Identification and Characterization of a Nuclease Specific for the 3' End of the U6 Small Nuclear RNA*

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The U6 small nuclear RNA is post-transcriptionally processed by the addition and removal of nontemplate uridylates (Us) at its 3' end prior to incorporation into the U4-U6 small nuclear ribonucleoprotein complex. An enzyme responsible for removing Us from the U6 3' end has not been previously identified. Here we biochemically isolate and characterize an exonuclease activity from HeLa cells that removes template and nontemplate 3'-nucleotides specifically from U6 RNA. We also report the isolation of an inhibitor of this U6 nuclease. U6 nuclease rapidly removes the four terminal 3' Us found in the human U6 coding sequence in a magnesium-dependent manner. Mutagenesis studies on the U6 RNA define regions essential for processing. U6 nuclease recognizes specific sequences on both strands near the base of the major intramolecular stem loop of the U6 RNA. The preprocessed 3' Us form part of the base of the stem loop, but neither specific sequences nor secondary structure at the four terminal nucleotides are required to achieve processing.

RNA polymerase (pol) III produces numerous small RNAs that have a variety of functions (1, 2). pol III transcripts include tRNAs, adenovirus VA RNA, U6 snRNA, 5 S rRNA, and other RNAs such as B1-Alu, which might be involved in retrotransposition of short interspersed elements. One common feature of all these RNAs is a tract of uridylic acids (Us) at their 3' ends that signals pol III to terminate transcription. Apart from pol III termination, these oligo(U) tracts have no known function, although the B1-Alu tail might hybridize with an internal A tract to provide a primer for reverse transcriptase (3–5).

In a number of documented cases, large portions of the 3' end of certain pol III transcripts are removed during the course of transcription by the pol III transcription complex (6–8). Binding of the nuclear autoantigen La protein to the oligo(U) tract of the nascent transcript appears to stabilize it against processing. In other reports of shortened transcripts, the last few nucleotides are absent, which might be due to pol III pausing (9). In this case, the La protein promotes proper termination and transcript release (9).

The U6 snRNA transcript undergoes major 3' end processing in vivo before it is incorporated into the U4-U6 snRNP splicing complex, and the 3' region is essential for splicing function (10). At the completion of U6 synthesis, the La protein associates with the 3' uridylate tract of U6 (UUUU-OH) as long as it contains a 3'-OH (11). What role the La protein serves in binding to the released transcript is not known, although it may stabilize U6 against degradation. The 3' end of La-associated U6 becomes heterogeneous through nontemplate 3' addition of Us (11–13). What happens next is unclear; however, the 3' end is eventually processed back to five Us in which the terminal U contains a 2',3'-cyclic phosphate (UUUU(U)P) (14). The activity responsible for the generation of the 2',3'-cyclic phosphate has not been biochemically identified. Formation of the 2',3'-cyclic phosphate is thought to release the La protein, thereby allowing the processed U6 RNA to become associated with the U4-U6 snRNP. Most of the processed U6 RNA, however, is not associated with either snRNP complexes or the La protein (11).

While U6 transcripts have been observed to be shortened by a nuclease in vitro (15), there has been no reported biochemical characterization of a nuclease that specifically targets U6 RNA for removal of its 3' Us. Here we report the isolation and initial characterization of a 3'-exonuclease that is specific for the U6 snRNA. We address whether the shortened U6 transcript is generated transcriptionally or post-transcriptionally. We assess how many nucleotides are removed and whether the removal is exo- or endonucleolytic. We identify from which end of the RNA the nucleotides are removed. Evidence for an inhibitor of the U6 nuclease is presented, and the kinetics of processing are examined. Finally, through mutagenesis of the U6 RNA we examine the specificity determinants of the U6 nuclease, both for U6 recognition and nucleotide removal.

