pH 10.4; conditions are described in Materials and Methods. A control primer-extension analysis was made without CMCT treatment (lane 1). Primer-extension analysis was made with an oligonucleotide complementary to positions 95–110 of U11 snRNA. No RT stop was detected after the alkaline treatment (lanes 3 and 4), even after a longer exposure of the X-ray film (lanes 2′, 3′, and 4′ in the insert on the right of **A**). Lanes U, G, C, A correspond to the RNA sequencing ladder. Nucleotide positions, starting from the 5′-terminal nucleotide bound to the cap structure, are indicated on the left. **B**: Localization of Ψ residues in HeLa cell's U12 snRNA. As in **A**, CMCT modifications were for 1, 10, and 20 min (lanes 2, 3, and 4, respectively), with (lanes 3 and 4) or without (lane 2) alkaline treatment. The oligonucleotide primer used for RT analyses was complementary to U12 snRNA from positions 60–79. The two RT stops, corresponding to residues Ψ_{19} and Ψ_{28}, are indicated. **C**: Verification of the presence of Ψ residues at positions 19 and 28, using hydrazine treatment. Ten micrograms of total RNA extracted from a HeLa cell nuclear extract were treated with hydrazine under the conditions described in Materials and Methods (lane 5). A control-extension experiment was made in the absence of hydrazine (lane 1). The absence of hydrazine reactivity at positions 19 and 28 confirmed the presence of Ψ residues at these two positions.

