In Vitro Transcribed RNA-based Luciferase Reporter Assay to Study Translation Regulation in Poxvirus-infected Cells

Pragyesh Dhungel¹, Fernando Cantu¹, Candy Hernandez¹, Zhilong Yang¹

¹Division of Biology, Kansas State University

Abstract

Every poxvirus mRNA transcribed after viral DNA replication has an evolutionarily conserved, non-templated 5'-poly(A) leader in the 5'-UTR. To dissect the role of 5'-poly(A) leader in mRNA translation during poxvirus infection we developed an in vitro transcribed RNA-based luciferase reporter assay. This reporter assay comprises of four core steps: (1) PCR to amplify the DNA template for in vitro transcription; (2) in vitro transcription to generate mRNA using T7 RNA polymerase; (3) Transfection to introduce in vitro transcribed mRNA into cells; (4) Detection of luciferase activity as the indicator of translation. The RNA-based luciferase reporter assay described here circumvents issues of plasmid replication in poxvirus-infected cells and cryptic transcription from the plasmid. This protocol can be used to determine translation regulation by *cis*-elements in an mRNA including 5'-UTR and 3'-UTR in systems other than poxvirus-infected cells. Moreover, different modes of translation initiation like cap-dependent, cap-independent, re-initiation, and internal initiation can be investigated using this method.

Keywords

Immunology and Infection; Issue 147; In vitro Transcription; Luciferase assay; Vaccinia virus; Poxvirus; Translation; 5'-UTR; 5'-poly(A) leader

Introduction

According to the central dogma, genetic information flows from DNA to RNA and then finally to protein^{1,2}. This flow of genetic information is highly regulated at many levels including mRNA translation^{3,4}. Development of reporter assays to measure regulation of gene expression will facilitate understanding of regulatory mechanisms involved in this process. Here we describe a protocol to study mRNA translation using an in vitro transcribed RNA-based luciferase reporter assay in poxvirus-infected cells.

Poxviruses comprise many highly dangerous human and animal pathogens⁵. Like all other viruses, poxviruses exclusively rely on host cell machinery for protein synthesis^{6,7,8}. To

Correspondence to: Zhilong Yang at zyang@ksu.edu.

Disclosures

The authors would like to declare no competing financial interest.

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efficiently synthesize viral proteins, viruses evolved many strategies to hijack cellular translational machinery to redirect it for translation of viral mRNAs^{7,8}. One commonly employed mechanism by viruses is to use *cis*-acting elements in their transcripts. Notable examples include the Internal Ribosome Entry Site (IRES) and cap-independent translation enhancer (CITE)^{9,10,11}. These *cis*-elements render the viral transcripts a translational advantage by attracting translational machinery via diverse mechanisms^{12,13,14}. Over 100 poxvirus mRNAs have an evolutionarily conserved *cis*-acting element in the 5'-untranslated region (5'-UTR): a 5'-poly(A) leader at the very 5' ends of these mRNAs^{15,16}. The lengths of these 5'-poly(A) leaders are heterogeneous and are generated by slippage of the poxvirus-encoded RNA polymerase during transcription^{17,18}. We, and others, recently discovered that the 5'-poly(A) leader confers a translation advantage to an mRNA in cells infected with vaccinia virus (VACV), the prototypic member of poxviruses^{19,20}.

The in vitro transcribed RNA-based luciferase reporter assay was initially developed to understand the role of 5'-poly(A) leader in mRNA translation during poxvirus infection^{19,21}. Although plasmid DNA-based luciferase reporter assays have been widely used, there are several drawbacks that will complicate the result interpretation in poxvirus-infected cells. First, plasmids are able to replicate in VACV-infected cells²². Second, cryptic transcription often occurs from plasmid DNA^{18,23,24}. Third, VACV promoter-driven transcription generates poly(A)-leader of heterogeneous lengths consequently making it difficult to control the poly(A)-leader length in some experiments¹⁸. An in vitro transcribed RNA-based luciferase reporter assay circumvents these issues and the data interpretation is straightforward.