MATERIALS AND METHODS

Buffers, DNA, and Proteins—H buffer contained 20 mM HEPES, pH 8, 5% glycerol, 2 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, and KCl. For all buffers in the H series, the number after the H denotes the molar KCl concentration. TSB+ contained 20 mM Tris acetate (50 mM citrato, pH 8), 5% glycerol, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 200 mM potassium glutamate, 0.1% Nonidet P-40, and 0.1 mM phenylmethylsulfonyl fluoride. The plasmid templates pU6 (human U6 snRNP promoter), pBRVA1, and G₄T₁ have been previously described (16–18). Oligonucleotides were synthesized using the Oligo 1000 DNA synthesizer (Beckman Instruments). T7 RNA polymerase and T7q DNA polymerase were purchased from New England Biolabs and Promega, respectively. HeLa nuclear extracts were prepared according to Dignam et al. (19), with slight modification (20). The human TATA binding protein was prepared as described (21).

U6 Nuclease Purification—HeLa nuclear extracts (1.3 g, 50 ml) were dialyzed to a conductivity equivalent to H.15 buffer and chromatographed on phosphocellulose (75-ml column), which was equilibrated with H.15 (18). Proteins were step eluted with buffers H.3 and H.5, and designated P.3 (130 mg, 90 ml) and P.5 (70 mg, 90 ml), respectively. Each fraction was dialyzed against TSB+ buffer. Fractions to be purified further were dialyzed against H.1 buffer. The P.5 fraction (35 mg,
45 ml was loaded onto a 1-ml Mono Q fast protein liquid chromatography column equilibrated with H.1 buffer at a flow rate of 0.5 ml/min. After washing the resin with H.1 buffer, the column was developed with a 20-ml linear gradient from 100 to 600 mM KCl in H buffer. Fractions (1 ml) containing U6 nuclease activity eluted between 350 and 390 mM KCl (0.5–8 ml). At this stage U6 nuclease was purified by 200-fold. Active fractions were pooled, dialyzed against TSB+ and stored at –80°C. Substantial purification of U6 nuclease was achieved by Mono S chromatography, eluting between 300 and 500 mM KCl. However, processing assays performed with the Mono S fractions displayed an unacceptably high level of nonspecific RNase activity and thus were not used for characterization.

U6 Nuclease Inhibitor Purification—The P.3 fraction was generated as described for the U6 nuclease purification and was precipitated with 0.42 g/ml ammonium sulfate. The precipitate was collected by centrifugation, solubilized with H.1 buffer, and dialyzed to a conductivity equivalent to H.1. A portion of the concentrated P.3 fraction (20 mg, 1 ml) was loaded onto a 1-ml Mono Q column, washed with H.1 buffer, and eluted with a 130-ml linear gradient from 100 to 600 mM KCl in H buffer. The U6 nuclease inhibitor eluted over six 1 ml fractions between 250 and 320 mM KCl. Active fractions were dialyzed against TSB+ and stored at –80°C.

Wild Type and Mutant U6 RNAs—The polymerase chain reaction (PCR) was used to fuse the T7 promoter to the U6 coding region. Primer combinations and sequences are shown in Table I. PCR reactions contained ~0.5 μm primer, 0.4 mM dNTP, 0.2 mM phenylmethylsulfonyl fluoride in a 50 mM Tris-Cl (pH 8), 1% polyvinyl alcohol, 60 mM KCl, 60 mM potassium glutamate, 0.5 mM spermidine, 0.5 mM GTP, 0.5 mM CTP, 0.5 mM UTP, 5 mM potassium glutamate, 0.5 mM spermidine (G6TI reactions used 4 mM GTP). The reactions were performed in a total volume of 20 μl and were 20°C.

RESULTS AND DISCUSSION

Inhibited Production of Short U6 Transcripts—Transcription of the 106-nucleotide human U6 snRNA gene can be reconstituted in HeLa nuclear extracts (Fig. 1A, lane 1). The majority of the transcript migrated several nucleotides shorter than expected (denoted by the large arrow). A minor amount migrated at the expected size (small arrow). The production of both transcripts required the U6 promoter and was inhibited by high but not low concentrations of α-amanitin (data not shown), which indicated that the transcripts were U6- and pol III-derived. These transcripts could be due to alternative pol III start or stop sites or could be due to post-transcriptional processing of the U6 RNA.