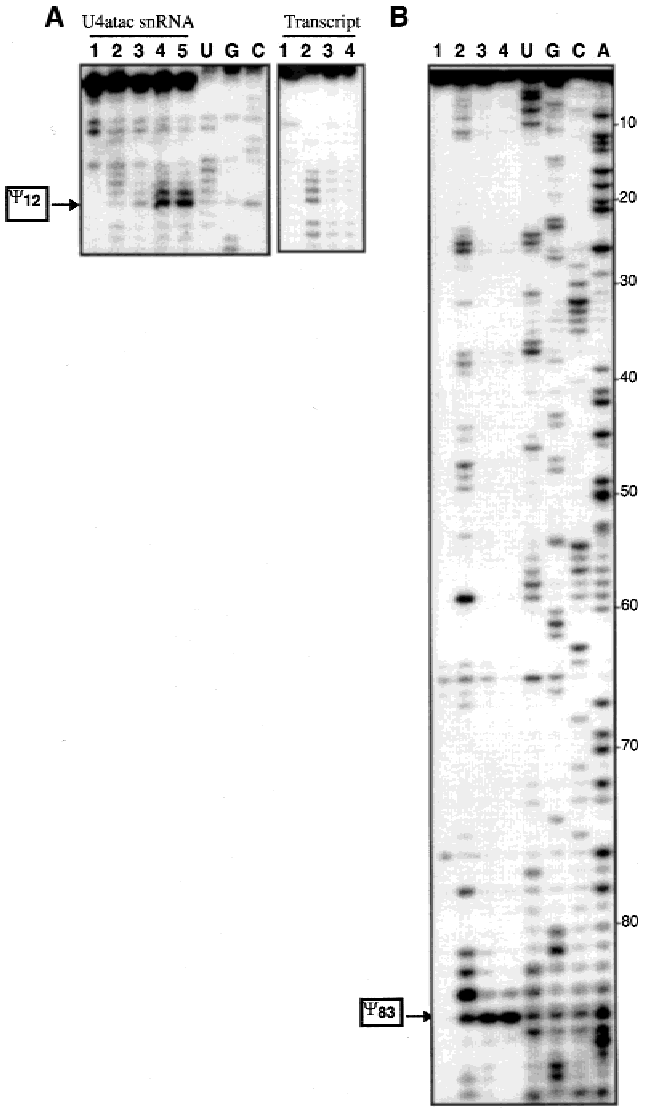

FIGURE 2. **A**: Localization of Ψ residues in the HeLa cell's U4atac snRNA. Fifty micrograms of total nuclear RNA (lanes marked U4atac snRNA) or 2 micrograms of in vitro-produced U4atac snRNA (lanes marked Transcript) were modified by CMCT for 2, 10, 20, and 30 min (lanes 2, 3, 4, and 5, respectively), with (lanes 3, 4, and 5) or without (lane 2) alkaline treatment. Primer-extension analyses were made with an oligonucleotide complementary to positions 70–82 of U4atac snRNA. The identified Ψ_{12} residue is indicated. Note the increased alkaline resistance of the CMCT modification at position 11, which can reflect either a partial conversion of U into Ψ or a stuttering phenomenon (Bakin & Ofengand, 1993). **B**: Localization of Ψ residues in the HeLa cell's U6atac snRNA. One hundred nanograms of purified U6atac UsnRNA (see Materials and Methods) were modified by CMCT for 1, 10, and 20 min (lanes 2, 3, and 4, respectively), with (lanes 3 and 4) or without (lane 2) alkaline treatment. Primer-extension analyses were made with an oligonucleotide complementary to positions 106–125 of the U6atac snRNA. The identified Ψ_{83} residue is indicated.

hydrazine analysis is only illustrated for U12 snRNA (Fig. 1C).

No Ψ residue was detected in U11 snRNA (Fig. 1A). All the RT stops, detected after a 1-min CMCT incubation without further alkaline treatment, disappeared when the alkaline treatment was achieved. Indeed, no band was detected in high yield after a 10- or 20-min CMCT incubation followed by an alkaline treatment, except at natural RT stops (Fig. 1A, lanes 3 and 4), and this even after a long exposure of the X-ray film (Fig. 1A, lanes 2′, 3′, and 4′). Taking into account the RNA sequence, the natural pauses of RT corresponding to positions 65 and 68 in the sequence ladder cannot mask RT stops due to CMCT modification of Ψ residues. Only the natural pause corresponding to position 18 in the sequence ladder took place at a position where an RT stop would be expected if U_{17} was converted into a Ψ residue. However, the intensity of the band observed at this position clearly decreased after the alkaline treatment, instead of being increased as expected for a Ψ residue.