There are four key steps in this method: (1) polymerase chain reaction (PCR) to generate the DNA template for in vitro transcription; (2) in vitro transcription to generate mRNA; (3) transfection to deliver mRNA into cells; and (4) detection of luciferase activity as indicator of translation (Figure 1). The resulting PCR amplicon contains the following elements in 5' to 3' direction: T7-Promoter, poly(A) leader or desired 5'-UTR sequence, firefly luciferase open reading frame (ORF) followed by a poly(A) tail. PCR amplicon is used as the template to synthesize mRNA by in vitro transcription using T7 polymerase. During in vitro transcription, m⁷G cap or other cap analog is incorporated in newly synthesized mRNA. The capped transcripts are transfected into uninfected or VACV-infected cells. The cell lysate is collected at the desired time after transfection to measure luciferase activities that indicate protein production from transfected mRNA. This reporter assay can be used to study translation regulation by *cis*-element present in 5'-UTR, 3'-UTR or other regions of an mRNA. Furthermore, the in vitro transcribed RNA-based assay can be used to study different mechanisms of translation initiation including cap-dependent initiation, cap-independent initiation, re-initiation and internal initiation like IRES.

Protocol

1. Preparation of DNA Template by PCR for In Vitro Transcription

1. To prepare the DNA template by PCR, design primers. When designing primers consider crucial characteristics like primer length, annealing temperature (T_m), GC content, 3' end with G or C etc.

NOTE: Discussed in detail in these literature^{25,26,27}.

2. Design primers to generate PCR amplicon containing the following elements in 5' to 3' direction: T7-Promoter, poly(A) leader, firefly luciferase ORF and a poly(A) tail referred hereafter to as T7_12A-Fluc. Design primers (Forward and Reverse) to encompass all the additional elements not present in the template DNA (Figure 2A).

NOTE: The sequence of all elements can be found in Table 1.

3. Include several extra nucleotides in forward primer (5'–3')²⁸, followed by T7-promoter, poly(A) leader or desired 5'-UTR sequence and approximately 20 nucleotides, adjust based on T_m , corresponding to the 5' end of the reporter gene's ORF. Ensure the corresponding region in the primer is identical to the sense strand (+ strand) of the gene.

NOTE: For long 5'-UTR, synthesize two DNA fragments: one with T7 promoter followed by long 5'-UTR and second with reporter gene's ORF. Join these two fragments using overlap extension PCR²⁹.

4. Design reverse primer (5'–3') to include poly(A) tail and approximately 20 nucleotides, adjust based on T_m , corresponding to the 3' end of the reporter gene's ORF. Ensure the corresponding region in the primer is identical to the anti-sense strand (- strand) of the gene and an in-frame stop codon is present before the poly(A) tail.

NOTE: The desired length of A's in a poly(A) leader or poly(A) tail can be customized in the primers. For example, to add 50 A's in the poly(A) tail, the reverse primer should entail 50 T's. Similarly, to add 20 A's in the poly(A) leader, the forward primer should entail 20 A's.

5. For internal control, design another set of primers containing the following elements in 5' to 3' direction: T7 Promoter, a random 5'-UTR coding sequence containing Kozak sequence, *Renilla* luciferase ORF and poly(A) tail referred hereafter to as T7_Kozak-Rluc.
6. In a PCR tube, add the reagents in the following order: DNase free water, 2x high-fidelity DNA polymerase, primers, and sequence confirmed luciferase template DNA (Table 2).

NOTE: Amounts of individual components in the mixture should be adjusted according to the reaction volume.

7. Use a standard 3-step (Denaturation, Annealing, Extension) PCR cycle to generate a DNA template as shown in Table 3.

NOTE: Annealing temperature X °C depends on the primer set being used and extension time T min depend on the PCR amplicon size and DNA polymerase used.

