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U2 Small Nuclear RNA 3′ End Formation Is Directed by a Critical Internal Structure Distinct from the Processing Site

MARTY R. JACOBSON,* MOLLY RHOADHOUSE, AND THORU PEDERSON

Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

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Mature U2 small nuclear RNA is generated by the removal of 11 to 12 nucleotides from the 3′ end of the primary transcript. This pre-U2 RNA processing reaction takes place in the cytoplasm. In this study, the sequences and/or structures of pre-U2 RNA that are important for 3′ processing have been examined in an in vitro system. The 7-methylguanosine cap, stem-loops I and II, the lariat branch site recognition sequence, the conserved Sm domain, and several other regions throughout the 5′ end of U2 RNA have no apparent role in the 3′ processing reaction. In fact, deletion of the entire first 104 nucleotides resulted in mini-pre-U2 RNAs which were efficiently processed. Similarly, deletion of the top two-thirds of stem-loop III or mutation of nucleotides in the loop of stem-loop IV had little effect on 3′ processing. Most surprisingly, the precursor's 11- to 12-nucleotide 3′ extension itself was of relatively little importance, since this sequence could be replaced with completely different sequences with only a minor effect on the 3′ processing reaction. In contrast, we have defined a critical structure consisting of the bottom of stem III and the stem of stem-loop IV that is essential for 3′ processing of pre-U2 RNA. Compensatory mutations which restore base pairing in this region resulted in normal 3′ processing. Thus, although the U2 RNA processing activity recognizes the bottom of stem III and stem IV, the sequence of this critical region is much less important than its structure. These results, together with the surprising observation that the reaction is relatively indifferent to the sequence of the 11- to 12-nucleotide 3′ extension itself, point to a 3′ processing reaction of pre-U2 RNA that has sequence and structure requirements significantly different from those previously identified for pre-mRNA 3′ processing.

The study of small nuclear ribonucleoproteins (snRNPs) has provided a wealth of information on several aspects of gene expression, including transcription (8, 19, 25–27, 41), nucleocytoplasmic traffic of RNA (10, 11, 24, 32–34, 37, 40, 63), RNA-RNA interactions (6, 7, 9, 17, 29, 36, 61, 62, 66), RNA-protein interactions (3, 4, 16, 30, 42, 53, 59), and RNA processing (23, 31–34, 47, 60).

Except for U6 RNA (25, 46), the abundant small nuclear RNAs (snRNAs) are synthesized by RNA polymerase II, and the primary transcripts carry 7-methylguanosine (^{7}mG) caps (8). Like most eukaryotic RNAs, human snRNAs are transcribed as precursor molecules having extra nucleotides extending beyond their mature 3′ ends. However, unlike eukaryotic pre-mRNAs, which usually have long 3′ extensions, snRNA precursors are only 8 to 20 nucleotides longer than the mature RNA (8). Once transcribed, the precursor snRNAs are transported to the cytoplasm, where they are assembled into snRNP complexes and the ^{7}mG cap becomes hypermethylated to 2,2,7-trimethylguanosine (37). After 3′ processing, the snRNP complexes are transported back into the nucleus, where they function in mRNA or rRNA processing (53).

Precursor U2 RNA molecules 11 to 16 nucleotides longer than mature U2 RNA have been detected both in rat liver (54) and HeLa cells (12, 60). Antibody selection and electrophoresis of U2 RNA isolated from HeLa cells pulse-labeled with [^{3}H]uridine indicate that once in the cytoplasm, pre-U2 RNA first is assembled with the Sm core snRNP proteins and then undergoes 3′ processing to mature size (32, 60). Consistent with this finding, endogenous pre-U2 RNA, in the form of pre-U2 snRNP complexes, undergoes 3′ processing to mature size when incubated in a cytoplasmic extract (47, 60). Similarly, when exogenous pre-U2 RNA is incubated in HeLa cytoplasmic extracts, assembly of U2 snRNP is found to precede pre-U2 RNA 3′ processing (22).

Considerable information is now available concerning the sequences of U2 RNA that are necessary for U2 snRNP formation and nuclear import (13, 15, 24, 37) and for the function of U2 snRNP in pre-mRNA splicing (2, 17, 20, 38, 53, 61, 65, 66). However, little is known about the sequences that influence 3′ processing of the U2 RNA precursor. In a broader context, our strategy has been to study the 3′ maturation of pre-U2 RNA as a case of eukaryotic RNA processing in miniature. We have anticipated that the apparent simplicity of the reaction and the relatively small size of the RNA might afford a clearer picture of a model eukaryotic RNA 3′ processing reaction than is feasible with larger RNAs such as pre-rRNA or pre-mRNA.

In this investigation, we have determined the sequences and structural features of human U2 RNA that are important for 3′ processing by constructing mutants at sites throughout the pre-U2 RNA molecule and examining their 3′ processing in an in vitro system. We have found that extensive portions of the pre-U2 RNA (surprisingly even the 11 nucleotides of the precursor tail itself) can be mutated without significantly affecting 3′ processing. Only mutations that altered base pairing in the bottom of stem III or in the stem of stem-loop IV of U2 RNA substantially affected 3′ processing. Compensatory mutations which restored base pairing at these sites resulted in normal levels of 3′ processing. Thus, the U2 RNA processing activity recognizes a distinct region of secondary structure consisting of the bottom third of stem III and the stem of stem-loop IV. However, the actual sequence of this region is much less important than structure. These results, together with the observation that the processing activity is

* Corresponding author.

surprisingly indifferent to sequence in the 3′ extension itself, point to a U2 RNA 3′ processing reaction whose requisite determinants reside in a compact internal site.

