Published in final edited form as:

Methods Mol Biol. 2018 ; 1823: 43–50. doi:10.1007/978-1-4939-8624-8_4.

***In Vitro* System for Coupling RNAP II Transcription to Primary microRNA Processing and a Three-way System for RNAP II Transcription/Splicing/microRNA Processing**

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Abstract

In the genome, primary microRNAs (pri-miRNAs) are encoded either as independent transcriptional units with their own promoters (intergenic miRNAs) or within the introns of other genes (intronic miRNAs). Here, we report two methods, one that we established for coupled RNAP II transcription and pri-miRNA processing and the other that is a three-way system for RNAP II transcription, pri-miRNA processing, and pre-mRNA splicing. In these systems, CMV-DNA constructs encoding the processing substrates are incubated in HeLa cell nuclear extracts in the presence of ^{32}P -UTP to generate the nascent RNAP II transcripts, which are processed efficiently by the endogenous RNA processing machineries in nuclear extracts.

Keywords

Primary microRNA processing; RNAP II transcription; pre-mRNA splicing; coupled steps in gene expression

Introduction

It is now well established that RNAP II transcription is functionally coupled to numerous steps in gene expression, including capping, splicing, polyadenylation, pri-miRNA processing, non-sense mediated decay and mRNA export^{1–13}. This coupling potently increases the rate and efficiency of the steps in gene expression and likely enhances the overall fidelity and efficiency of gene expression. MiRNAs are small, non-coding RNAs with critical roles in post-transcriptional control of gene expression^{14–17}. In mammalian cells, most miRNAs are transcribed by RNAP II to generate primary miRNAs (pri-miRNAs) transcripts¹⁴. The majority of pri-miRNAs are found within introns of other genes, and these intronic miRNAs are either processed directly from the intron or the intronic miRNAs have their own promoters. In some cases, pri-miRNAs are encoded as individual transcription units, and these intergenic miRNAs are transcribed by their own promoter. Pri-miRNAs are first processed in the nucleus by the microprocessor complex (Drosha and DGCR8) into ~70 nt hairpin precursor miRNAs (pre-miRNAs), which are then exported to the cytoplasm and

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further processed by Dicer to generate mature miRNAs^{18–22}. The steps of the pri-miRNA processing reaction were first elucidated using *in vitro* systems in which naked pri-miRNA transcripts generated by T7 RNA polymerase were processed in whole cell lysates or by purified Drosha/DGCR8^{20–22}. *In vivo* studies subsequently reported that pri-miRNA processing is coupled to RNAP II transcription²³. To understand the mechanisms underlying this coupling, we previously developed an *in vitro* system in which RNAP II transcription is functionally coupled to pri-miRNA processing¹². In addition, we have also developed a three-way system for RNAP II transcription/pri-miRNA processing/splicing²⁴. In the first system, pri-miRNA is transcribed by RNAP II and processed in HeLa cell nuclear extracts¹². In the second, a miRNA is present in the intron of a splicing substrate. The pre-mRNA containing the intronic miRNA is transcribed by RNAP II and then undergoes both splicing and pri-miRNA processing²⁴. These systems provide useful tools for investigating the mechanisms involved in linking RNAP II transcription, splicing and pri-miRNA processing.

2. Materials

All solutions are prepared using analytical grade reagents and ultrapure water (Milli-Q purified deionized water at a sensitivity of 18 MΩ cm at 25 °C). Storage temperature of each reagent is listed below.