8. Detect the PCR product by running 5–10% of PCR reaction in 1% agarose Tris-acetate-EDTA (TAE) gel electrophoresis (containing 0.1 µg/ml ethidium

bromide) along with commercially available molecular weight standard. Visualize the gel under a UV illuminator to determine the size of the PCR product.

9. After determining the correct size of the PCR product, ~1.7 kb for T7_12A-Fluc and ~1.0 kb for T7_Kozak-Rluc, purify it using a commercially available PCR purification kit. Elute the DNA using 100 µL nuclease free water (Figure 2B).
10. Once purified, check the concentration of the DNA using a spectrophotometer and determine the A260/A280 ratio (~1.8–2.0 is acceptable).
11. Store purified DNA at –20 °C or use for in vitro transcription immediately.

2. Generate mRNA by In Vitro Transcription

1. Synthesize RNA from the PCR product in vitro, using an in vitro transcription kit (Figure 3A).

NOTE: T7_12A-Fluc and T7_Kozak-Rluc DNA templates are used to synthesize 12A-Fluc and Kozak-Rluc mRNAs, respectively.

1. To do this, take a microcentrifuge tube and add the reagents in the following order: DNase-RNase free water, NTP Buffer Mix, Cap Analog, Template PCR Product, T7-RNA polymerase Mix (Table 4).

NOTE: Other capping systems can also be used to cap RNA sequentially after in vitro transcription following the manufacturer's instruction.

2. Mix thoroughly and incubate at 37 °C for 2 h.
3. Proceed to the purification of the synthesized RNA using an RNA purification kit.
2. Run purified RNA in 1.5% agarose Tris-borate-EDTA (TBE) gel (containing 0.5 µg/mL ethidium bromide) to check the RNA. Visualize the gel under a UV illuminator (Figure 3B).
3. Check the concentration of the RNA using a spectrophotometer and determine the A260/A280 ratio (~1.8–2.0 is acceptable).
4. Aliquot the purified RNA and store at –80 °C.

3. Transfect mRNA to Cells

1. Seed HeLa cells in a 24-well plate (to be approx. ~80–90% confluent next day) and incubate overnight in an incubator at 37 °C with 5% CO₂.
2. Infect HeLa cells with vaccinia virus (VACV) at a Multiplicity of Infection (MOI) of 5 or keep uninfected HeLa cells for comparison.
NOTE: MOI is the number of infectious viral particles per cell.
3. MOI of X = $\{[(\text{Number of cells} \times X) / \text{Virus Titer}] \times 1000\}$ µl of virus per 1 mL medium.

4. After desired h post infection (hpi) (in this experiment at 10–12 hpi), transfect mRNA (500 ng of total mRNA per well of 24-well plates) using a cationic lipid transfection reagent as shown in Figure 3C.
 1. For one well of a 24-well plate, mix 480 ng of 12A sequence bearing firefly luciferase (12A-Fluc) mRNA and 20 ng of Kozak sequence bearing *Renilla* luciferase (Kozak-Rluc) mRNA in one microcentrifuge tube. In another microcentrifuge tube add 1.1 03BCL of cationic lipid transfection reagent.
 2. Add 55 μ L of reduced serum medium in both tubes. Mix and incubate at room temperature for 5 min.
 3. After 5 min of incubation, add 55 μ L cationic lipid transfection reagent containing reduced serum medium in mRNA containing tube.
 4. Mix gently but thoroughly, and incubate at room temperature for 15 min.
 5. During the incubation, remove the cell culture medium and add 400 μ L of reduced serum medium per well of 24-well plates.
 6. After incubation, add 100 μ L of the mixture dropwise and evenly to one well of 24-well plates.