MATERIALS AND METHODS

Mutagenesis. The parent human U2 RNA plasmid used in this study, pMRG3U2 (Fig. 1B), was constructed as follows. Plasmid pG2U2^{pre} (22) was digested with *Hin*dIII and then made blunt by filling in the resulting 5′ overhang with the Klenow fragment of DNA polymerase as described by the suppliers (Promega). An *Eco*RI linker (5′-d[pGGAATTCC]-3′; New England Biolabs) was ligated into this site by using T4 DNA ligase, recircularizing the plasmid. The resulting plasmid (containing two *Eco*RI sites separated by the U2 RNA sequence) was next digested with *Eco*RI and *Bam*HI. The approximately 230-bp U2 DNA-containing fragment was gel purified and ligated into the *Eco*RI-*Bam*HI site of the vector pGEM-3Zf(+) (Promega), generating plasmid pGEM-3Zf(+)U2. The 18 nucleotides located between the U2 RNA sequence and the T7 RNA polymerase transcription start site were subsequently replaced by three nucleotides (GGG), using oligonucleotide-mediated site-directed mutagenesis of a uridine-containing single-stranded pGEM-3Zf(+)U2 DNA template as previously described (28, 52). The oligonucleotide was extended with T4 DNA polymerase (Promega), and the resulting double-stranded DNA was transformed into *Escherichia coli* BMH 71-18 (International Biotechnologies, Inc.). Construction of pMRG3U2 was verified by dideoxy sequencing (50) in both orientations of double-stranded DNA templates, using the Sequenase version 2.0 DNA sequencing kit as described by the supplier (United States Biochemical). Upon confirmation of the authenticity of the parent plasmid pMRG3U2, separate mutant plasmids (except pMRG3U2-50 and pMRG3U2-54), each having a specific mutation within the MRG3U2 RNA coding sequence (Table 1), were constructed by oligonucleotide-mediated site directed mutagenesis of a uridine-containing single-stranded pMRG3U2 DNA template (28, 52). Plasmid pMRG3U2-50 was constructed by deletion of the 104-bp *Sac*I fragment from plasmid pMRG3U2-49. Plasmid pMRG3U2-54 was similarly constructed by deletion of the 104-bp *Sac*I fragment from plasmid pMRG3U2-53.

In vitro transcription of pre-U2 RNAs. Pre-MRG3U2 RNAs were transcribed in vitro from their respective *Bam*HI-digested plasmids (except for MRG3U2-25 and MRG3U2-26, whose corresponding plasmids were digested with restriction enzymes *Nco*I and *Ase*I, respectively) by T7 RNA polymerase (Bethesda Research Laboratories), using a modification of the procedure previously described (39). A typical 50-μl reaction mixture containing approximately 2 to 5 μg of linearized plasmid DNA, 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl, 20 mM dithiothreitol, 40 U of RNasin (Promega), 500 μM each ATP, GTP, and UTP, 50 μM CTP, 20 μCi of [α-^{32}P]CTP (New England Nuclear), 1 mM 7-mGpppG (New England Biolabs), and 50 U of T7 RNA polymerase was incubated at 30°C for 60 min. After 60 min, 1 U of RQ1 DNase (Promega) was added, and the reaction mixture was incubated at 30°C for an additional 10 min. The in vitro-transcribed RNA was subsequently purified by extraction with an equal volume of phenol-chloroform-isoamyl alcohol (24:24:1) followed by ethanol precipitation. When necessary, unlabeled pre-U2 RNAs were synthesized as described above except that each of the four nucleotides was present at a final concentration of 500 μM and [α-^{32}P]CTP and 7-mGpppG were omitted from the transcription reaction.

5′ end labeling of U2 RNA. In vitro-transcribed pre-U2 RNAs were dephosphorylated by using calf intestinal alkaline phosphatase as described by the suppliers (Promega) and subsequently 5′ end labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; New England Nuclear) by T4 polynucleotide kinase (Promega) as described previously (49).

Preparation of a cytoplasmic extract for U2 RNA processing. HeLa cells were grown in suspension culture, harvested, and fractionated into nuclei and cytoplasm as described previously (43, 44). The cytoplasmic U2 RNA processing activity was prepared by centrifugation of a 40% ammonium sulfate-cut, cytoplasmic S100 fraction through a 10 to 30% glycerol gradient, all as previously described (23). Following fractionation, the active fractions (~15S) were pooled, and 200-μl aliquots were rapidly frozen in liquid nitrogen and stored at −80°C. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) as described by the suppliers. Although these preparations were relatively stable for several months when stored in this manner, some loss in 3′ processing activity was observed over time. ^{32}P-labeled pre-U2 RNAs were incubated with the processing extract as described previously (23), with only minor modifications. In a standard processing reaction, approximately 2 ng of U2 RNA was incubated in a 100-μl reaction mix containing 10 μl of extract (~4 to 6 μg of protein), 10 mM Tris-HCl (pH 8.1), 6.5 mM $MgCl_2$, and 62 mM NaCl. Processing of 5′-end-labeled RNA was examined under similar conditions; however, a total reaction mixture volume of 2.0 ml containing 200 μl of extract and 60 ng of 5′-end-labeled U2 RNA was necessary to visualize the reaction products. Unless otherwise indicated, all reaction mixtures were incubated for 30 min at 37°C prior to phenol-chloroform extraction and ethanol precipitation as previously described (23). RNA was analyzed by electrophoresis through 6% polyacrylamide gels (19:1 ratio of acrylamide to bisacrylamide) containing 8.3 M urea followed by autoradiography. Band intensities of autoradiographs were determined by using a flat-bed scanner for densitometry controlled by PDI Quantity One Gelscan software (PDI, Huntington Station, N.Y.), run on a Sun SPARCstation work station (Sun Microsystems, Inc., Mountain View, Calif.). The relative efficiency of 3′ processing of each mutant RNA, calculated as the percentage of the wild-type value observed on the same day with the same extract, was determined as follows: [(band intensity of mature mutant product/total intensity of mutant RNA)/(band intensity of mature wild-type product/total intensity of wild-type RNA)] × 100 = processing index.

