

U2.3 Precursor Small Nuclear RNA *in vitro* Processing Assay

Chan Lin[#], Yujie Feng[#], Xueyan Peng, Jiaming Wu, Weili Wang* and Yunfeng Liu*

State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, College of Life Science and Technology, Guangxi University, 100 Daxue Road, Nanning, Guangxi 530004, China

[#]Contributed equally to this work

*For correspondence: wangwl0209@163.com; yunfengliu_bio@126.com

[Abstract] Small nuclear RNAs (snRNAs) are vital for eukaryotic cell activities and play important roles in pre-mRNA splicing. The molecular mechanism underlying the transcription of snRNA, regulated via upstream/downstream cis-elements and relevant trans-elements, has been investigated in detail using cell-free extracts. However, the processing of precursor snRNA (pre-snRNA), which is required by 3' end maturation of pre-snRNA, remains unclear as a proper processing assay is difficult to develop *in vitro*. Here, we present an *in vitro* method using synthetic labeled RNA as substrates to study the 3' cleavage of pre-snRNA.

Keywords: snRNA, Pre-snRNA, Processing, *Arabidopsis*, *In vitro*

[Background] As critical components of spliceosome for the processing and splicing of precursor mRNAs (pre-mRNAs), small nuclear RNAs (snRNAs) are essential and fundamental non-coding small RNAs in eukaryotic cells. The transcription complex relying on the DNA-dependent RNA polymerase II (Pol II) is required for most snRNA transcription (including U1, U2, U4, and U5), except for U6, which depends on RNA polymerase III (Pol III) (Carbon *et al.*, 1987; Vankan and Filipowicz, 1988). The Pol II mediated transcription of snRNA requires a set of integrator factors (INTs) in metazoans (Baillat *et al.*, 2005). INTs contain at least 14 subunits in metazoans (Baillat *et al.*, 2005; Chen and Wagner, 2010), associated with C-terminal domain (CTD) of Pol II to form the transcription complex. Recently, five plant INT factors involved in snRNA biosynthesis were identified in *Arabidopsis* and termed Defective in snRNA Processing (DSP), including DSP1-4 and CPSF73-I (Cleavage and Polyadenylation Specificity Factor 73 kDa) (Liu *et al.*, 2016). The INTs/DSPs are recruited to the promoter of snRNA and associate with Pol II to synthesize the pre-snRNA, transcribing it beyond the 3' end of mature snRNAs. The nascent pre-snRNA transcripts contain the snRNA sequence and the excessive 3' box RNA fragment. Consequently, pre-snRNAs are subject to 3' maturation, a process involving endonucleolytic cleavage of the nascent transcript at the cleavage site located on the upstream of the 3' box, to produce mature snRNA (Uggen and Murphy, 2003 and 2004; Baillat *et al.*, 2005; Chen and Wagner, 2010).

The molecular mechanism underlying the initiation and inhibition of snRNA transcription has been well studied both *in vivo* and *in vitro*. The functions of cis-elements, including the distal sequence element (DSE), proximal sequence element (PSE) in humans and upstream sequence element (USE) in plants, and trans-elements, including INTs/DSPs, have been investigated in detail (Hernandez, 2001).

Compared with the well-studied regulation of snRNA transcription, the processing steps of pre-snRNA

remain poorly understood (Hernandez, 2001; Jawdekar and Henry, 2008). Most studies on pre-snRNA processing were performed by evaluating the relative content of pre-snRNA in specific genotype material. An efficient system for detecting the processing of pre-snRNA using cell extracts will facilitate the study of the maturation of pre-snRNA in specific genotypic plants and reveal the influence of specific factors on pre-RNA maturation after transcription.

Accordingly, we applied a synthetic pre-U2.3 snRNA and created a system to analyze the *in vitro* processing activities of pre-U2.3 using the production of cell extracts. This *in vitro* processing analysis provides a method to detect the activation of pre-snRNA maturity.