4. Measure Luciferase Activities

1. Five-hours post-co-transfection of 12A-Fluc and Kozak-Rluc mRNA, measure luciferase activity using a luciferase assay system capable of performing two reporter assays (e.g., Dual Luciferase Reporter assay kit).
2. Remove the reduced serum medium and lyse the cells by adding 150 μ L 1x lysis buffer, a component of the luciferase assay kit.
3. After 10 min incubation at room temperature, collect the lysate by scrapping the cells and transfer to a microcentrifuge tube.
4. Centrifuge the lysate at 12,000 $\times g$ for 10 min at 4 °C to pellet cell debris.
5. Add 30 μ L of supernatant in opaque-walled 96 well white assay plate with a solid bottom.
6. Measure the dual luminescence using the luciferase assay kit and a multimode plate reader luminometer.
7. Perform the measurement using kinetics function (on a per-well basis) using the settings described in Table 5.

NOTE: The reading can also be taken using manual luminometer. Add an equal volume of lysate and substrate for Fluc in a cuvette. Wait for 2 s and measure for 10 s using luminometer. Following Fluc measurement, quickly take out cuvette from luminometer and add an equal volume of the substrate for Rluc manually. Again, wait for 2 s and measure for 10 s using luminometer.

8. Export the luminescence reading data into a desirable file format.
9. Determine relative translation rate from 12A-Fluc mRNA in uninfected and VACV infected HeLa cells by dividing Fluc value by internal control Rluc value.

NOTE: Supplementary Figure 1 shows the step-by-step analysis of raw data to get relative Fluc activity.

Representative Results

The four steps of in vitro transcribed RNA-based luciferase reporter assay: PCR to generate DNA template for In vitro transcription, in vitro transcription to generate mRNA, mRNA transfection, and luciferase measurement, can be seen in the schematic diagram (Figure 1). Designing of primers for both DNA templates (Fluc and Rluc) and the general scheme of overhang extension PCR is illustrated in the schematic (Figure 2A). After PCR, the correct sized PCR product was detected by TAE agarose gel electrophoresis (Figure 2B). Subsequently, the PCR product is used as the template to synthesize RNA in vitro (Figure 3A), which is purified and run in TBE gel electrophoresis to verify the size (Figure 3B). The purified and verified mRNA is transfected into cells using cationic lipid transfection reagent (Figure 3C). Primers used in this protocol are listed in Table 6.

The in vitro transcribed RNA-based luciferase reporter assay was developed to understand the role of 5'-poly(A) leader in mRNA translation during poxvirus infection. Using this assay, we tested the translation efficiency of a Fluc mRNA that contains a 5'-poly(A) leader (12 nt) in uninfected and VACV-infected cells. The Fluc value was normalized using Rluc value in both uninfected and VACV-infected cells to determine the relative Fluc activity (i.e. Fluc activity/Rluc activity) (Figure 4A). The division of Fluc by Rluc normalized the transfection efficiency and RNA stability in a particular well. Using this analysis approach, we determined that a 5'-poly(A) leader containing mRNA has a translational advantage during VACV infection (Figure 4B). The advantage in infected cells was not due to differential transfection efficiency or mRNA stability as the RNA level was similar in uninfected and VACV infected cells 5 h post mRNA transfection¹⁹.

Discussion

All four-core steps are critical to the success of the in vitro transcribed RNA-based luciferase reporter assay. Special attention should be given to primer design, especially for the T7 promoter sequence. T7 RNA polymerase starts transcription from the underlined first G (GGG-5'-UTR-AUG-) in T7 promoter added before the 5'-UTR sequence. Although the transcription start site (TSS) starts from the first G at the 5' end, decreasing the number of G's less than three in T7 promoter region decreased the RNA yield/output from in vitro transcription. During the experiment, we observed that gel purified DNA product was not the best for in vitro transcription as both yield and quality of RNA were lower. We only ran 5–10% of the PCR reaction in 1% agarose gel electrophoresis to determine the size and purified the rest 90–95%, using a PCR purification kit, to be used for in vitro transcription. In the case of non-specific amplification from PCR, cutting the desired sized band from gel

and using gel-purified DNA fragment is recommended. As the yield might be low, we suggest increasing the reaction volume for the in vitro transcription reaction.

