A 3′-Terminal Minihelix in the Precursor of Human Spliceosomal U2 Small Nuclear RNA*

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U2 RNA is one of five small nuclear RNAs that participate in the majority of mRNA splicing. In addition to its role in mRNA splicing, the biosynthesis of U2 RNA and three of the other spliceosomal small RNAs is itself an intriguing process involving nuclear export followed by 5′-cap hypermethylation, assembly with specific proteins, 3′ end processing, and then nuclear import. Previous work has identified sequences near the 3′ end of pre-U2 RNA that are required for accurate and efficient processing. In this study, we have investigated the structural basis of U2 RNA 3′ end processing by chemical and enzymatic probing methods. Our results demonstrate that the 3′ end of pre-U2 RNA is a minihelix with an estimated stabilization free energy of ~6.9 kcal/mol. Parallel RNA structure mapping experiments with mutant pre-U2 RNAs revealed that the presence of this 3′ minihelix is itself not required for in vitro 3′-processing of pre-U2 RNA, in support of earlier studies implicating internal regions of pre-U2 RNA. Other considerations raise the possibility that this distinctive structural motif at the 3′ end of pre-U2 RNA plays a role in the cleavage of the precursor from its longer primary transcript or in its nucleocytoplasmic traffic.

The major spliceosome that operates on most eukaryotic pre-mRNAs contains five small RNAs, U1, U2, U4, U5, and U6. Beyond their role in mRNA splicing, the biosynthesis of these small spliceosomal small RNAs is itself an interesting process. U6 small nuclear is transcribed by RNA polymerase III (1, 2) and associates with spliceosomes without extensive 3′-processing or nucleocytoplasmic transit (3). In contrast, the other four major spliceosomal RNAs, U1, U2, U4, and U5, are transcribed by RNA polymerase II in mammalian cells as precursor molecules extended at their 3′ ends that are then exported to the cytoplasm where they undergo cap hypermethylation and ribonucleoprotein assembly followed by 3′ processing and nuclear import (4–13).

The mammalian precursor molecules of U1, U2, U4, and U5 RNAs have been defined in several studies (4, 6, 8–11, 14), and their 3′-processing has been well characterized particularly for U2 RNA (11, 13, 15–17). A distinct region of pre-U2 RNA lying near the 3′ end of mature RNA has been shown to be critical for accurate and efficient 3′-processing (16). It was also found that sequences in the 5′-half of pre-U2 RNA play no role in the 3′-processing reaction (16), suggesting that in the folded structure of pre-U2 RNA, the 5′-half of the molecule is not interactive with the 3′ end, at least not in a way that influences the 3′-processing reaction. The 3′ end of pre-U2 RNA also has been implicated in the nucleocytoplasmic traffic of U2 RNA. Non-processed 3′ end variants of pre-U2 RNAs display impaired nuclear import in both Xenopus oocytes (18) and human cells (19), indicating that cytoplasmic 3′-processing is a key step in the nuclear import pathway.

The 3′ end of the precursor of human U2 RNA consists of an 11-nucleotide element (11, 14, 20, 21), but the structure of this 3′ tail is not known. The secondary structures of the processed mature forms of U2 RNA from human and other organisms as well as that of U1, U4, and U5 RNAs have been experimentally determined in several previous investigations (22–32). In this study, we have investigated the structure of the 3′ end of human pre-U2 RNA by chemical and enzymatic probing methods and find that it exists as a stably folded minihelix. Additional results suggest that this 3′ end minihelix of pre-U2 RNA may be more relevant to the initial cleavage of pre-U2 RNA from its primary transcript or its intracellular traffic rather than its 3′-processing reaction.