2.1 Materials for preparation of CMV-DNA templates from plasmids

1. CMV-DNA plasmids encoding pri-let-7a-1 (CMV-let-7a) or FTZ pre-mRNA containing pri-let-7a in the intron (CMV-FTZ-let) should be stored at –20 °C in 1× TE buffer (Tris HCl, pH 8.0, 1 mM EDTA). Dilute the plasmids to a working concentration of 5 ng/μl before use.
2. CMV-DNA templates are generated by PCR. The PCR primers are CMV_F: 5'-TGGAGGTCGCTGAGTAGTGC-3' and CMV_R: 5'-TAGAAGGCACAGTCGAGG-3'. Make 100 μl aliquots at a final concentration of 10 μM and store at –20 °C.
3. 10 mM dNTPs. Store at –20 °C.
4. 50 mM MgSO₄. Store at –20 °C.
5. Platinum Taq HiFi and 10× HiFi Buffer provided by supplier (Invitrogen). Store at –20 °C.
6. 10× TBE: Combine 432 g Tris Base, 220 g boric acid, and 37.2 g EDTA. Add water to a final volume of 4 liters. Store at room temperature.
7. 10 mg/ml ethidium bromide in H₂O. Ethidium bromide is light sensitive, so store in a dark container.
8. Agarose HS standard/high melt. Store at room temperature.
9. 3 M sodium acetate. Store at room temperature.
10. 100 % and 70 % ethanol diluted from 200 proof pure ethanol. Store at room temperature.

11. Phenol/chloroform, pH 7.9. Store at 4 °C.
12. 1 kb DNA ladder marker. Store at –20 °C.

2.2 Materials for Coupled Transcription/Pri-miRNA Processing Reaction

1. 12.5 mM ATP. Filter, make 100 µl aliquots, and store at –20 °C.
2. 0.5 M Creatine Phosphate di-Tris salt: Filter, prepare 100 µl aliquots, and store at –20 °C.
3. 80 mM MgCl₂: Filter, prepare 100 µl aliquots and store at –20 °C.
4. CMV-DNA templates: Make 50 µl aliquots at 200 ng/µl. Store at –20 °C.
5. ³²P-UTP (800 Ci/mmol, 250 µCi). Store at 4 °C.
6. HeLa cell nuclear extract (*see* Note 1). Store at –80 °C.
7. α-Amanitin: Dilute to 10 ng/µl with water from 1 mg/ml stock. Store at –20 °C. Prepare 100 µl aliquots, and use a fresh aliquot for each experiment.
8. Glycogen (Roche), 20 mg/ml. Store at –20 °C.
9. Formamide gel-loading dye: Combine 16 ml formamide, 0.4 ml 0.5 M EDTA, 0.8 ml 2.5 % xylene cyanol, and 0.8 ml 2.5 % bromophenol blue. Mix well and make 1 ml aliquots. Store at –20 °C.

2.3 Denaturing Polyacrylamide Gel

1. Denaturing gel solution (15 %). Mix 215 g urea with 50 ml of 10× TBE and 187.5 ml 40 % acrylamide–bisacrylamide (w/v 29:1). Bring the volume up to 500 ml with water. Filter and store at 4 °C.
2. N, N, N', N'-Tetramethylethylenediamine (TEMED). Store at 4 °C.
3. 10 % ammonium persulfate (APS). Store at 4 °C.
4. Model V16 polyacrylamide gel electrophoresis system, PROTEAN II xi spacers 0.5 mm, PROTEAN II xi comb (Bio-Rad).
5. Gel-loading tips (Bioexpress): Flat orifice, 83 mm × 0.33 mm diameter.
6. Whatman paper 3 MM Chr.
7. Gel dryer vacuum system.
8. PhosphorImager cassette: Mounted General Purpose, 20 cm × 25 cm, screen and cassette or equivalent.
9. PhosphorImager

¹In some preparations of nuclear extract, RNAP II transcribes end to end in a promoter-independent manner. If you are using your own DNA template, use the smallest possible PCR fragment that contains your sequence of interest to avoid large end-to-end transcription products.

3. Methods

Carry out all procedures on ice unless otherwise specified.