The absence of Ψ residue in U11 snRNA is in contrast to the high phylogenetic conservation of two Ψ residues in the 5′-terminal segment of U1 snRNA that interacts with the intron (Fig. 3) (for review, see Massenet et al., 1998). In connection with this observation, it should be noticed that the analogy between U1 and U11 is not complete. Although the U1 snRNA–intron interaction involves the whole sequence that is conserved at the 5′ extremity of U2-dependent introns (for review, see Nilsen, 1998), the interaction between the 5′ extremity of U12-dependent introns and U11 snRNA only involves residues 4–8 of the intron (Kolossova & Padgett, 1997; Yu & Steitz, 1997). In addition, the U11 segment implicated in the interaction contains no U residue.

Upon inspection of the CMCT and hydrazine analyses of U12 snRNA and based on the same reasoning as for the analysis of U11 snRNA, we concluded that U12 snRNA contains 2 Ψ residues (Fig. 1B,C). Here again, this is in contrast with the 12 and 13 Ψ residues found in the rat hepatoma and the *Vicia faba* plant U2 snRNAs, respectively (Reddy et al., 1981; for review, see Massenet et al., 1998), Interestingly, the Ψ residue found at position 19 is involved in one of the two bp that bulge out the A residue responsible for the nucleophilic attack in the first step of the splicing reaction (Fig. 3C). A Ψ residue was detected at the equivalent position of U2 snRNA seems to be conserved (position 34 in the human and plant U2 snRNAs and position 35 in the *S. cerevisiae* U2 snRNA). The presence of a Ψ residue at equivalent positions in the interactions formed between U12 snRNA and the BS sequence of U12-type introns on the one hand and U2 snRNA and the BS sequence of U2-type introns on the other hand is a strong hint for a functional importance of this posttranscriptional modification. Mutations at position 35 in the *S. cerevisiae* U2 snRNA resulted in a strong decrease in the in vitro splicing efficiency (McPheeters & Abelson, 1992) and is lethal in vivo (Pascolo & Séraphin, 1997). The effect of the generation of some compensatory mutations in the BS sequence and its U2 snRNA-recognition ele-