**EXPERIMENTAL PROCEDURES**

Full-length wild-type human pre-U2 RNA was transcribed from plasmid pMRG3U2 (16). Human pre-U2 RNA with a wild-type 3′ tail and lacking nucleotides 1–104 (non-essential for 3′-processing) was transcribed from the plasmid pMRG3U2–54 (16). Pre-U2 RNAs with mutant 3′ tails, U2–75 and U2–80, were transcribed from the plasmids described previously (17). The RNAs were labeled at their 5′ ends with [γ-32P]ATP and polynucleotide kinase or at their 3′ ends with [32P]pCp and RNA ligase. The RNA samples containing 10–100 ng/μl reaction (2 × 104 cpm/μl) were subjected to Pb2+-mediated cleavage in 50 mM potassium acetate, 10 mM magnesium acetate, 50 mM HEPES-KOH, pH 7.5, containing 4 or 10 mM PbCl2. The reactions were carried out at 20 °C for 6 min. Nuclelease VI and RNase T2 and T1 digestions were done in 50 mM KCl, 10 mM MgCl2, 50 mM Tris-HCl, pH 7.5, for 6 min at 20 °C. Nuclease VI was used at 5 or 10 units/ml, RNase T2 was used at 10 or 25 units/ml, and RNase T1 was used at 5 or 25 units/ml. In all of the experiments, the RNA was preincubated for 5 min at 70 °C followed by 15 min at 20 °C in the absence of PbCl2 or enzyme to renature the RNA. Yeast tRNA (Roche Molecular Biochemicals) was present at 200 μg/ml in all enzymatic digestions and chemical probing with the exception of RNase T1 digestions where it was present at 20 mg/ml. After chemical cleavage, the reactions were stopped by the addition of EDTA (20 mM), and the RNAs were recovered by ethanol precipitation. After enzymatic

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cleavages, the RNAs were recovered by phenol/chloroform extraction in the presence of EDTA (25 mM final concentration). In the case of RNase T1, the reactions were also stopped by the addition of an excess of tRNA (2 mg/ml). The RNAs were dissolved in deionized formamide containing 20 mM EDTA, 0.05% (w/v) cyanol blue, and 0.05% (w/v) bromphenol blue and were displayed by electrophoresis on 15% polyacrylamide gels containing 8M urea followed by autoradiography. Sequencing ladders were obtained by alkaline hydrolysis of labeled RNA (90 min at 96 °C in 20 ml of deionized H2O) followed by ethanol precipitation. RNase T1 sequencing was performed by the digestion of heat-denatured labeled RNA with 1000 units/ml RNase T1 in the presence of 2.5 mg/ml tRNA for 30 min at 55 °C in 7 M urea, 1 mM EDTA, 20 mM sodium citrate, pH 5.0. Diethylpyrocarbonate sequencing was performed for 9 min at 96 °C in 500 mM sodium acetate, 1 mM EDTA, pH 4.5, in the presence of tRNA (50 mg/ml) followed by ethanol precipitation and aniline treatment (33).

RESULTS AND DISCUSSION

Fig. 1A shows both the full-length human pre-U2 RNA, and Fig. 1B shows the miniature pre-U2–54 RNA used in this investigation. We have previously shown that the 3'-processing of pre-U2–54 RNA is identical to that of full-length pre-U2 RNA (17). To determine the structure of the 3' end of both pre-U2 RNAs, the pre-U2 RNAs were labeled at their 3' (both RNAs) or 5' (pre-U2–54) ends with 32P and subjected to chemical (Pb2+) or enzymatic digestion methods that produce RNA structure-dependent phosphodiester bond cleavages (34). Fig. 2 shows a representative RNA sequencing gel of the pre-U2–54 RNA cleavage products. Fig. 2, A and R, shows Pb2+ and enzymatic cleavages obtained with 5' end-labeled pre-U2–54 RNA, and Fig. 2C shows the probing of 3' end-labeled pre-U2–54 RNA. Fig. 3 presents the RNA structure deduced from the experimental data. The results indicate that the 3' extension of the pre-U2–54 RNA has a minihelix structure with an estimated stability (change in free energy upon its formation) of approximately −6.9 kcal/mol (35). In keeping with the Roman numeral-based nomenclature used for the previously determined secondary structures of mature spliceosomal small nuclear RNAs, we term the 3' minihelix "stem-loop V." We noted in particular that there was no indication in the structure probing results for the previously entertained pseudoknot interaction between stem-loop V and an internal region at the base of stem-loop IV (see Fig. 10 in Ref. 17). Indeed, our results indicate that the internal sequence previously considered as the partner for the contemplated pseudoknot is actually a part of stem-loop III itself and thus is unavailable for pseudoknot formation.