Materials and Reagents

1. Nylon membrane (Invitrogen, catalog number: LC2003)
2. DNaseI (NEB, catalog number: M0303S)
3. Nuclease-free water (ThermoFisher Scientific, catalog number: AM9932)
4. Trizol (ThermoFisher Scientific, catalog number: 10296028)
5. Chloroform (Sigma-Aldrich, catalog number: 288306)
6. Isopropanol (Sigma-Aldrich, catalog number: 563935)
7. Ethanol (Sigma-Aldrich, catalog number: 51976)
8. 2-mercaptoethanol (Gibco, catalog number: 21985023)
9. Hexylene glycol (Sigma-Aldrich, catalog number: 112100)
10. Triton X-100 (Fisher Scientific, Acros organics, catalog number: 215680010)
11. dNTP (10 mM each) (ThermoFisher Scientific, catalog number: R0192)
12. NTP (10 mM each) (ThermoFisher Scientific, Molecular biology grade NTPs, catalog number: R0481)
13. Glycerol (Sigma-Aldrich, catalog number: G5516)
14. HEPES (Sigma-Aldrich, catalog number: H3375)
15. Various salts: NaCl, KCl, MgCl₂, and MnCl₂ (Sigma-Aldrich, catalog numbers: S9888, P9541, M8266, and M1787, respectively)
16. Creatine phosphate (Sigma-Aldrich, catalog number: 10621714001)
17. Polyvinyl alcohol (Sigma-Aldrich, catalog number: 341584)
18. DTT (ThermoFisher Scientific, catalog number: R0861)
19. Pepstatin A (Sigma-Aldrich, catalog number: P5318)
20. Phenylmethylsulfonyl (PMSF) (Sigma-Aldrich, catalog number: 52332)
21. Protease inhibitor (Roche, catalog number: 11697498001)
22. Diethyl pyrocarbonate (DEPC) (Sigma-Aldrich, catalog number: D5758)
23. [γ -³²P]-ATP (3,000 Ci/mmol, 10 mCi/ml) (PerkinElmer, catalog number: BLU002A)
24. T7 RNA polymerase (ThermoFisher, catalog number: 18033019)
25. T4 polynucleotide kinase (PNK) (NEB, catalog number: M0201S)
26. High-fidelity DNA polymerase (ThermoFisher, catalog number: F530L)

27. pCR-Blunt (ThermoFisher, catalog number: K270020)

A map of pCR-Blunt can be found on the website of the manufacturer:
www.thermofisher.com/order/catalog/product/K270020#/K270020.

28. RNA marker (Abnova, catalog number: R0002)

29. Bradford reagent (Bio-Rad, catalog number: 5000205)

30. Gel Purification kit

31. PCR Extraction Kit

32. RNase away

33. Primers:

U2.3-RNA-F: atacccttcggccctttggc

U2.3-RNA-R: ctgcgttaacatataaaatctctg

T7-U2.3-F: TAATACGACTCACTATAGGGatacccttcggc

PolyG-U2.3-R: CCCCCCCCCCCctgcgttaacatataaa

34. Processing buffer (see Recipes)

35. M1 buffer (see Recipes)

36. M2 buffer (see Recipes)

37. M3 buffer (see Recipes)

38. 2× formamide loading buffer (see Recipes)

39. 6% denaturing polyacrylamide gel containing 6 M urea (see Recipes)

Equipment

1. PhosphorImager (GE Health Care, model: Typhoon FLA 9500)

2. Suitable space for working with ^{32}P radioactivity

3. Geiger counter (Thermo, 900 mini)

4. Plexiglass shield to protect user from radioactivity

5. Plexiglass box for ^{32}P waste

6. Sephadex G-25 spin column (Merck, 11273990001)

7. PCR Machine

8. Phosphor Screen (Molecular Dynamics)

9. Cell strainer (Biofil, catalog number: CSS013100)

10. Vertical electrophoresis gel box and glass plates with spacers and combs (Bio-Rad, model: Mini-PROTEAN Tetra)

11. Spectrophotometer (any UV absorption spectrophotometry including Bradford available)

Software

1. Quantity One (Bio-Rad Laboratories) or ImageJ

Procedure

A. Assemble pre-U2.3 templates

1. Add the following to the PCR reaction:

36.5 µl ddH₂O

10 µl 5× Phusion HF buffer

1 µl 10 mM dNTP mix

200 ng DNA of *Arabidopsis*

0.5 µl U2.3-RNA-F and U2.3-RNA-R primers (10 pmol/µl)