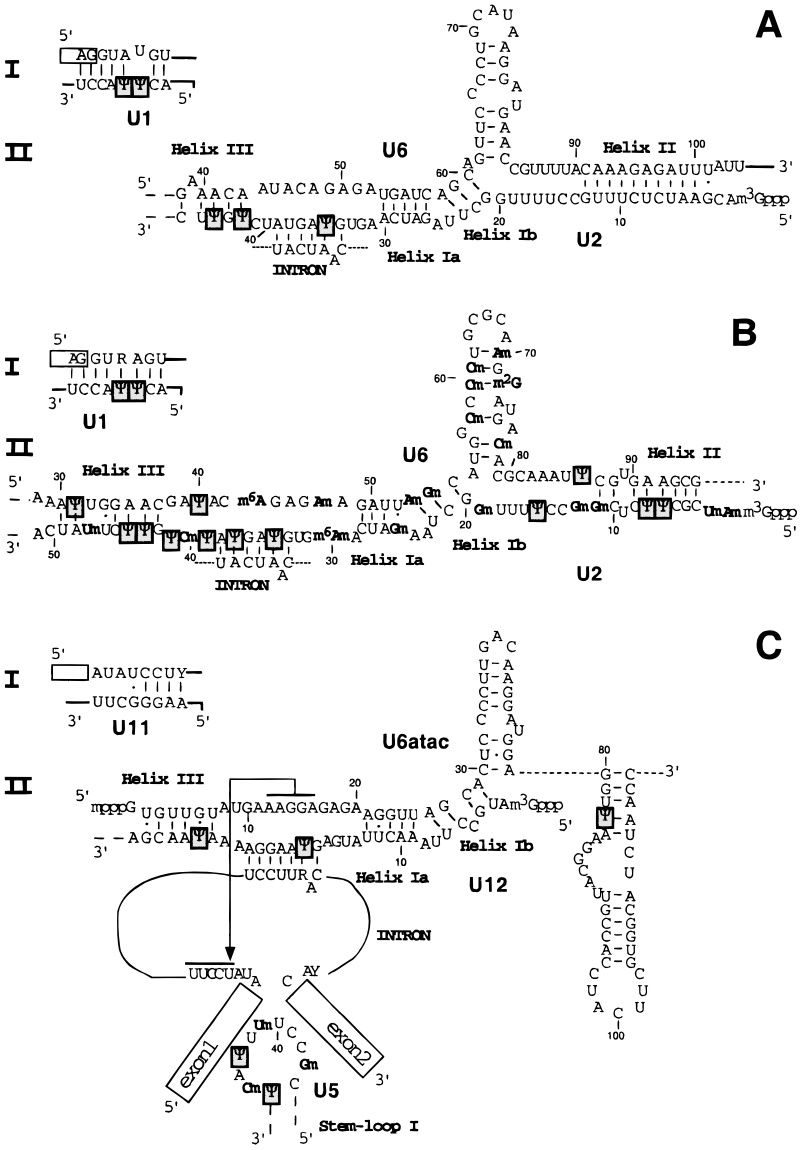

FIGURE 3. Positions of Ψ residues within the network of RNA–RNA interactions of the *S. cerevisiae* spliceosome (**A**), the vertebrate major form of spliceosomes (**B**) and the vertebrate minor form of spliceosomes (**C**). The interaction of the 5′ splice site with U1 (**A** and **B**) or U11 snRNAs (**C**) is shown (Scheme I of each panel). UsnRNA–UsnRNA and UsnRNA–pre-mRNA interactions at the catalytic center of the spliceosomes are shown in schemes II of the three panels. Helices I, II, and III formed between the U2 and U6 snRNAs or between the U12 and U6atac snRNAs are represented, as well as the base-pair interactions between U2 or U12 snRNAs and the BS sequences. The interaction between U6atac snRNA and a sequence close to the 5′ splice site is indicated by overlined residues joined by an arrow. The interaction between the terminal loop I of U5 snRNA and the exon extremities is also shown for the minor form of spliceosomes (**C**) (for a review of these interactions in the major form of spliceosomes, see Nilsen, 1998, and for the minor form of spliceosomes, see references in this article). The Ψ residues are boxed, base and ribose methylations are indicated in bold (this study; Massenet et al., 1999; for review, see Reddy, 1988; Massenet et al., 1998). Nucleotide positions, starting from the 5′ extremity of each snRNA, are indicated.

ment was tested in *S. cerevisiae* (Pascolo & Séraphin, 1997). However further experiments are needed to get a clear answer on the functional importance of a Ψ residue at position 35. Presence of a Ψ residue may stabilize the interaction between U2 or U12 snRNA with the BS sequence or may be involved in recognition of the heterologous helix by protein factors.

The second Ψ residue that we detected in U12 snRNA (Ψ_{28}, Fig. 1) is located 9 nt upstream of the one found in the BS recognition sequence. A Ψ residue is also found at the equivalent position of all the studied U2 snRNAs (Ψ_{43} in the vertebrate RNA and Ψ_{44} in the *S. cerevisiae* RNA; for review, see Massenet et al., 1998). Residue Ψ_{28} in U12 snRNA and the corresponding Ψ residues of the vertebrate and yeast U2 snRNAs belong to segments of the U12 and U2 snRNAs that can form an intermolecular helix III with the U6atac and U6 snRNAs, respectively (Fig. 3). A functional importance of helix III was demonstrated for the U2-type spliceosome of HeLa cells (Sun & Manley, 1995), but not for those of *S. cerevisiae* (Yan & Ares, 1996). Up to now, helix III was not detected by cross-linking experiments in the U12-type spliceosome (Tarn & Steitz, 1996a). In addition, recent data suggest that it cannot be formed in the plant atac UsnRNAs (Shukla & Padgett, 1999). Independent of helix III formation, the segments of the U2 and U12 snRNAs, containing the second highly conserved Ψ residue, have an important function for protein association. Indeed, this U2 snRNA segment binds proteins Prp9, Prp11, Prp21, and Cus1 in yeast and their counterparts, which form the SF3a and SF3b factors, in HeLa cells (for review, see Krämer, 1996). Although the absence of U to Ψ conversion at position 44 of the *S. cerevisiae* U2 snRNA was found to have no effect on growth (Massenet et al., 1999), the presence of a Ψ residue at this position in U2 and U12 snRNAs

may facilitate protein recognition. The nucleotide sequence of the *Arabidopsis thaliana* U12 snRNA has been deduced from a cDNA sequence (Shukla & Padgett, 1999); no identification of posttranscriptional modification was achieved. Interestingly, a U residue is present at position 19, which opens the possibility of its conversion into a Ψ residue. However, a C residue is found at position 28, so that a Ψ residue is not expected at this position of the *A. thaliana* U12 snRNA.

Our Ψ mapping of the U4atac snRNA revealed the presence of a Ψ residue at position 12 (Fig. 2A). The RT stop at this position is followed by a stop of lower amplitude at the following U residue. Either this corresponds to a partial conversion of U into Ψ at position 11, or this is an analysis artefact due to stuttering of RT, as already reported for Ψ residues located within a series of U residues (Bakin & Ofengand, 1993). The two stops were absent when the experiment was performed on an in vitro-produced U4atac snRNA (Fig. 2A, lanes 1–4 marked Transcript). As previously proposed for the U4 and U6 snRNAs, a heteroduplex can be formed between the U4atac and the U6atac snRNAs and this is supported by results of cross-linking and genetic experiments (Tarn & Steitz, 1996a; Shukla & Padgett, 1999). According to the proposed models (Fig. 4), stem II in the U4atac/U6atac duplex is extended compared to the U4/U6 stem II. Based on nucleotide sequence homology, the part of stem II formed by the U4atac sequence from positions 12 to 23 and the U6atac sequence from positions 28 to 39 is the counterpart of the U4/U6 stem II. Hence, residue Ψ_{12},