RESULTS

We previously demonstrated that human pre-U2 RNA is accurately and efficiently processed at its 3′ end by a fractionated HeLa cytoplasmic extract in the absence of snRNP assembly (23). Hence, this 3′ processing reaction is dependent on the sequence and/or secondary structure of the pre-U2 RNA itself. To further investigate the sequence and structure requirements for pre-U2 RNA 3′ processing, we constructed a new T7 promoter-human U2 gene, pMRG3U2 (see Fig. 1B and Materials and Methods). This U2 RNA gene produces a transcript that lacks the 18-nucleotide 5′ leader that was present in our initial studies (23). MRG3U2 RNA transcripts contain three extra G's at their 5′ end, necessary for efficient transcription by T7 RNA polymerase, and carry

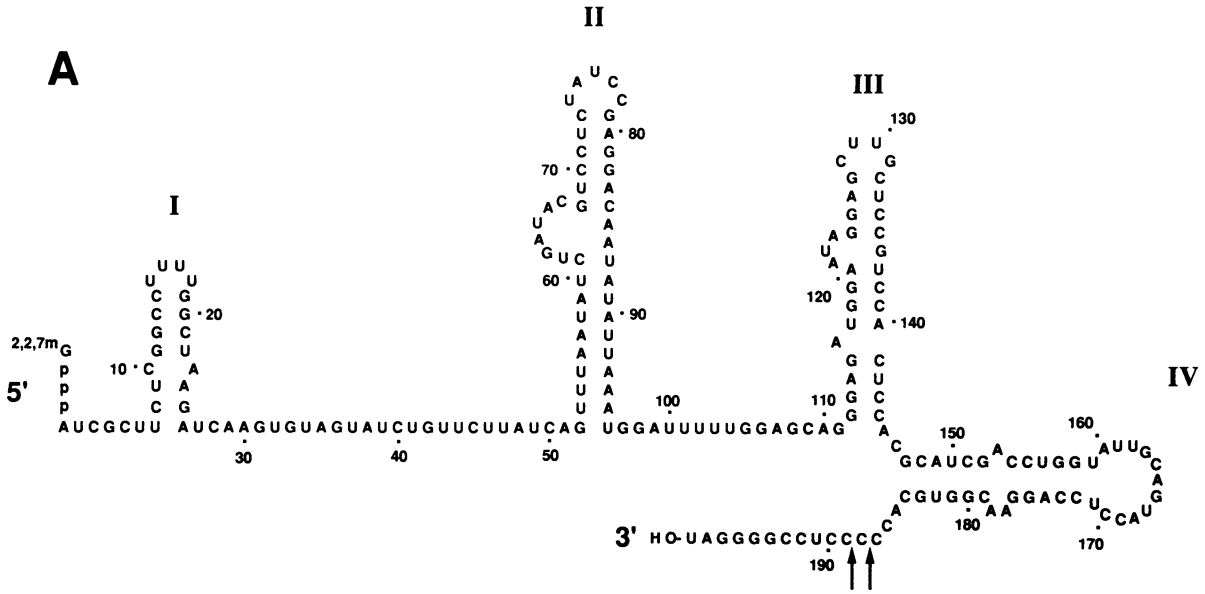

FIG. 1. (A) Sequence and structure of human pre-U2 RNA. The proposed secondary structure is that of Keller and Noon (21). Arrows indicate the 3′ ends of mature U2 RNA. The 3′ extension of the pre-U2 RNA is drawn in single-stranded form although it can be folded into a small stem-loop structure. (B) Sequence of the human U2 RNA gene insert in plasmid pMRG3U2. The sequence encoding mature human U2 RNA is underlined. The T7 and SP6 RNA polymerase promoter sequences (boxed nucleotides) and transcription start sites (arrows) are indicated, as are restriction sites of the remaining polylinker region.

the U2 RNA precursor's 11- to 12-nucleotide 3′ extension (Fig. 1). Incubation of MRG3U2 RNA in the in vitro processing system (see Materials and Methods) resulted in the accumulation of an RNA species 11 to 12 nucleotides shorter than the precursor molecule (Fig. 2, lanes 3 and 4). 5′-end-labeled MRG3U2 RNA was similarly reduced in length by 11 to 12 nucleotides upon incubation (Fig. 2, lanes 1 and 2), showing that processing occurs at the 3′ end.

We observed that 3′ end processing of MRG3U2 RNA began without a lag and that approximately 70% of the RNA

TABLE 1. Human U2 RNA plasmids used

Plasmid	U2 RNA gene mutation[a]
pMRG3U2	Wild type
pMRG3U2-1	A_{29}→T
pMRG3U2-2	ATATTAAAT$_{88-96}$ →TATAATTTG
pMRG3U2-3	A_{90} →T
pMRG3U2-4	TTTTT$_{100-104}$ →AAAAA
pMRG3U2-5	A_{149} →G
pMRG3U2-6	TATCA$_{47-51}$ →ATAGT
pMRG3U2-7	dl 112–144
pMRG3U2-8	AG$_{110-111}$ →TC
pMRG3U2-9	TATTGCAGTACCT$_{159-171}$ →ATTGCACTCC
pMRG3U2-10	dl 15–18
pMRG3U2-11	T_{58} →A
pMRG3U2-12	T_{58} →A + A_{90} →T
pMRG3U2-13	GGCT$_{19-22}$ →ACAC
pMRG3U2-14	dl 46–49
pMRG3U2-15	CCCCC$_{186-190}$ →GGGGG
pMRG3U2-16	C_{155} →G
pMRG3U2-17	G_{175} →C
pMRG3U2-18	T_{182} →C
pMRG3U2-19	dl 34–37
pMRG3U2-20	CCAGG$_{172-176}$ →GGTCC
pMRG3U2-21	G_{147} →C
pMRG3U2-22	CCTGG$_{154-158}$ →GGACC
pMRG3U2-23	C_{184} →G
pMRG3U2-24	GAGGAC$_{79-84}$ →CTCCTG
pMRG3U2-25	CCCCCTCCGGGGA TCC$_{186-201}$ →GCGGGAGGCCCCATGG
pMRG3U2-26	GGGGATCC$_{194-201}$ →AAAATTAA
pMRG3U2-27	CCT$_{189-191}$ →GGG
pMRG3U2-28	CCTGG$_{154-158}$ →GGACC + CCAGG$_{172-176}$ →GGTCC
pMRG3U2-29	GTCCTC$_{68-73}$ →CAGGAG
pMRG3U2-30	dl 53–95
pMRG3U2-31	GTCCTC$_{68-73}$ →CAGGAG + GAGGAC$_{79-84}$ →CTCCTG
pMRG3U2-32	CCTCCGG$_{189-195}$ →AAAAAAA
pMRG3U2-33	dl 63–66
pMRG3U2-34	CCTCCG$_{189-194}$ →GCGCGC
pMRG3U2-35	CCTCCGG$_{189-195}$ →TTTTTTT
pMRG3U2-36	dl 123–136 + insertion of TC at deletion junction
pMRG3U2-37	dl 116–140 + insertion of CATG at deletion junction
pMRG3U2-38	C_{151} →G
pMRG3U2-39	G_{180} →C
pMRG3U2-40	C_{151} →G + G_{180} →C
pMRG3U2-41	A_{149} →G + T_{182} →C
pMRG3U2-42	dl 153 + dl 177–178
pMRG3U2-43	dl 153
pMRG3U2-44	dl 177–178
pMRG3U2-45	GGAG$_{112-115}$ →CCTC
pMRG3U2-46	GGAG$_{112-115}$ →CCTC + CTCC$_{141-144}$ →GAGG
pMRG3U2-47	CTCC$_{141-144}$ →GAGG
pMRG3U2-48	dl 1–26
pMRG3U2-49	TCG$_{2-4}$→GCT + AG$_{110-111}$ →TC
pMRG3U2-50	dl 1–104 + AG$_{110-111}$→TC
pMRG3U2-51	TCTGTTCT$_{39-46}$→AAAAAAAA
pMRG3U2-52	GGAG$_{112-115}$→CTC
pMRG3U2-53	TCG$_{2-4}$ →GCT + AG$_{110-111}$ →TC + dl 116–140 + insertion of CATG at dl 116–140 junction
pMRG3U2-54	dl 1–104 + AG$_{110-111}$ →TC + dl 116–140 + insertion of CATG at dl 116–140 junction