The presence of alternative start sites is unlikely to account for the two transcripts since prior studies have demonstrated only a single start site for the U6 gene (22, 23). pol III will pause near the end of the gene when the La protein is absent (9). The La protein allows for efficient and accurate termination of transcription. Inasmuch as the La protein is present in nuclear extracts (24–26) and we fail to observe such pausing at other pol III genes such as Va (Fig. 1A, lane 2, and data not shown), pausing is unlikely to account for the shortened transcript, although this possibility cannot be ruled out. The shorter U6 transcript is unlikely to be caused by a nonspecific RNase, since other pol III- and pol II-derived transcripts were not shortened (lanes 2 and 3).

In an effort to identify the source of the shortened U6 transcript, nuclear extracts were passed over phosphocellulose and step eluted with 0.3 and 0.5 M KCl (P.3 and P.5 fractions, respectively). The P.5 fraction reconstituted U6 transcription (although, in general, only weakly) but yielded only the shorter transcript (Fig. 1B, lane 3). Comparison to size standards on a high resolution sequencing gel revealed a distribution of transcripts having a median size of 101 nucleotides. When increasing amounts of the P.3 fraction were included in the U6 transcription reaction, longer transcripts centered around 106 nucleotides were observed (lanes 1 and 2). This suggested that a factor present in the P.3 fraction inhibited the production of the shorter transcripts. This inhibitor was purified further by

| Table I Oligonucleotide primers for generating T7 promoter-U6 fusion constructs |
|---------------------------------|-----------------|-----------------|
| 5′ primer (5′-3′) mutant name | 3′ primer (5′-3′) mutant name |
| 5′TGTCGCTGGATCCCAU102G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAATATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCAU103G | AAAATAATGGAACGCTT |
| 5′TGTCGCTGGATCCCATG103G | AAAATAATGGAACGCTCAGTAGCTACATAG. T7 RNA polymerase initiates transcription immediately 3′ to this sequence. Since T7 RNA polymerase prefers to initiate transcription at a G, it was necessary to include a G at the transcription start site of all constructs. Those initiating Gs that are not found in the wild type gene are indicated by italics. The mutated nucleotides are underlined.
Mono Q chromatography. As shown in Fig. 1C, an activity eluting across fractions 15–19 (280–320 m M KCl) inhibited the production of the shorter U6 transcripts. From these data we conclude that a distinct biochemical entity inhibits the production of shortened U6 transcripts. At this point we do not know if this inhibitor is related to the La protein. We have deferred further characterization of the inhibitor until the nature of the shortened U6 transcript is more fully defined.

Post-transcriptional Shortening of the U6 snRNA in Vitro—To address directly whether the shorter U6 transcript was generated during or after transcription, we took advantage of the P.3 inhibitor to produce full-length U6 transcripts in the P.5 fraction. Transcripts were purified from a high resolution denaturing gel and incubated in the P.5 fraction for varying lengths of time. As shown in Fig. 2A, the transcript became progressively shorter over a period of a few minutes. This result indicated that the processing activity was post-transcriptional. Similar experiments performed with other pol III- or pol II-derived transcripts yielded no detectable processing, which confirms that the processing activity is specific (data not shown). The issue of specificity for U6 is explicitly addressed below.

In Fig. 2A, RNA lengths of 102 and 103 nucleotides predominated. We do not know why these transcripts are slightly shorter than those shown in Fig. 1B, lane 3. The fact that the RNA produced in Fig. 1B was generated and available for processing over a course of 30 min, while the RNA in Fig. 2A was exposed to the processing activity for only a few minutes, might account for the difference.

To study the U6 processing reaction in more detail, large quantities of purified U6 RNA were generated in vitro using T7 RNA polymerase. To fuse the T7 promoter to the U6 coding sequence, an oligonucleotide containing the T7 promoter plus the 5’-coding sequences of U6 and an oligonucleotide containing the 3’-coding sequences were used to amplify the U6 gene by PCR. The resulting DNA was purified and incubated with T7 RNA polymerase to generate U6 RNA. In addition, the processing activity was further purified by Mono Q chromatography. As shown in Fig. 2B, this synthetic 106-nucleotide U6 RNA was rapidly processed to 102 nucleotides.