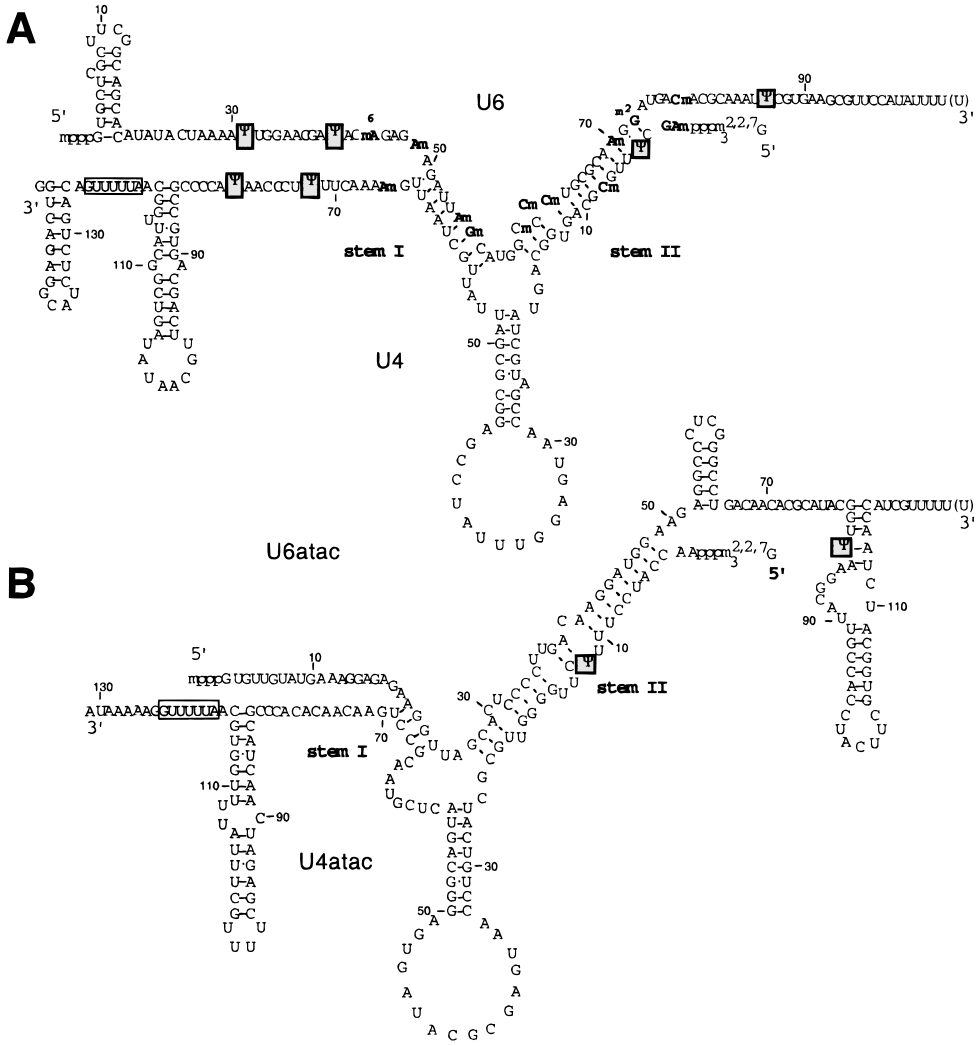

FIGURE 4. Positions of Ψ residues in the U4–U6 (**A**) and U4atac-U6atac (**B**) snRNA duplices. For the U4/U6 RNA duplex, the nucleotide sequences and posttranscriptional modifications shown are from rat (Harada et al., 1980; Krol et al., 1981; for review, see Reddy, 1988), the heterologous stems I and II are as proposed by Brow and Guthrie (1988). Pseudouridine residues are squared in black. For the U4atac/U6atac RNA duplex, nucleotide sequences are from human as determined by Tarn & Steitz (1996a), Ψ residues (this study) are squared in black. The U4atac/U6atac heterologous stems I and II were proposed by Tarn & Steitz (1996a).

which we detected in the U4atac snRNA, is likely to be the counterpart of residue Ψ_4 in the vertebrate U4 snRNAs (Fig. 4). It should be noted that formation of a Ψ residue was not observed at the equivalent position of the *Schizosaccharomyces pombe* and *S. cerevisiae* U4 snRNAs (Gu et al., 1996, Massenet et al., 1999). The two other Ψ residues, present in the vertebrate U4 snRNA segment extending from positions 70 to 80, have no counterpart in the U4atac snRNA.

In a previous review on UsnRNA posttranscriptional modifications that we published before completion of this work, we mentioned that we had detected no Ψ residue in the U6atac snRNA (Massenet et al., 1998). However, by using the partial purification of U6atac snRNA described in this article and an oligonucleotide primer that allowed analysis of the 3′-terminal region, one Ψ residue was detected at position 83 (Fig. 2B). As compared to U6 snRNA, U6atac snRNA contains an additional sequence in its 3′-terminal region. This additional sequence can be folded into a stem-loop structure (Tarn & Steitz, 1996a) and the Ψ residue that we detected is formed in one of the helices (Figs. 2B and 4). The Ψ residue, present in the 3′-terminal region of the vertebrate U6 snRNA, is located in a different context relative to sequence and secondary structure (Fig. 4). Interestingly, the *Influenza* virus NS1 protein was recently shown to inhibit formation of the U12/U6atac complex (Wang & Krug, 1998). Protein NS1 binds to the additional stem-loop structure of the U6atac snRNA and Wang and Krug (1998) proposed that this additional stem-loop structure participates in additional interactions with U12 snRNA. The two other Ψ residues found in vertebrate U6 snRNA, which have no counterpart in yeast U6 snRNA, are not found in the U6atac snRNA either (Fig. 4).

Thus, our study reveals a low level of pseudouridylation of the atac UsnRNAs. Altogether, U11, U12, U4atac, and U6atac snRNAs contain 4 Ψ residues, whereas their counterparts in the major spliceosomes contain 20 Ψ residues altogether. Three of the four detected Ψ residues correspond to posttranscriptional modifications common to the two splicing machineries, this in spite of a high level of sequence divergence of the two UsnRNA series.

Interestingly, the patterns of pseudouridylation of the *S. cerevisiae* UsnRNAs and vertebrate atac UsnRNAs show similarities. Tarn and Steitz (1996a) already pointed out that the *S. cerevisiae* spliceosomes, which are members of the U2-type spliceosome, share several common features with the vertebrate U12-type spliceosome. The list of common properties is increasing with the present data and other recent data:

1. The U4atac snRNA and the *S. cerevisiae* U4 snRNA lack a 3′ terminal stem-loop structure.
2. The secondary structure models, proposed for the isolated *S. cerevisiae* U6 snRNA and for the U6atac snRNA, show similarity, and differ from that proposed for the vertebrate U6 snRNA (Tarn & Steitz, 1996a).
3. Helix II, formed between U2 and U6 snRNAs, is essential for splicing in mammalian cells (Datta & Weiner, 1991; Wu & Manley, 1991), whereas it is only required when helix Ib cannot be formed in *S. cerevisiae* (Field & Friesen, 1996). It cannot be formed in the atac spliceosomes, as the 5′ end of U12 is truncated compared to U2 snRNA (Fig. 3).
4. Helix III, formed between U2 and U6 snRNAs (Fig. 3), is needed for active spliceosome formation in HeLa cells (Sun & Manley, 1995), but not in *S. cerevisiae* (Yan & Ares, 1996). This helix was not detected by cross-linking experiments in the vertebrate atac spliceosome (Tarn & Steitz, 1996a) and it cannot be formed with the plant atac UsnRNAs (Shukla & Padgett, 1999).
5. Both *S. cerevisiae* and atac UsnRNAs contain a limited number of Ψ residues, as compared to vertebrate UsnRNAs from the main form of spliceosome.
6. The 5′ and BS sequences of the *S. cerevisiae* introns and of the atac introns show a very low level of deviation when compared to the consensus sequences, whereas a high degree of divergence is observed for the vertebrate U2-type introns.

Points 5 and 6 may be related one to the other: a high degree of posttranscriptional modification of the UsnRNAs may be required in vertebrate and plant UsnRNAs of the major spliceosomes to stabilize the interactions between the pre-mRNA and the UsnRNAs, which are of low energy in the U2-type spliceosomes. Such common properties may reflect characteristic features of a common ancestor for the U2-dependent and the U12-dependent spliceosomes. According to this hypothesis, the U2-dependent spliceosomes of vertebrates would have diverged more rapidly from the common ancestor than the vertebrate U12-dependent spliceosomes and the *S. cerevisiae* U2-dependent spliceosomes. Based on the observation of U12-dependent introns in the plant *A. thaliana*, it appears that the two spliceosomal machineries have existed side by side for more than a billion years (for review, see Sharp & Burge, 1997). This means that over all this time, the two systems should have preserved enough distinctive features to ensure fidelity of intron excision. Differences in the pattern of UsnRNA posttranscriptional modifications may help ensure the specificity of each splicing machinery.

How can there be numerous conversions of U residues into Ψ residues in the UsnRNAs from the major form of spliceosomes and a low number of such conversions in the atac UsnRNAs, when both RNA series are synthesized in the same cell? One main explanation is the replacement of the U residues, which are converted into Ψ residues in the major UsnRNAs, with

other residues in the atac UsnRNAs. Another important question is whether a given Ψ synthase can act both on one of the main UsnRNAs and on its counterpart of the atac spliceosomes, for instance at positions 19 of U12 snRNA and 34 of U2 snRNA. Until now, little was known about UsnRNA-Ψ synthases. Only one of them was identified in yeast, the Pus1p tRNA-Ψ synthase, which catalyzes Ψ_{44} formation in the *S. cerevisiae* U2 snRNA (Massenet et al., 1999). Based on in vitro assays performed in the presence of 5-fluorouridine with HeLa cell nuclear extract or S100 extract, Patton concluded that distinct Ψ synthases are needed for formation of the various Ψ residues of the main UsnRNAs (for review, see Patton, 1994). Furthermore, formation of Ψ residues in U4 and U6 snRNA transcripts was found to be increased upon formation of the U4/U6 snRNA duplex (Zerby & Patton, 1996, 1997). The involvement of distinct Ψ synthases for Ψ formation in UsnRNAs is also supported by the absence of consensus sequence at the site of pseudouridylation. The UsnRNA–Ψ synthase specificity may depend upon RNA secondary-structure recognition. In spite of nucleotide-sequence dissimilarity, U2 and U12 snRNAs can be folded into very similar structures (Wassarman & Steitz, 1992). Thus, the enzyme that converts U_{34} into Ψ in U2 snRNA may be the same one that converts U_{19} into Ψ in U12 snRNA. The same is true for Ψ_{43} formation in U2 snRNA and Ψ_{28} formation in U12 snRNA. As mentioned above, Ψ_{12} in the U4atac snRNA has a position in stem II of the U4atac/U6atac duplex very similar to that of Ψ_4 of U4 snRNA in the U4/U6 duplex. Here also, a common Ψ synthase recognizing the heterologous stem II, may modify both U4 and U4atac snRNAs. It is difficult to extend this reasoning to the formation of Ψ_{83} in the U6atac snRNA, as the Ψ residue found in the counterpart region of U6 snRNA is located in a completely different context of sequence and secondary structure. Interestingly, we noticed that Ψ_{83} in U6atac snRNA and Ψ_{19} in U12 snRNA are formed in sequences showing a strong degree of similarity (UApyGPuNΨAAGGNA). This opens the question of the existence of a Ψ synthase common to U12 and U6atac snRNAs. Can it be a RNA-guided Ψ synthase? Answers to these various questions on UsnRNA-Ψ synthases require further progress in the identification of these enzymes in vertebrates.