[a] Nucleotide numbering corresponds to the previously published human U2 RNA sequence (5, 55, 57, 58). dl, deletion.

was converted to the mature U2 RNA size after 25 min (Fig. 3). We noted that the pre-U2 RNA appeared to first be shortened by two to three nucleotides (Fig. 3; compare lanes 2 and 3 with lane 1). The progressive increase in the amount of mature product with time appears to correlate with the disappearance of the +11 to +9 bands, suggesting that the latter are precursors of the final mature RNA product. Although in some instances faint intermediate bands (+1 to +8) were observed approximately 10 min after the initiation of processing, no change in the intensity of these bands was observed during subsequent incubation, suggesting that they are not true intermediates in the processing reaction pathway.

To examine the importance of sequence, secondary structure, or both in the 3′ end maturation of U2 RNA, we constructed mutants throughout the molecule (Table 1) and tested their 3′ processing (Table 2). We found that deletion or mutation of stem-loop I or II, or of several other regions throughout the 5′ end of U2 RNA, and mutation of the Sm domain had little or no effect on 3′ processing of the

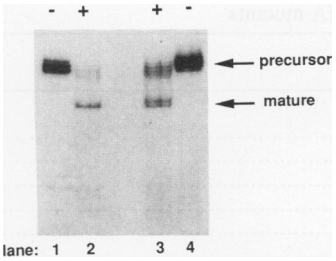

FIG. 2. In vitro processing of pre-U2 RNA. Precursor U2 RNAs were transcribed from linearized pMRG3U2 DNA and incubated in the in vitro processing system as described in Materials and Methods. Lanes: 1 and 2, pre-U2 RNA 5′ end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (Materials and Methods); 3 and 4, pre-U2 RNA uniformly labeled with [α-^{32}P]CTP. Precursor (+12 nucleotides) and mature (+0) U2 RNA molecules are indicated. The presence (+) or absence (−) of fractionated cytoplasmic HeLa cell extract in the reaction is indicated above the gel lanes, buffer being substituted for extract in lanes 1 and 4.

respective RNAs (i.e., 3′ processing was 80 to 110% of the wild-type value; Table 2, sections A to C). Typical data for some of these mutants are shown in Fig. 4. Similarly, deletion of the upper two-thirds of stem-loop III (Table 2, section D, dl 123–136 and dl 116–140; Fig. 4, lanes 11 and 12) or mutation of the loop of stem-loop IV (Table 2, section E, line 14; Fig. 4, lanes 13 and 14) also had no significant effect on 3′ end processing. Most surprisingly, even the nucleotides in the 3′ extension itself could be replaced by completely different sequences with little effect on 3′ processing (Table 2, section F; Fig. 5).

In contrast to these various regions, the bottom third of stem-loop III and the stem of stem-loop IV were of critical importance to the 3′ processing reaction. Mutations that reduced base pairing in the stem of the stem-loop IV resulted in either a complete inhibition of 3′ processing or a significant reduction (boxed values in section E of Table 2; Fig. 6A, lanes 3 to 6; Fig. 6B, lanes 3 to 6). Compensatory mutations that restore base pairing in stem IV resulted in the restoration of 3′ processing (circled values in section E of Table 2; Fig. 6A, lanes 7 and 8; Fig. 6B, lanes 7 and 8). Deletion of the entire stem and loop III reduced 3′ processing to 66% of the wild-type value (section D of Table 2, dl 112–144). Since deletion of the top two-thirds of stem-loop III did not lead to reduced 3′ processing (dl 123–136 and dl 116–140 in section D of Table 2; Fig. 4, lanes 11 and 12), the bottom of stem III must be the feature that is important.

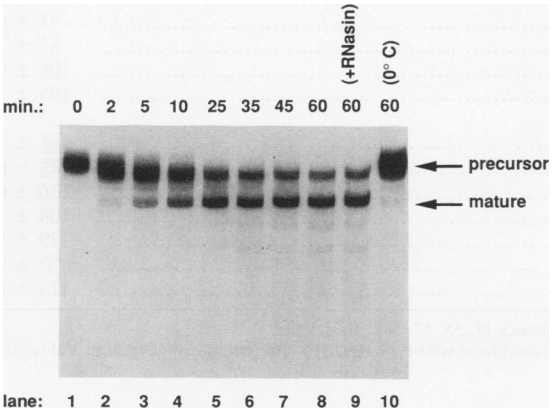

FIG. 3. Time course of in vitro pre-U2 RNA processing. In vitro-transcribed pre-U2 RNA, uniformly labeled with [α-^{32}P]CTP, was incubated in the presence of fractionated cytoplasmic HeLa cell extract at 37°C for various lengths of time as indicated above the gel lanes. The RNase inhibitor RNasin (Promega) was present in the reaction in lane 9. The reaction in lane 10 was incubated 60 min at 0°C.