Similar to other transfection-based methods, DNA/RNA may stimulate DNA/RNA sensing pathways that may globally or selectively suppress translation. Therefore, data should be interpreted with cautions, although we did not experience problems potentially caused by this issue in our experiments.

The proposed method is suitable for use in different model systems with some modifications like the method of mRNA delivery, internal control to be used, a suitable time for translation, sample preparation and analysis of data. The main limitation of this method is that it is a reporter assay to quickly test translation regulation by *cis* elements that do not completely reflect physiological conditions. Therefore, this method should be corroborated by other complementary experiments, if possible.

Compared to DNA modification, the roles of RNA modifications are less well understood. However, with the discovery of enzymes that write, read and erase RNA modifications^{30,31,32,33,34,35}, it is now possible to study the influence of RNA modification in gene expression^{30,31,32,33,34,35}. The in vitro transcribed RNA-based luciferase reporter assay may be modified to incorporate different RNA modifications and used to test their effects on RNA translation. For example, this method can incorporate different cap analogs that have various modifications^{30,31}. Additionally, supplementing an internal RNA modifying enzyme during or after in vitro transcription can possibly incorporate internal RNA modification. Addition of a modification to cap 0, cap 1, and an internal RNA modification will provide a tool to study the roles of these RNA modifications in translation.

The in vitro transcribed RNA-based luciferase reporter assay has great potential and broad application in understanding basic biology about RNA translation. Different mechanisms for the initiation of translation, including cap-dependent initiation, cap-independent initiation, re-initiation and internal initiation such as IRES can be studied using this method. On top of these advantages, this assay can be employed to test translation regulation by *cis*-elements at 5'-UTR and 3'-UTR in an mRNA. The described protocol uses the PCR product, which provides the advantage to avoid lengthy cloning and quickly examine the effects of RNA elements on translation. To minimize potential errors during PCR, high fidelity polymerase and low PCR cycle number should be used. Alternatively, if a template is used frequently, the desired 5'-UTR and luciferase ORF can be cloned into a plasmid as the template of in vitro transcription. Together, the protocol consolidates transcription and mRNA capping in a single reaction and utilizes conventional transfection and analysis that make in vitro transcribed RNA-based luciferase reporter assay a userfriendly, quick, and straightforward method to study mechanisms of mRNA translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The project was funded by the National Institutes of Health (AI128406 www.nih.gov) to ZY and in part by Johnson Cancer Research Center (<http://cancer.k-state.edu>) in the form of Graduate Student Summer Stipend to PD.

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Figure 1: Schematic of the experimental procedure.

PCR is used to generate a DNA template with the desired elements. mRNA encoding a luciferase reporter gene is synthesized in vitro using a T7 RNA polymerase-based system. A Firefly luciferase (Fluc) mRNA is co-transfected with a Renilla luciferase (Rluc) mRNA into uninfected or VACV-infected cells. Luciferase activities are measured using a luminometer with dual luciferase capability.

Figure 2: Primer design and PCR-based DNA amplification.

(A) The forward primer is synthesized to include an 8nt random sequence, T7 promoter followed by a desired 5'-UTR and part of the 5' end of the luciferase reporter gene, while the reverse primer includes a T-tract to generate poly(A) tail and the 3' end of the luciferase reporter gene. By overhang extension PCR using a plasmid template containing a luciferase gene, a DNA template is generated. (B) DNA band of the desired size from PCR reaction was detected using 1% agarose TAE gel electrophoresis.

Figure 3: mRNA synthesis and transfection.

(A) Schematic of in vitro transcription. DNA amplified by PCR containing the luciferase gene downstream from the 5'-UTR of interest and the T7 promoter is used as the template. The T7 RNA polymerase is recruited to the promoter and adds ribonucleotides, shown in white, from 5' to 3' direction. Once mRNA is 25–30 nt long, m⁷G cap is added using an anti-reverse cap analog, ARCA. (B) RNA bands from in vitro transcription were detected using 1.5% agarose TBE gel electrophoresis. (C) Schematic demonstrating the transfection of reporter mRNA into cells. Medium containing either the reporter mRNA or cationic lipid transfection reagent in separate tubes is allowed to equilibrate at room temperature for 5 min. The solutions are then mixed followed by incubation at room temperature for 15 min after which the RNA/transfection reagent mixture is added into cells in culture plates.