0.5 µl High-Fidelity DNA Polymerase

2. Perform PCR to generate pre-U2.3 fragment with the following cycles:

98°C for 30 s to denature the DNA

95°C for 10 s

58°C for 30 s

72°C for 15 s

Repetition of PCR for 31 cycles

72°C for 10 min

4°C for storage

3. Electrophoresis PCR-generated DNA fragments in 1.5% agarose gel.

4. Verify the pre-U2.3 (355 bp) fragment and purify PCR product from agarose gel using Gel Purification kit following manufacturer's instructions.

5. Cloning of the amplified PCR products to pCR-Blunt.

6 µl purified pre-U2.3 PCR product

1 µl pCR-Blunt vector

2 µl 5× T4 DNA Ligase buffer

1 µl T4 DNA Ligase

6. Incubate the ligation reaction at room temperature for 10 min. Transform the construct into competent *Escherichia coli*. Extract the positive vector and confirm the pre-U2.3 fragment using singer sequencing.

7. Assemble pre-U2.3 templates and fuse with T7 promoter and Poly-G. Add the following to the reaction:

74 µl ddH₂O

20 µl 5× Phusion HF buffer

2 µl 10 mM dNTP mix

1 µl pre-U2.3 pCR-Blunt vector (50 ng/µl)

1 µl T7-U2.3-F and PolyG-U2.3-R primers (10 pmol/µl)

1 µl High-Fidelity DNA Polymerase

Perform PCR with the following cycles:

98°C for 30 s to denature the DNA

95°C for 10 s

58°C for 30 s

72°C for 15 s

Repetition of PCR for 33 cycles

72°C for 10 min

4°C for storage

8. Load a small aliquot of PCR product (5 µl) in 1.5% agarose gel for checking the quality of PCR fragments. If the product contains a single band, the rest of the product (95 µl) can be purified directly using the PCR Extraction Kit without gel purification.
9. Elute the PCR product in the final 50 µl DEPC water, used as *in vitro* transcription template.

B. Synthesize pre-U2.3-polyG snRNA substrates

1. *In vitro* RNA transcription. Add the following to the reaction:

22 µl DEPC water

10 µl 5× Transcription Buffer (Ambion)

2.5 µl NTPs (10 mM ATP, CTP, UTP, GTP)

1 µl RNasin (Promega 40 units/µl)

1 µl 1 M DTT

5 µl Transcription Template (200 ng/µl)

1 µl T7 RNA Polymerase (50 unit/µl)

Incubate for 1 h at 37°C

2. Add 1 µl of DNase I (10 units/µl) and incubate for 15 min at 37°C.
3. Add 50 µl 2× formamide loading buffer to the reaction solution, boil for 5 min, and load onto a pre-run 5% denaturing polyacrylamide gel containing 6 M urea. Separate RNAs at 20 mA/gel, ~1 h.
4. After electrophoresis, visualize the PAGE gel with ethidium bromide and cut out the RNA bands directly with a clean razor blade. The gel slice is crushed into a fine slurry, which is soaked in the TE buffer. The slurry is then centrifuged and the supernatant recovered with a pipette. Polyacrylamide fragments carried over into the supernatant can be removed by filtration through a 0.2 mm filter.
5. Add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M and use 3 volumes of absolute ethanol to precipitate RNA. Pellet the RNA by centrifuging at 12,000 × g for 15 min.
6. Wash small pellets with 75% ethanol to remove undesired salts.
7. Dissolve the RNA substrates in 20 µl DEPC water.
8. Radioactive labeling of RNA substrates. Add the following to the reaction:
3 µl DEPC H₂O
20 µl RNA substrate
3 µl T4 PNK Reaction Buffer (10×)
1.5 µl T4 PNK

1.5 μ l RNasin (Promega 40 units/ μ l)

1 μ l [γ - 32 P]-ATP

Incubate at 37°C for 30 min. Add 30 μ l DEPC water (This volume can be varied if more or less pre-snRNA probe is desired).