3.1 Preparation of CMV-DNA templates

1. PCR reaction: Mix 2 μ l of the CMV-DNA plasmid (CMV-let-7a or CMV-FTZ-let) (*see Note 1*), 2 μ l 10 mM dNTPs, 2 μ l 50 mM MgSO₄, 5 μ l 10 $\times$ HiFi Buffer, 5.0 μ l of each primer, 28.6 μ l of Milli-Q water, and 0.4 μ l Platinum Taq HiFi. Start the PCR reaction at 94 $^{\circ}$ C for 5 min followed by 32 cycles at 94 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, and 68 $^{\circ}$ C for 2 min.
2. When the PCR reaction is finished, bring the volume up to 150 μ l with 1 $\times$ TE buffer and check the CMV-DNA template by running a small aliquot (1 μ l) on a mini-agarose gel next to the 1 kb DNA size marker.
3. Ethanol precipitate the CMV-DNA template. To do this, add 2 μ l of glycogen; mix well. Add 1/10 volume 3 M sodium acetate; mix well. Add 3 volumes of 100 % ethanol. Centrifuge at 16,000 $\times$ g for 15 min to pellet the DNA. Remove the supernatant, without disturbing the pellet. Wash once with 1 ml 70 % ethanol. Air-dry the pellet and dissolve it in 100 μ l of water.
4. Determine the concentration of the CMV-DNA template using a NanoDropTM spectrophotometer or a UV spectrophotometer. Confirm the size and quality of the DNA by running 2 μ l on an agarose gel next to the 1 kb DNA size marker.

3.2 Coupled RNAP II transcription/pri-miRNA processing system

1. Preheat two water baths, one at 30 $^{\circ}$ C and another at 37 $^{\circ}$ C.
2. For assembly of pre-initiation complexes (PICs), incubate the CMV-let-7a DNA template (4 μ ls containing 800 ng DNA) with 1 μ l MgCl₂ (80 mM), 3 μ l 15% polyvinyl alcohol (PVA) and 15 μ l HeLa nuclear extract (*see Note 2*) at 30 $^{\circ}$ C for 20 min.
3. To initiate RNAP II transcription, add 1 μ l ³²P-UTP (800 Ci/mmol; Perkin Elmer Life Sciences), 1 μ l ATP (12.5 mM) and 1 μ l Creatine Phosphate di-Tris salt (500 mM) to the reaction mixture.
4. After transcription for 5 min at 30 $^{\circ}$ C, add 1 μ l of α -amanitin (10 ng/ μ l) per 25 μ l reaction mixture, and mix gently.
5. Place 5 μ l of the reaction mixtures in a new tube and add 18.2 μ l fresh nuclear extract and 1.8 μ l 80 mM MgCl₂ to allow pri-miRNA processing at 37 $^{\circ}$ C for specified times indicated in Figure 1.
6. Remove a 4 μ l aliquot of the reaction mixture at each time point and transfer it to a microfuge tube containing 1 ml TRIzol (Invitrogen) (*see Note 3*).

²-HeLa nuclear extract was prepared as described²⁶except that the final centrifugation following dialysis was omitted, which results in increased efficiency of RNAP II transcription.

7. Repeat step 6 for all subsequent time points.
8. Add 200 μ l of chloroform pH 6.6 to each sample and mix well by vortexing. Centrifuge for 15 min at $16,000 \times g$.
9. Transfer 400 μ l of the aqueous phase to a new tube containing 2 μ l of glycogen. Mix by vortexing.
10. Add 500 μ l of 100 % isopropanol and mix by vortexing.
11. Spin at $16,000 \times g$ for 15 min. The RNA pellet should look white/translucent.
12. Carefully remove the supernatant using a pipet.
13. Do a quick spin at $16,000 \times g$ and remove any remaining supernatant using a P200 pipetman (*see* Note 4).
14. Resuspend the pellet in 15 μ l formamide gel loading dye. Heat samples in hot water (75–90°C) for 10 min.
15. Prepare a gel by combining 15 ml of 8 % denaturing gel solution with 15 μ l TEMED and 150 μ l 10 % APS. Pour the gel and wait 10 min until it is polymerized.
16. Load 7.5 μ l of each sample on the denaturing gel. Run the gel at 20 mAmps, constant current for 45–60 min.
17. Transfer the gel to Whatman paper and dry the gel on a gel dryer for 30 min at 80°C.
18. Put the gel in a PhosphorImager cassette, expose 1–12 hr, and scan the gel using a PhosphorImager.