To further define the role of sequence and/or secondary structure in this critical region, mutants MRG3U2-45 and MRG3U2-47 were constructed (Table 1). Whereas mutation of nucleotides 112 to 115 had only a moderate effect (reducing processing to 72% of the wild-type value; $GGAG_{112–115}$ →CCTC in section D of Table 2), mutation of nucleotides 141 to 144 (base paired to nucleotides 112 to 115 in the bottom of stem III) reduced processing to 38% of the wild-type value (boxed value in section D of Table 2). Typical results are shown in Fig. 6C. Since both of these mutations disrupt the secondary structure of the bottom third of stem III, this result would at first glance suggest that the sequence of nucleotides 141 to 144 and not the structure at the bottom of stem III is important for 3′ processing. However, near-normal 3′ processing was observed for a double mutant (MRG3U2-46) in which the sequences of both regions 112 to 115 and 141 to 144 are mutated in a compensatory fashion, restoring base pairing (circled value in section D of Table 2; Fig. 6C, lanes 7 and 8). Thus, although the sequence of nucleotides 141 to 144 is important, the observed reduction in 3′ processing resulting from the mutation of these four nucleotides can be overcome by a compensatory mutation which restores base pairing.

The foregoing data suggest that the pre-U2 RNA 3′ processing activity recognizes a distinct region of secondary structure embracing the bottom third of stem III and the stem of stem-loop IV. As mentioned earlier, the results for other mutants establish that several regions in the 5′ end of the U2 RNA molecule have little or no apparent role in the 3′ processing reaction. However, we have not mutated every nucleotide in the 5′ half of U2 RNA, and thus it is not possible to know whether some region of importance for 3′ processing might lie therein. To examine this possibility, mutants MRG3U2-50 and MRG3U2-54 were constructed (Table 1). The first 104 nucleotides of U2 RNA are deleted in each of these mutant U2 RNA genes. In addition, the top two-thirds of stem-loop III is also deleted in mutant 54 (Table 1). As shown in Fig. 7, these mini-pre-U2 RNAs were both accurately and efficiently processed. Hence, the 5′ end of U2 RNA (nucleotides, 1 to 104) has no apparent role in the 3′ processing of pre-U2 RNA, suggesting that all of the sequences or structure important for accurate and efficient 3′ processing reside within the 3′ half of the U2 RNA molecule.

DISCUSSION

Previous work has characterized DNA sequences, called 3′ end signals, located approximately 15 bp downstream from the presumed transcription termination site that are important in specifying the 3′ ends of pre-U1 RNA (18, 19) and pre-U2 RNA (64). Here we are concerned instead with the reaction that converts the pre-snRNA into the mature molecule. This is an extremely transcription-distal event, since it takes place in the cytoplasm (32–34, 40, 60). This intriguing feature of snRNA biosynthesis led us to suspect that the 3′ processing reaction would have unusual features as well, as has proven to be the case.

In vivo, pre-U2 RNA is assembled into U2 snRNP in the cytoplasm prior to 3′ processing (32, 60). Moreover, in our in

TABLE 2. 3′ end processing of human U2 RNA mutants

Region	Processing Index (% wt ± σn)[b]
A. Stem-loop I and single-stranded region (nucleotides 1–46[a])	
(1)[c] A_{29}→U	94 ± 7
(10) dl 15–18	89 ± 13
(13) $GGCU_{19-22}$→ACAC	97 ± 8
(19) dl 34–37	107 ± 12
(48) dl 1–26	88 ± 2
(51) $UCUGUUCU_{39-46}$→AAAAAAAA	107 (n = 1)
B. Stem-loop II (nucleotides 46–96)	
(2) $AUAUUAAAU_{88-96}$→UAUAAUUUG	102 ± 7
(3) A_{90}→U	90 ± 6
(6) $UAUCA_{47-51}$→AUAGU	100 ± 13
(11) U_{58}→A	99 ± 10
(12) U_{58}→A + A_{90}→U	89 ± 10
(14) dl 46–49	109 ± 13
(24) $GAGGAC_{79-84}$→CUCCUG	97 ± 7
(29) $GUCCUC_{68-73}$→CAGGAG	96 ± 6
(30) dl 53–95	88 ± 2
(31) $GUCCUC_{68-73}$→CAGGAG + $GAGGAC_{79-84}$→CUCCUG	79 ± 9
(33) dl 63–66	87 ± 5
C. Sm domain (nucleotides 97–111)	
(4) $UUUUU_{100-104}$→AAAAA	94 ± 4
(8) $AG_{110-111}$→UC	96 ± 14
D. Stem-loop III (nucleotides 112–144)	
(7) dl 112–144	66 ± 7
(36) dl 123–136 + insertion of UC at deletion junction	120 ± 14
(37) dl 116–140 + insertion of CAUG at deletion junction	130 ± 12
(45) $GGAG_{112-115}$→CCUC	72 ± 9
(46) $GGAG_{112-115}$→CCUC + $CUCC_{141-144}$→GAGG	(88) ± 6
(47) $CUCC_{141-144}$→GAGG	[38] ± 7
(52) $GAGG_{141-144}$→CUC	91 ± 0
E. Stem-loop IV (nucleotides 145–184)	
(21) G_{147}→C	[64] ± 5
(23) C_{184}→G	[58] ± 17
(5) A_{149}→G	[25] ± 4
(18) U_{182}→C	[27] ± 7
(41) A_{149}→G + U_{182}→C	(97) ± 9
(38) C_{151}→G	[25] ± 7
(39) G_{180}→C	[30] ± 13
(40) C_{151}→G + G_{180}→C	(98) ± 8
(22) $CCUGG_{154-158}$→GGACC	[31] ± 7
(20) $CCAGG_{172-176}$→GGUCC	[65] ± 9
(28) $CCUGG_{154-158}$→GGACC + $CCAGG_{172-176}$→GGUCC	(82) ± 8
(16) C_{155}→G	78 ± 2
(17) G_{175}→C	59 ± 13
(9) $UAUUGCAGUACCU_{159-171}$→AUUGCACUCC	93 ± 6
(43) dl 153	85 ± 3
(44) dl 177–178	106 ± 9
(42) dl 153 + dl 177–178	105 ± 11
F. Mature 3′ end and 3′ extension (nucleotides 185–200)	
(15) $CCCCC_{186-190}$→GGGGG	[60] ± 4
(25) $CCCCCUCCGGGGAUCC_{186-201}$→GCGGGAGGCCCCAUGG	[85] ± 6
(26) $GGGGAUCC_{194-201}$→AAAAUUAA	136 ± 6
(27) $CCU_{189-191}$→GGG	108 ± 16
(32) $CCUCCGG_{189-195}$→AAAAAAA	129 ± 5
(34) $CCUCCG_{189-194}$→GCGCGC	(77) ± 2
(35) $CCUCCGG_{189-195}$→UUUUUUU	113 ± 2

[a] Nucleotide numbering corresponds to the previously published human U2 RNA sequence (5, 55, 57, 58). dl, deletion.