With this experimental setup, we consistently observed a low level of shortening to 103 nucleotides, and a high level of shortening to 102 nucleotides, but no shorter. From day to day, low levels of contaminating nonspecific RNases in the system caused varying degrees of nonspecific degradation of the RNA. Thus quantitation of processing on an absolute scale was not feasible. However, as presented below, we were able to quantitate the fractional extent of processing.

U6 Nuclease Trims Four Nucleotides from the 3’ End of U6—Because of earlier reports of in vivo shortening of the U6 3’ end (11, 14), we examined whether nucleotides were being removed from its 3’ end. We reasoned that if three or four nucleotides were enzymatically removed from the 3’ end of
the wild type 106-nucleotide U6 RNA, then a 3'-truncated RNA that is missing the last two Us should have only an additional one or two nucleotides removed. Indeed, as shown in Fig. 3, the wild type and 104-nucleotide U6 RNA, termed U6(1–104), were processed to the same length. Therefore, the processing activity appears to be a nuclease that trims three to four nucleotides from the 3' end of wild type U6 RNA. The results with U6(1–104) suggest that the nuclease removes nucleotides up to a specified position, in this case predominately nucleotide 102, as opposed to removing a fixed number of nucleotides from the 3' end of any competent substrate RNA. That 105-, 104-, and 103-nucleotide intermediates are observed on the wild type U6 (e.g., see Figs. 1B, 2A, and 4) suggests that the nuclease is a 3',5'-exonuclease as opposed to a site-specific endonuclease.

**Kinetics of Processing**—Having a kinetic assay in hand for monitoring the processing of the U6 RNA, we sought to obtain more information on the mechanism of processing. The rates of processing were measured at various concentrations of U6 nuclease (Fig. 4A). The relative level of processing was determined by the equation: processing (%) = 100P/(U + P), where P denotes the level of processed RNA and U denotes the level of unprocessed RNA. At the highest concentration of nuclease, the processing reaction possessed a t_{1/2} of ~4 s, suggesting that processing is relatively rapid. As expected, the rate of processing increased linearly with the amount of U6 nuclease added (Fig. 4B). However, with the lowest amount of U6 nuclease added, the reaction did not proceed to completion. A significant proportion of the U6 RNA was either not processed or only slowly processed. This type of behavior would be expected of an
enzyme that slowly turns over. In other words, when there is more U6 nuclease than substrate, then the RNA is saturated and the processing reaction is rapid. When there is less U6 nuclease than RNA substrate, then to obtain processing of all the RNA, the nuclease must dissociate from the processed product and reassociate with unprocessed substrate. This turnover or product release appears to be inherently slow and overall rate-limiting.

Many nuclease reactions require magnesium. As shown in Fig. 5, the U6 nuclease also requires magnesium for activity. In the presence of magnesium, the U6 RNA is completely processed within 40 s. In the absence of magnesium, no processing was observed over a period of 60 s. When magnesium was added after preincubation of RNA and nuclease, the kinetics of processing were very slow. It is unlikely that the U6 nuclease was generally inactivated, since on longer incubations the reactions went to completion (data not shown). Instead, the kinetics were reminiscent of the slow enzyme turnover described in Fig. 4. One plausible interpretation of the data in Fig. 5 is that the U6 nuclease binds U6 RNA in the absence of magnesium but must dissociate from the RNA in order to chelate the magnesium that is necessary for processing. We emphasize that these mechanistic interpretations are tentative until more detailed studies are performed to rigorously establish whether product release is rate-limiting in multiple rounds of processing and whether magnesium binding must proceed by an ordered mechanism.

Substrate Specificity of U6 Nuclease—The observed processing of the U6 RNA leads us to pose the following questions with regard to substrate specificity. 1) What features of the U6 RNA target it for processing and not other RNAs? 2) What features of the U6 RNA cause processing to terminate at nucleotide 102? 3) What role does secondary structure play? 4) Since the U6 RNA ends with four Us, is U6 nuclease uridylate-specific? While we cannot provide complete answers to all of these questions, we have begun to address them through mutagenesis of the U6 RNA.