Figure 4: Increased translational efficiency of mRNA containing a 5'-poly(A) leader. (A) Fluc mRNA containing a poly(A) leader in the 5'-UTR and Rluc mRNA with the Kozak consensus sequence in the 5'-UTR are co-transfected into cells. (B) Fluc mRNA with 5'-poly(A) leader was transfected in uninfected and VACV-infected cells along with Rluc mRNA. 5 hpi, luciferase activity was measured using a luminometer. Rluc normalized Fluc activity is represented in uninfected and VACV-infected cells. Error bars indicate the standard deviations (SD) of at least three repeats. Student's t-test was used to determine P-values; ***P-value < 0.001.

Table 1:

Sequences used in the method – the table contains the sequences of the T7 promoter, poly(A) leader, Kozak sequence, poly(A) tail.

Elements	Sequence
T7 Promoter	TAATACGACTCACTATAGGG
Poly(A) leader	AAAAAAAAAAAAA, ranging from 3 to 51 As
Kozak sequence	GCCACC
Poly(A) tail	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA.....

Table 2:

PCR reaction – the order and the volume of components added in the PCR reaction.

Components	Volume
DNase free water:	38 μ L
2X High-fidelity DNA polymerase Master mix:	50 μ L
Forward Primer (10 μ M):	4 μ l
Reverse Primer (10 μ M):	4 μ l
Luciferase DNA Template (1–10 ng/ μ L):	4 μ l
Total:	100 μL
Source for Fluc DNA template is pGL3-Fluc plasmid	
Source for Rluc DNA template is pRL-Rluc plasmid	

Table 3:

PCR Program – the steps for PCR program along with temperature, time and cycle.

Step	Temperature	Time	Cycle
Initial denaturation	95 °C	2 min	(1x Cycle)
Denaturation	95 °C:	15 s	
Annealing	X °C:	30 s	(25x Cycle)
Extension	72 °C:	T min	
Final Extension	72 °C:	7 min	(1x Cycle)
Hold	4 °C:	∞	

Table 4:

In vitro transcription reaction – the order and the volume of components added for in vitro transcription reaction.

Components	Volume	
DNase-RNase free water:	up to 20 μ L	
NTP Buffer Mix (20 mM of each rNTP):	2 μ L	
Cap Analog (40 mM):	4 μ L	
Template PCR Product (400 ng) [*] :	X μ L	(PCR Product Concentration dependent)
T7-RNA polymerase Mix:	2 μ L	
Total:	20 μL	

^{*} T7_12A-Fluc and T7_Kozak-Rluc template used to synthesize 12A-Fluc and Kozak-Rluc mRNA, respectively.

Table 5:

Luciferase Measurement Settings – the steps for luciferase measurement with the recommended volume or time.

Steps	Volume/Time
Inject Luciferase Assay Substrate (Fluc):	30 μ L
Wait / Incubation time:	2 s
Luminescence Measurement (Fluc):	10 s
Stop & Glo Substrate (Rluc):	30 μ L
Wait / Incubation time:	2 s
Luminescence Measurement (Rluc):	10 s

Table 6:

Primers – the primers used in this method with complete sequences.

Primers	Sequence
T7-12A-Fluc Forward	ATCGACGATAATACGACTCACTATAGGGGaaaaaaaaaaa ATGGAAGACGCCAAAAACATAAAG
T7-12A-Fluc Reverse	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACACGGCGATCTTCCGC
T7-Kozak-Rluc Forward	ATCGACGATAATACGACTCACTATAGGGGatcgtagccacc ATGACTTCGAAAGTTTATGATC
T7-Kozak-Rluc Reverse	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTATGTTTCATTTT GAGAACTCGCTC