[b] Processing index = [(mature mutant product/total mutant RNA)/(mature wild-type product/total wild-type RNA)] × 100. σn, mean deviation. Values that are circled or boxed are discussed in the text.

[c] Numbers in parentheses correspond to the mutant plasmid numbers in Table 1.

vitro system (23), the assembly of exogenous pre-U2 RNA into an snRNP kinetically precedes 3′ processing when ATP is present (22). However, pre-U2 RNA 3′ processing clearly does not require prior in vitro snRNP assembly because it occurs efficiently in the absence of ATP, even though the substrate RNA is not assembled into Sm antibody-reactive snRNPs under these conditions (22). Clearly then, the U2 RNA 3′ processing machinery does not depend on binding to assembled snRNP proteins for its action.

Defining the mechanism of the pre-U2 RNA 3′ processing reaction as exonucleolytic, endonucleolytic, or both will require further purification and characterization of the activ-

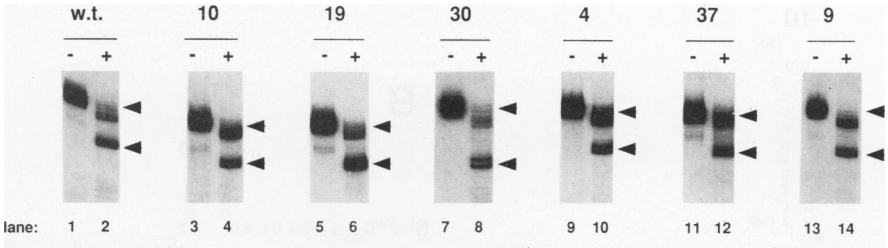

FIG. 4. 3′ end processing of the wild-type (w.t.; lanes 1 and 2) and pre-U2 RNAs containing mutations in stem-loop I (lanes 3 and 4), the lariat branch point recognition sequence (lanes 5 and 6), stem-loop II (lanes 7 and 8), the Sm domain (lanes 9 and 10), stem loop III (lanes 11 and 12), and the loop of stem-loop IV (lanes 13 and 14). Numbers above each pair of lanes correspond to the mutant plasmid numbers in Table 1. The presence (+) or absence (−) of fractionated cytoplasmic HeLa cell extract in the in vitro processing reaction is indicated above the gel lanes.

ity itself. Initial studies reveal that this activity behaves in gel filtration as a large complex (native molecular weight of ≥700,000 [20a]). Our results (Fig. 3) suggest that the 3′ processing reaction begins with the removal of the last 2 or 3 nucleotides of the 11- to 12-nucleotide precursor tail, followed promptly by the appearance of the final reaction product bearing the mature U2 RNA 3′ end(s). Although in some instances faint intermediate bands were observed representing the +8 through +1 molecules, no change in their intensity was observed over the course of a processing reaction even when the reaction was deliberately slowed down by incubation at a reduced temperature (25°C; data not shown). While it is possible that a strictly exonucleolytic reaction occurs so rapidly even under the deliberately slowed conditions that we cannot detect the ladder of expected intermediates, we consider it more likely that following the initial removal of the last two to three nucleotides of pre-U2 RNA, there is an endonucleolytic cleavage at the mature 3′ end. We will revisit the important issue of reaction mechanism when a more purified 3′ processing activity is at hand.

Although our collection of pre-U2 RNAs obviously does not include mutations at every nucleotide in the molecule, on the basis of our results, several regions can be eliminated as being critical for 3′ processing. These dispensable or relatively unimportant regions include ones important for the binding of the Sm core snRNP proteins (nucleotides 100 to 104), sequences near the 5′ end that are involved in pre-mRNA recognition and splicing (nucleotides 6 to 26 and 34 to 37), and a region of U2 RNA (nucleotides 47 to 96) that is theoretically capable of adopting multiple stem-loop structures, including a pseudoknot (1, 14). Similarly, the accurate and efficient 3′ processing of mini-pre-U2 RNAs which lack nucleotides 1 to 104 rules out important contacts by the 3′ processing machinery in the 5′ end of the RNA. This result also rules out a requirement for any structural interactions between the 5′ and 3′ ends of pre-U2 RNA for 3′ processing. This contrasts with a recently described case, the 3′ processing of *Drosophila* 5S rRNA, in which a base-pairing interaction between the 5′ end and the region adjacent to the 3′ processing site is important for processing (45). We also found that uncapped pre-U2 RNA that had been 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase was accurately and efficiently processed, demonstrating that neither a ^{7}mG cap nor its hypermethylation is important for 3′ processing. In addition, since we know that pre-U2 RNA does not undergo pseudouridine formation in our in vitro system in the absence of exogenous ATP (22) and since, as mentioned above, all of our assays were carried out in the

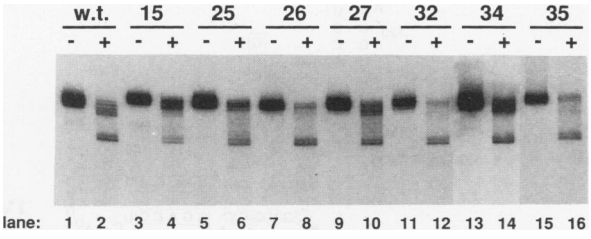

FIG. 5. 3′ processing of the wild-type (w.t.) and 3′ extension pre-U2 RNA mutants. Numbers above each pair of lanes correspond to the mutant plasmids in Table 1. The presence (+) or absence (−) of fractionated cytoplasmic HeLa cell extract is indicated above the gel lanes. All samples were run on the same gel; however, lanes 13 and 14 required a lighter exposure.