Our first step in addressing these questions was to perform deletion analyses on the U6 RNA. In Fig. 6A, time courses of processing were presented for wild type U6, a 3’ deletion U6(1–76), and a 5’ deletion U6(58–106). Above the wild type U6 reaction is shown a proposed secondary structure for the human U6 snRNA as described by Epstein et al. (27). Above the two deletion constructs are representations of the truncated RNAs. No effort was made to determine their potential secondary structure; they serve only to delineate the deletion end points. Processing was not detected for either of these truncated RNAs, which confirms that the U6 nuclease is not a general nuclease but recognizes specific determinants on the U6 RNA. Since U6(1–104) was readily processed (Fig. 3), we can conclude that an important sequence or structural element lies between nucleotides 76 and 104. In addition, an important sequence or structural element lies within the first 57 nucleotides of the U6 RNA.

As shown in Fig. 6A, the U6 RNA appears to have two major hairpin structures. The four 3’ Us that are processed lie at the base of the large hairpin. We were curious as to whether the region complementary to the processed nucleotides was important for processing. To this end, we compared the processing of two RNAs, one termed U6(27–106), which was truncated from the 5’ end up to the hairpin and the other termed U6(33–106), which was truncated six nucleotides into the hairpin. As shown in Fig. 6B, U6(27–106) was readily processed while U6(33–106) was not processed. This result indicated that the 5’ end of an important sequence and/or structural element for U6 RNA processing resides between nucleotides 27 and 33. From this data alone we cannot discern whether this region provides important structural and/or sequence information.

To address the importance of secondary structure in the processed region and characterize the sequence and length requirements in this region, a battery of 5’ and 3’ mutants was generated. A summary of the data is presented in Fig. 7. We point out the following observations.

1) When one or three nucleotides were added to the end of U6 (mutants 107G and 107U3), efficient processing back to nucleotide 102 was observed. Thus, U6 nuclease possesses the necessary activity to remove the added nontemplate 3’ Us that are normally found on U6 in vivo. The fact that short 3’ deletions and extensions were fully processed back to nucleotide 102 indicated that the nuclease is not measuring four nucleotides. Instead it appears simply to stop at position 102, which from the 3’ end is the first position to contain an A.

2) When the secondary structure at the 3’ end was partially or fully disrupted with one or more mutations between nucleotides 103 and 106 (mutants U3103C3, U4103G4, U4103A4, U3103C3, U4103G4), efficient processing back to nucleotide 102 was observed, although in some cases much less efficiently. Surprisingly, some of these mutations included complete replacement of the four terminal Us with either As or Gs. Thus, neither a stretch of Us nor secondary structure at the 3’ end of U6 appears to be essential for processing. The observation that some of these mutations are significantly depressed for processing, such as U3103C3, suggests that this region does provide some important sequence information.

3) U6 RNAs containing clustered mutations extending from nucleotide 28 to 30 (A28G3), which forms the upstream base of the major stem loop, were efficiently processed, demonstrating that this region provides neither sequence nor secondary structure information for processing. When secondary structure in this region was restored with Gs (mutant A28G3-U3103C3), instead of AUs, processing was relatively unaffected when compared with U3103C3.

4) Since nucleotide 102 was the first position from the 3’ end in U6(wild type) that contained an A, we initially wondered whether the presence of an A signified a stop. However, mutant U6(U3103A3), which replaces the four terminal Us with As, was efficiently processed back to position 102, thus indicating that an A anywhere along the path of the nuclease does not suffice to terminate processing. To examine what role A102 played, we
mutated it to a U. The mutation caused two dramatic effects; first, U6(A102U) was poorly processed, indicating that A102 was important for the overall level of processing, and second, U6(A102U) was processed back to A100 and not to the expected nucleotide 102. Thus, it appears that an A at nucleotide 102, but not at a site downstream of this position, is important for terminating the processing. When A102 is replaced with a U, U6 nuclease continues on through U102 and U101 and finally terminates at A100. Apparently, an A at or just upstream of nucleotide 102 signifies a stop for U6 nuclease.