It is noted that the conditions employed for both Pb2+ and enzymatic cleavage are ones that have been calibrated in numerous previous studies to generate a very low frequency of hits (a maximum one cut per molecule and often less than one). The expected low yield of cleaved molecules in the present experiments is indicated by the large amounts of uncleaved RNA remaining at the origins of the sequencing gels (Fig. 2). The deliberate use of these low frequency cleavage conditions minimizes or eliminates intermolecular interference by fragments or cleavage-induced alterations of the intramolecular folded structure.
To determine whether stem-loop V, as detected in pre-U2–54, is also present in wild-type full-length pre-U2 RNA, similar Pb²⁺ or enzymatic probing experiments were carried out. As shown in Fig. 4, the results indicated a structure virtually identical to that obtained for pre-U2–54. In addition to demonstrating that stem-loop V is a feature of the normal cellular pre-U2 RNA, these results show that the overall secondary structure of the mature region of the molecule is congruent with the previously determined secondary structure of mature U2 small nuclear RNA itself (23). To our knowledge, this is the first experimentally based description of the secondary structure of the precursor form of any of the spliceosomal small RNAs.

We next investigated the 3’ end structure of a previously described mutant of pre-U2 RNA (pre-U2–75) that is deficient in 3’-processing (17). U2–75 has an altered 3’ end in which the previously contemplated pseudoknot would not form. As can be seen in Fig. 5, even though U2–75 is not processed efficiently (17), its 3’ end nevertheless has a minihelical structure. Based on this finding, we went on and determined the 3’ end of another mutant, pre-U2–80, which has a compensatory base-pairing mutation designed to restore the aforementioned hypothetical pseudoknot and which is processed nearly as efficiently as wild-type pre-U2 RNA (17). As can be seen in Fig. 6, the 3’ end of pre-U2–80 has a structure very different from the 3’ end of pre-U2–54. Instead of stem-loop V, the 3’ end forms a base-paired configuration with an internal region near the 5’ base of stem-loop III.

The results of this study provide an important step in defining the molecular structure of the 3’ end of human pre-U2 RNA and also offer new insights into its 3’-processing reaction. The
A major conclusion is that the 3'-terminal nucleotides of human pre-U2 RNA form a stable minihelix. Such a structure had been suggested previously by computational RNA-folding algorithms but could not be singularly considered because of a then-plausible alternative structure in which the pre-U2 RNA 3' tail was potentially engaged in a pseudoknot with a single-stranded region lying just upstream from the 5' edge of stem-loop III (see Fig. 10 in Ref. 17). The second conclusion from this study is that the stem-loop V minihelix is not essential for 3'-processing, because it is present in a processing-defective
mutant, viz. pre-U2–75, and is absent from a mutant that does process well, viz. pre-U2–80. However, in the present study, the structures of the 3' end of pre-U2 RNA were studied in the absence of cellular proteins including the processing activity itself, and it is possible that in vivo the 3' ends of pre-U2–55 and pre-U2–80 would adopt different structures. This notwithstanding, the results of this study are compatible with the idea that the 3' minihelix has some other function in U2 RNA biosynthesis of which the following examples are the two most plausible possibilities. One possibility is that this structure serves as a recognition element for the machinery that cleaves pre-U2 RNA from the initial U2 RNA primary transcript. It is known that the primary transcript of human U2 RNA extends 250 nucleotides or more beyond the 3' end of the penultimate pre-U2 RNA molecule (36). It is not known whether the sequence that forms stem-loop V in the penultimate pre-U2 RNA has the same structure in the primary transcript as opposed to being single-stranded because of other dominant secondary structure or by being itself tied up in a longer range structure. Nonetheless, it is conceivable that the stem-loop V sequence does adopt the same minihelical structure and thus potentially plays a role in the 3' cleavage of the primary transcript to form pre-U2 RNA.

A second and not mutually exclusive possibility is that the 3' minihelix of pre-U2 RNA plays a role in its nuclear export. This idea is based on the indication in an earlier study that a mutant
pre-U2 RNA, which could not have possibly formed the presently defined 3’ minihelix, was exported less efficiently from the nucleus to the cytoplasm (17). Moreover, a recent investigation has revealed that 3’ minihelix structures are essential for the nuclear export of other small RNAs (37), and 3’ minihelix extensions built onto chimeric tRNA-ribozyme RNAs have also been shown to enhance export from the nucleus (38).

The next step in understanding the structure of pre-U2 RNA will probably come from crystallography, because pre-U2 RNA (~200 nucleotides) is considerably beyond the size of RNA that can be studied by NMR at present. Crystallization efforts using mature-length U-small nuclear RNAs have not yielded diffracting crystals so far, but such efforts should certainly be pursued and encouraged. Meanwhile, our present study reinforces the continuing important role of chemical and nuclease cleavage based methods for RNA structure determination (34).

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