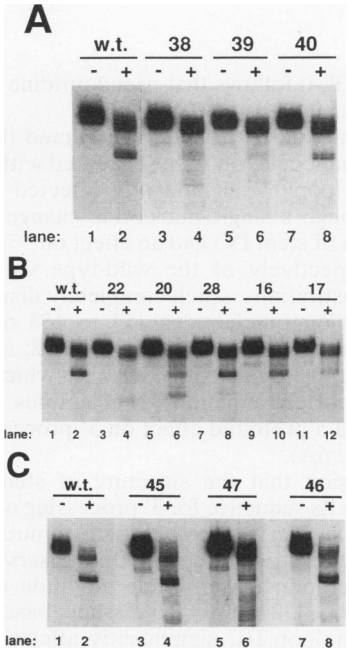

FIG. 6. 3′ processing of the wild-type (w.t.) and pre-U2 RNAs mutated in the bottom half of stem IV (A), the top half of stem IV (B), and the bottom third of stem III (C). Numbers above each pair of lanes correspond to the mutant plasmids in Table 1. The presence (+) or absence (−) of fractionated cytoplasmic HeLa cell extract is indicated above the gel lanes.

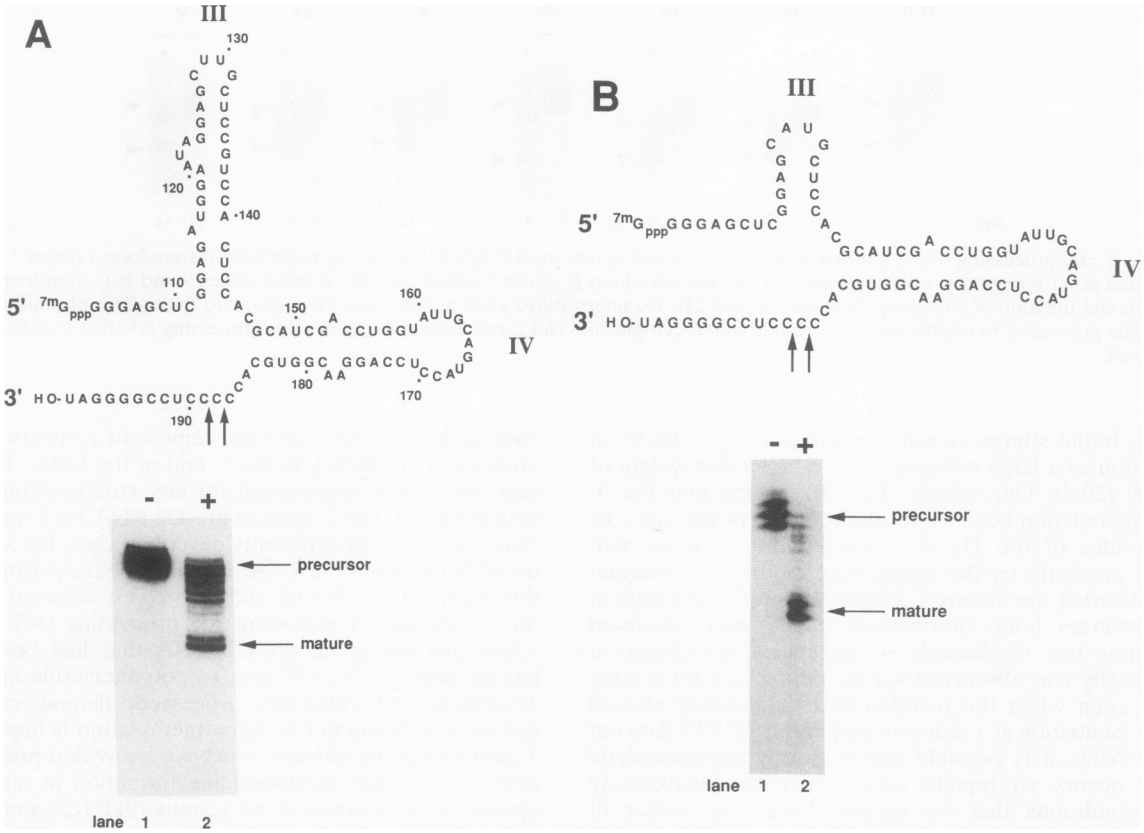

FIG. 7. 3′ processing of mini-pre-U2 RNAs MRG3U2-50 (A) and MRG3U2-54 (B). Numbers in panel A correspond to the locations of nucleotides in the full-length U2 RNA molecule (see Fig. 1). Arrows in the upper portions of panels A and B correspond to the locations of the 3′ ends of mature U2 RNA. In vitro 3′ processing of the two mini-pre-U2 RNAs is shown in the lower portion of each panel. The presence (+) or absence (−) of fractionated cytoplasmic HeLa cell extract is indicated above the gel lanes.

absence of ATP, it follows that pseudouridine is not a factor in 3′ processing.

Of all the mutations that we constructed throughout the pre-U2 RNA molecule, only those located within or near the stem of stem-loop IV significantly affected 3′ processing (Fig. 8). Although a single-nucleotide change of nucleotide 155 or 175 (top of stem IV) had an effect on 3′ processing (78 and 59%, respectively of the wild-type value), multiple-nucleotide substitutions which completely disrupt base pairing in this region (nucleotides 154 to 158 or 172 to 176) significantly affected 3′ processing (Table 2, section E; Fig. 6B). Similarly, single-nucleotide changes which disrupt base pairing in the bottom of stem IV (nucleotides 149 to 151 and 180 to 184) had a profound effect on 3′ processing (Table 2, section E; Fig. 6A).