MATERIALS AND METHODS

RNA preparation

HeLa nuclear extracts from the Computer Cell Culture Center S.A. (Belgium) were used as the source of atac UsnRNAs. To this end, the nuclear extract was successively treated by equal volumes of phenol, phenol/chloroform (1:1) and chloroform/isoamyl alcohol (24:1). The extracted total nuclear RNA was ethanol precipitated and used for CMCT or hydrazine modifications or for purification of the U6atac snRNA.

U6atac snRNA purification

U6atac snRNA was purified from a total nuclear RNA extract by affinity selection on streptavidin-agarose beads, using the following oligonucleotide: 5′-$(dU^*)_5$GAAGTAGGTGGCAATG CCTTAACCGT-3′ (* denotes a biotinylated 2′-deoxyuridine), which is complementary to nucleotide positions 78–103 of U6atac snRNA. The affinity selection was performed as described in Ségault et al. (1995), using 50 μg of total nuclear RNA and 1 μg of oligonucleotide. Hybridization of the oligonucleotide was performed at 65 °C for 10 min. After affinity selection, the streptavidin-agarose beads were treated with 160 μg/mL of proteinase K (Boehringer) in 50 μL of buffer containing 5 mM EDTA, 300 mM NaCl, 1.5% SDS, 50 mM Tris-HCl, pH 7.5. Then, RNA was phenol extracted, ethanol precipitated, and quantified with a Gene Quant apparatus.

Identification of Ψ residues in the atac UsnRNAs

The CMCT-modification protocol was adapted from that of Bakin & Ofengand (1993), as previously described (Massenet et al. 1999). CMCT modifications were performed on 10 μg of total nuclear RNA for 1, 10, and 20 min for U11 and U12 snRNA analyses, on 50 μg of total nuclear RNA for 2, 10, 20, and 30 min for U4atac snRNA analysis, on about 100 ng of affinity-purified U6atac snRNA for 1, 10, and 20 min for U6atac snRNA analysis, and on 1 μg of in vitro-produced U4atac and U6atac UsnRNAs for 1 or 2, 10, and 20 min. The alkaline treatment was for 3 h, as described previously (Massenet et al., 1999). Hydrazine modifications were performed as previously described by Massenet et al. (1999), on 10 μg of total nuclear RNA.

Positions of CMCT and hydrazine modifications were identified by primer-extension analyses, using the AMV RT (Life Science, USA), in the conditions described by Mougin et al. (1996). The oligonucleotides, complementary to the following regions of the atac UsnRNAs, were used as primers: U4atac: nt 41–61, nt 70–82, and nt 108–131; U6atac: nt 106–125; U11: nt 95–110; and U12: nt 60–79 and nt 121–146. Primers were 5′-end labeled with [γ-^{32}P] ATP (3,000 Ci/mmol) and T4 polynucleotide kinase. RNA sequencing ladders were done on 40 μg of total nuclear RNA for U11 and U12 snRNAs and on in vitro-produced U4atac and U6atac snRNAs. U4atac and U6atac coding regions, under the control of a T7 promoter, were provided by A. Mougin (unpubl. results).

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