5) Thus far deletion analyses have located the 5′ end of an important element between nucleotides 27 and 33. Mutations at positions 28, 29, and 30 indicate that these nucleotides are relatively unimportant. This suggests that the 5′ end of the important element lies between nucleotides 31 and 33. Nucleotide U31 is paired with the important A102 in the proposed secondary structure. To address whether U31 was important for processing, it was changed to an A (mutant U31A) and was found to significantly depress processing. To address whether potential loss of secondary structure at this position was inhibiting processing, a complementary mutation was made (U31A:A102U). No processing above that with each of the individual mutants was observed, which indicated that secondary structure alone at this position was not sufficient to support processing. Instead, it appears that, minimally, nucleotides 31 and 102 provide important sequence informa-
tion for processing. Whether secondary structure at this position is also important cannot be addressed by the data. However, that U31 and A102 are both important and are normally basepaired would suggest that U6 nuclease is recognizing this region in the context of secondary structure. Interestingly, U31 is one of only three Us in the U6 RNA that is post-translationally modified to pseudouridine (27). The significance of this modification is not known.

6) We note that in the presence of high levels of U6 nuclease all 3’ mutants were processed as well as wild type. Only at lower levels of nuclease were differences in processing apparent. The concentration dependence suggested that the 3’ region might stabilize the binding of U6 nuclease to U6 RNA.

**Model for U6 3’ End Processing—** Compiling the findings presented here leads us to suggest an initial working model for processing of the U6 3’ terminus. Many details are still sketchy and need to be worked out. This view is intended solely as a guide for designing future experiments to refine the mechanism. The U6 nuclease in complex with magnesium targets U6 through RNA interactions in the vicinity of U31 and A102.

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**FIG. 7. Mutation analysis of U6.** U6 RNA containing one or more point mutations were generated as described under “Materials and Methods.” The location of each mutation is illustrated in the context of the structure of the wild type U6 RNA and is shown in a larger type face. The presence or absence of secondary structure in the mutated region is arbitrary. No attempt was made to model alternative secondary structures. The point to which the RNA is processed back to is indicated by an arrow. Where processing was sufficiently weak that assignment of the processing end point could not be ascertained with confidence, the arrow was omitted. The relative level of processing was determined as described in the text and grouped into four categories relative to wild type, as indicated. 5’ mutants, shown to the left, were generated in the context of U6(27–106). wt, wild type.
These two nucleotides are base paired in the proposed secondary structure of U6, when not complexed with U4. Other unmapped regions of interactions are likely, possibly including some interactions with the 3’-terminal U tract. U6 nuclease then nonspecifically hydrolyzes the nearby 3’ end of the RNA, one nucleotide at a time until it reaches position 102 where it stops. Critical interactions of the nuclease with A102 might prevent the nuclease from hydrolyzing this nucleotide. In the absence of A at 102, the nuclease selects the next upstream A, albeit inefficiently, and hydrolysis proceeds up to that point. At the completion of the reaction, the nuclease slowly dissociates from the processed RNA and is available to process other U6 RNAs. The U6 processing reaction can be regulated by a distinct inhibitor located in the P.3 fraction.

It is unclear why the cell processes the 3’ end of U6. At least in Xenopus oocytes, there appears to be an excess of U6 snRNA complex. Removal of most of the terminal Us and formation of a 2’,3’-cyclic phosphate appear to be prerequisites for incorporation into the snRNP complex (11, 14). Since U4 snRNA hybridizes with the U6 snRNA through U6’s major stem loop, a major unwinding of the U6 stem loop would be necessary to transit into the mature state. Unwinding of the U6 stem loop and subsequent incorporation into the U4-U6 snRNP complex might initiate at the 3’ end of U6. If so, then the 3’ end of U6 would be expected to be a highly regulated region. Processing of the U6 3’ end may be one step along the pathway toward initiating its incorporation into the U4-U6 snRNP.

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