The conclusion that the structure of stem IV is more important than its sequence for 3′ processing of pre-U2 RNA derives from the fact that compensatory mutations of stem IV mutants restored processing. One observation that deserves consideration is that while mutation of nucleotides 141 to 144 (MRG3U2-47), which disrupts base pairing in the bottom of stem-loop III, significantly affects 3′ processing (Table 2, section D; Fig. 6C), mutation of nucleotides 112 to 115 (5′ side of the bottom of stem III; MRG3U2-45) had much less of an effect on 3′ processing (38 and 72%, respectively). There are several possibilities which could explain these results. First, as has previously been mentioned, both the sequence of nucleotides 141 to 144 and the structure at the bottom of stem III might be important. However, it is also possible that nucleotides 141 to 144 (CUCC) of mutant MRG3U2-45 could base pair either with nucleotides 106 to 109 (GGAG) or with nucleotides 124 to

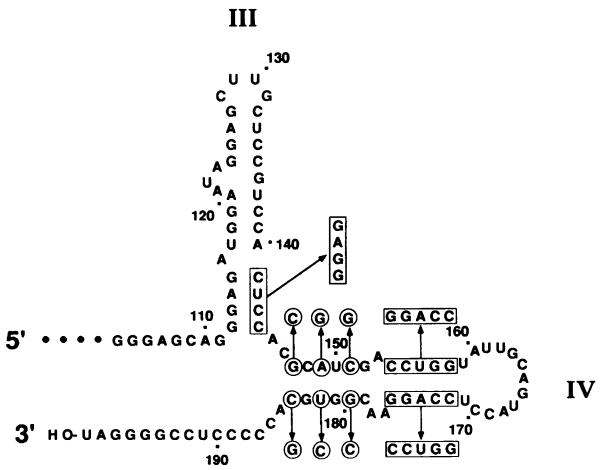

FIG. 8. Composite illustration of critical mutations in the bottom of stem III and in stem IV. Nucleotide changes which resulted in a significant reduction in 3′ processing of pre-U2 RNAs are indicated (circles and boxes).

127 (GGAG), thereby restoring structure which could be recognized by the processing activity. Similarly, alternate structures might explain the significant decrease in processing observed for mutant MRG3U2-47 ($CUCC_{141-144}$ →GAGG). Mutated nucleotides 141 to 144 could pair with nucleotides 189 to 192 (CCUC), or nucleotides 140 to 147 (AGAGGACG) of MRG3U2-47 might pair with nucleotides 67 to 74 (CGUCCUCU), thereby forming a structure which is not recognized by the processing activity. Alternatively, nucleotides 112 to 115 (GGAG) of MRG3U2-47 could pair either with nucleotides 132 to 135 (CUCC) or with nucleotides 190 to 193 (CUCC), again forming a structure which is not recognized by the processing activity. In any case, compensatory mutations of stem III mutants which reestablish base pairing of the bottom of stem III restore processing. This finding, together with the observation that the complete loss of stem-loop III results in a significant reduction in 3′ processing, indicates that the structure at the bottom of stem III is important for 3′ processing of pre-U2 RNA.

It has been demonstrated that stem-loop IV of U2 RNA is essential for the binding of the U2 snRNP-specific proteins A′ and B″ (3, 4, 16, 22). Mutation of loop IV (nucleotides 159 to 171) eliminated binding of the B″ protein in snRNP assembly studies (20a), in keeping with previous observations (15). However, this U2 RNA mutant (MRG3U2-9) underwent 3′ processing at 93% of the wild-type U2 level, indicating that sequences important for B″ binding are not a significant aspect of recognition by the 3′ processing activity. Thus, it is unlikely that the U2 snRNP-specific B″ protein is involved in the 3′ processing of pre-U2 RNA. Our finding that sequences in and adjacent to stem IV are important for 3′ processing is compatible with a previous suggestion (51), although this suggestion was based on the interpretation that the observed impairment of 3′ processing reflected an inability of these RNAs to bind U2 snRNP-specific proteins rather than the direct involvement of this region in the 3′ processing reaction as we have shown here.

One might suspect that the presence of a bulge in the middle of stem IV constitutes a structural determinant for binding of the 3′ processing machinery, similar to that observed for the interaction of Tat protein with the TAR region of human immunodeficiency virus RNA (48). However, removal of the stem IV bulge had no effect on 3′ processing (last entry of section E, Table 2), showing that this structural factor is not involved in the recognition of pre-U2 RNA by the 3′ processing activity.

One of the more surprising findings in this investigation is that mutation of the 11- to 12-nucleotide 3′ extension itself had relatively little effect on 3′ processing (section F of Table 2). Yet it is interesting to note that mutation of nucleotides which span the mature 3′ site (nucleotides 187 and 188) resulted in a moderate reduction in 3′ processing (boxed values in section F of Table 2). Similarly, a moderate reduction in 3′ processing was also observed for mutant MRG3U2-34 ($CCTCCG_{189-194}$ → GCGCGC; circled value in section F of Table 2). Although additional studies will be required to clarify this point, it is possible that the nucleotides at (187 and 188) or immediately adjacent to (189) the mature 3′ site are of importance. That the effects of mutation at these latter positions is manifest as a decrease in processing efficiency (rather than processing beyond the mature 3′ site or instability of the product) suggests that these nucleotides influence the binding of the processing activity or the initiation of the cleavage reaction.

We have noted a possible fifth stem-loop structure in pre-U2 RNA, wherein nucleotides 186 to 189 (5′-CCCCC-3′), which span the mature 3′ end of U2 RNA, would be paired with nucleotides 194 to 197 (5′-GGGGG-3′) of the 3′ extension. Since our results clearly demonstrate that accurate and efficient 3′ processing of pre-U2 RNA is dependent upon structure, it was surprising that mutations which would disrupt this potential stem-loop V structure did not significantly reduce 3′ processing. In fact, greater than wild-type activity was observed in most of these cases (section F of Table 2). This finding suggests that destabilizing this potential stem-loop V structure might actually enhance processing, perhaps by making the 3′ extension more readily available (single stranded).

We conclude that in the binding step of the reaction, the pre-U2 RNA 3′ processing activity recognizes primarily the aforementioned critical structure comprising stem IV and the bottom of stem III and that the catalytic step of the reaction is quite indifferent to the nucleotide sequence of the substrate, viz., the 3′ extension. This situation offers a sharp contrast with the cases of pre-mRNA 3′ processing prior to polyadenylation and the 3′ cleavage of histone pre-mRNA. The former reaction involves, in addition to the AAUAAA sequence 10 to 20 nucleotides 5′ of the poly(A) addition site, a GU- or U-rich sequence 3′ of this site (56). In the latter case, the reaction requires a stem-loop and purine-rich sequence 9 to 14 nucleotides 3′ of the cleavage site (35). As mentioned above, we hope to expand our understanding of this intriguing U2 RNA 3′ processing reaction at both the cellular and biochemical levels. These endeavors are well under way in our laboratory.

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