9. Unincorporated nucleotides are removed using a Sephadex G-25 quick spin column.

C. Synthesize radioactive RNA marker

1. Take 15 μ l commercial RNA marker into Sephadex G-25 column to remove the salt and dye.
2. For radioactive labeling of the RNA marker, add the following to the reaction:
 - 10 μ l filtered RNA marker
 - 6 μ l DEPC water
 - 2 μ l T4 PNK Reaction Buffer (10 \times)
 - 1 μ l T4 PNK
 - 1 μ l [γ - 32 P]-ATPIncubate at 37°C for 30 min
3. Remove unincorporated nucleotides using a Sephadex G-25 quick spin column.
4. Prepare a dilution series of the labeled RNA marker (1:5, 1:15, 1:50, and 1:150). Drip 2 μ l of series diluted marker and pre-U2.3-polyG RNA substrate onto the nylon membrane. Fix the RNAs spot to the membrane by cross linking using the UV crosslinker with a setting of 120,000 μ J cm $^{-2}$.
5. Place the plastic-wrapped nylon membrane on the storage phosphor imaging screen and expose for 8 h. The phosphor screen is then scanned with PhosphorImager.
6. Dilute RNA marker using RNA loading buffer to same radiation level with pre-U2.3-polyG RNA substrate according to the radioactive signal of the series dilution spot.

D. *In vitro* processing assay of pre-U2.3-polyG snRNA

1. Carefully grind 2 g inflorescence to fine powder in liquid N₂ and pour the powder into 15 ml tube.
2. Set the tube on ice, add 8 ml M1 buffer, and invert several times.
3. Filter the solution through cell strainer (100 μ M) into a 50 ml tube.
4. Centrifuge at 12,000 \times g for 10 min at 4°C.
5. Discard the supernatant, add 4 ml M2 buffer, and mix by pipetting. Transfer the solution into a 5 ml tube. Wash with 3 ml M2 buffer and 3 ml M3 buffer, consecutively, each wash by spin 1 min at 13,200 \times g. Remove all of the solution with a small tip pipette.
6. Add 200 μ l processing buffer (without creatine phosphate) to suspend the pellet.
7. Take 10 μ l nuclear protein into 190 μ l Bradford reagent, let stand for 5 min, and measure the protein concentrations at 595 nm.
8. Dilute the nuclear protein of each sample to 50 ng/ μ l using processing buffer (without creatine phosphate).
9. For a total of 50 μ l reaction volume for each repeat, incubate 40 μ l of nuclear protein, 5 μ l of

labeled DNA, and 5 μ l of 200 mM creatine phosphate stock solution (20 mM final concentration) at 30°C. (The number of repeats can be varied. We did four repeats here).

10. Stop reaction by adding 450 μ l TRIzol at various time points (we stopped at 10, 30, 60, and 90 min reaction time points). RNAs were extracted following the manufacturer's instructions.
11. Dissolve the RNA pellet in 10 μ l DEPC water. Add 10 μ l formamide loading buffer to the solution and boil for 5 min.
12. Load half of the RNA extraction, as well as 0.5 μ l pre-U2.3 substrate (used as input) and 5 μ l RNA marker, onto a pre-run 6% denaturing polyacrylamide gel containing 6 M urea.
13. Run at 120 V in 1x TBE until the loading dye reaches 2/3 of the gel length.
14. Remove the gel from the electrophoresis apparatus after electrophoresis. Transfer gel onto plastic wrap carefully. Absorb solution with filter paper. Wrap the gel carefully using plastic wrap.
15. Place the plastic-wrapped gel on the storage phosphor imaging screen and expose for 6-10 h. The phosphor screen is then scanned with the PhosphorImager.
16. Export the image of the radioactive signal in the 'tiff' format (Figure 1).