3.3 Coupled RNAP II transcription/ splicing/pri-miRNA processing system

1. Preheat a water bath to 30 °C.
2. For assembly of pre-initiation complexes (PICs), incubate the CMV-FTZ-let DNA template (4 μ l containing 800 ng DNA) with 1 μ l $MgCl_2$ (80 mM), 3 μ l 15% polyvinyl alcohol (PVA) and 15 μ l HeLa nuclear extract at 30°C for 20 min.
3. To initiate RNAP II transcription, add 1 μ l ^{32}P -UTP (800 Ci/mmol; Perkin Elmer Life Sciences), 1 μ l ATP (12.5 mM) and 1 μ l Creatine phosphate di-Tris salt (500 mM) to the reaction mixture.
4. After transcription for 10 min at 30 °C, add 1 μ l of 10 ng/ μ l α -amanitin and 1 μ l of $MgCl_2$ (80 mM) per 25 μ l reaction mixture, and mix gently.
5. Incubate the reaction mixtures at 30°C for specified times indicated in Figure 2.

³. Samples in TRIzol are stable at room temperature and can be stored until all of the time points have been collected. At this step, samples can also be stored at 4 °C overnight and processed later.

⁴. Ensure that the pellets are dry before adding the formamide loading dye because any remaining ethanol will add to the volume of the sample as well as distort migration of the bands on the gel.

6. Follow steps 6–18 in Subheading 3.2. For step 16, run the gel at 20 mAmps, constant current for 60 or 120 min.

Acknowledgements

We are grateful to members of the Reed lab for comments on the manuscript. This work was supported by an NIH grant GM122524 to RR.

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Figure 1.

Coupled RNAP II transcription/pri-miRNA processing in vitro. (A) Structure of the CMV-let-7a DNA template. The sizes of 5' flanking region, 3' flanking region, and pre-let-7a are indicated. The thick line indicates the natural pri-miRNA sequences and the thin lines indicate the vector sequences. (B) CMV-let-7a DNA template was incubated in nuclear extract for 20 min to assemble a pre-initiation complex followed by addition of ^{32}P -UTP, ATP, and Creatine Phosphate and continued incubation for 5 min. α -Amanitin was added to stop transcription and incubation was continued for the indicated times. Pri-miRNAs, pre-miRNAs and 5'/3' flanks are indicated. The endogenous U6 snRNA and tRNA in the extract are labeled by ^{32}P -UTP²⁵ Markers (in base pairs) are indicated, and Ori indicates the gel origin. (C) A dark exposure of (B).

Figure 2.

Coupled RNAP II transcription/splicing/pri-miRNA processing in vitro. (A) Structure of the CMV-FTZ-let DNA template. The sizes of FTZ pre-mRNA exons, intron, 5' flanking region, 3' flanking region, and pre-let-7a are indicated. The thick line indicates pri-miRNA sequences and the thin lines indicate intron sequences. (B) CMV-FTZ-let DNA template was incubated in nuclear extract for 20 min to assemble a pre-initiation complex followed by addition of ^{32}P -UTP, ATP, and Creatine Phosphate and continued incubation for 10 min. α -Amanitin was added to stop transcription and incubation was continued for the indicated times. Full length transcript, spliced mRNA, exon1, pre-miRNAs and 5'/3' flanks are indicated. Markers (in base pairs) are indicated and Ori indicates the gel origin. Samples were run for 1 hour. (C) Same as (B), except that samples were run for 2 hours to obtain a better separation of pre-mRNA and 5'/3' flanks.