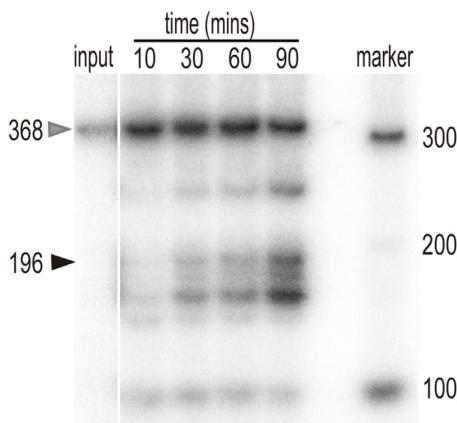


Figure 1. *In vitro* processing of pre-U2.3-polyG. *In vitro* transcribed pre-U2.3-polyG RNAs were processed in the nuclear protein extracts from *Arabidopsis* for various time points, as indicated at the top of the figure. After extraction, RNAs were resolved on a PAGE gel and detected with a PhosphorImager. The position of the pre-U2.3-polyG RNA is indicated by the grey arrow, while the processed mature snRNAs are indicated by the black arrow.

Data analysis

Band intensities were quantified with ImageJ or Quantity One software following these steps (we used Quantity One):

1. Open the gel image using Quantity One.
2. Open the 'Volume Tools' and select the 'Volume Rect Tool.' Select minimum areas of target bands from the image, including the processed mature snRNA and input (Figure 2).

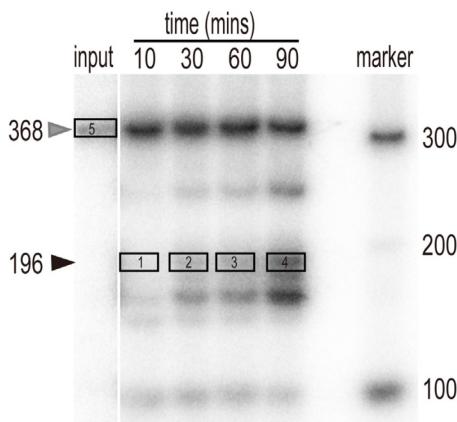


Figure 2. The selected frame of target bands in Quantity One software. Successively select the right processed snRNA bands of various reactions (frame 1-4), according to the relevant position of RNA markers and the pre-U2.3-polyG input (frame 5), using the 'Volume Rect Tool.'

3. Click the 'Volume Analysis Report' and select report options including area name, concentration, mean value, and density (Table 1).

Table 1. Intensity of relevant bands

Index	Band Name	Volume	Mean Value	Density
		INT*mm ²	INT	INT/mm ²
1	10 min	307.75	33.13	4621.05
2	30 min	476.26	58.59	8173.07
3	60 min	694.97	73.95	10315.99
4	90 min	1095.37	116.56	16259.60
5	Input	829.91	72.58	10125.62

4. Use the value of Density to quantify the relative processing efficiency. The intensities of the radioactive signals of various mature U2.3 were normalized to the input.

Recipes

1. Processing buffer
10 mM HEPES, pH 7.9
50 mM KCl
10% glycerol
20 mM creatine phosphate
3 mM MnCl₂
2.5% Polyvinyl alcohol
1 mM DTT

Add PMSF, Pepstatin A, and protease inhibitor just before use.

Note: Be sure to make the solution on the day of use.

2. M1 buffer

10 mM Phosphate buffer, pH 7.8

0.1 M NaCl

10 mM 2-Mercaptoethanol

1 M Hexylene glycol

Add PMSF, Pepstatin A, and protease inhibitor just before use.

3. M2 buffer

10 mM Phosphate buffer, pH 7.8

0.1 M NaCl

10 mM 2-mercaptopropanoic acid

1 M Hexylene glycol

10 mM MgCl₂

Add PMSF, Pepstatin A, and protease inhibitor just before use

4. M3 buffer

10 mM Phosphate buffer, pH 7.8

0.1 M NaCl

10 mM 2-Mercaptoethanol

Add PMSF, Pepstatin A, and protease inhibitor just before use

5. 2× formamide loading buffer

95% Deionized formamide

0.025% (w/v) Bromophenol blue

0.025% (w/v) Xylene cyanol FF

5 mM EDTA, pH 8.0

6. 6% denaturing polyacrylamide gel containing 6 M urea (10 ml)

2 ml 30% polyacrylamide solution (29:1)

Urea 3.6 g

1ml 5× TBE

9.9 ml water

80 μl 10% APS

10 μl TEMED

Before preparing the gel, clean all components with 70% ethanol and RNase away.

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Competing interests

The authors declare no competing interests.

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