Video Article

Toeprinting Analysis of Translation Initiation Complex Formation on Mammalian mRNAs

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Abstract

Translation initiation is the rate-limiting step of protein synthesis and represents a key point at which cells regulate their protein output. Regulation of protein synthesis is the key to cellular stress-response, and dysregulation is central to many disease states, such as cancer. For instance, although cellular stress leads to the inhibition of global translation by attenuating cap-dependent initiation, certain stress-response proteins are selectively translated in a cap-independent manner. Discreet RNA regulatory elements, such as cellular internal ribosome entry sites (IRESes), allow for the translation of these specific mRNAs. Identification of such mRNAs, and the characterization of their regulatory mechanisms, have been a key area in molecular biology. Toeprinting is a method for the study of RNA structure and function as it pertains to translation initiation. The goal of toeprinting is to assess the ability of *in vitro* transcribed RNA to form stable complexes with ribosomes under a variety of conditions, in order to determine which sequences, structural elements, or accessory factors are involved in ribosome binding—a precursor for efficient translation initiation. Alongside other techniques, such as western analysis and polysome profiling, toeprinting allows for a robust characterization of mechanisms for the regulation of translation initiation.

Video Link

The video component of this article can be found at https://www.jove.com/video/57519/

Introduction

As translation consumes most cellular energy, it makes sense that translation is tightly regulated[1]. Conversely, dysregulation of translation-and the consequent alterations in protein output-is often observed in stress-response and disease states, such as cancer[1,2]. A major advantage of translational control is the speed with which cells can alter their protein output in order to respond to various stimuli[3]. Translation regulation thus represents an important mechanism that can influence cell survival and death[1,2,3]. Of the steps of translation, initiation is the most highly regulated and complex[3]. Briefly, most eukaryotic mRNAs contain a 5' m^7G cap that is almost always essential for their translation. Cap-dependent initiation requires eukaryotic initiation factors eIF4E, eIF4A, and eIF4G (the cap-recognition complex) to interact with the 5' end of the mRNA. The 43S preinitiation ribosome complex, which contains eIF2-bound initiator tRNA and eIF3, is recruited to the 5' end of the mRNA *via* an interaction of eIF4G with eIF3. The preinitiation complex is thought to scan mRNA, aided by eIF4A (an RNA helicase) until the start codon (AUG) is located. The 48S initiation complex is subsequently formed and tRNA is delivered into the P-site of the ribosome. Finally, the 60S and 40S ribosome subunits are united to form the 80S initiation complex, followed by translation elongation[1,3,4]. In contrast, internal ribosome entry sites (IRESes) bypass the requirement for a 5' cap by recruiting the 40S ribosomal subunit directly to the initiation codon[3]. Physiological stress conditions attenuate global mRNA translation due to modifications of key general eukaryotic initiation factors (eIFs). However, non-canonical translation initiation mechanisms allow for selective translation of certain mRNAs which often encode stress-response proteins, and dysregulation of non-canonical translation initiation is implicated in disease states like cancer[1,2]. Discreet RNA regulatory elements, such as cellular IRESes, allow for the translation of such mRNAs[2,3].

One particularly interesting aspect of translational control is to understand mechanisms of canonical versus non-canonical translation of a given mRNA. Toeprinting is a technique that allows the detailed mechanistic study of translation initiation of specific RNAs *in vitro*. The overall goal of toeprinting is to assess the ability of an RNA of interest to nucleate the formation of a translation initiation complex with the ribosome under a variety of conditions, in order to determine which sequences, structural elements, or accessory factors are required for efficient translation initiation. For instance, ribosome recruitment might be hindered in the absence of a 5' cap but stimulated by the presence of an IRES.

The principle of the technique is to *in vitro* transcribe an RNA of interest, incubate it in the presence of cellular extracts containing translation components (or the purified components) to allow initiation complexes to form, and to reverse transcribe the RNA with a specific primer. Stable RNA-ribosome complexes will cause reverse transcription to stall at the 3' edge of the ribosome-the so-called 'toeprint'[5,6,7].

In this protocol, the ribosomal subunits, eIFs, tRNAs, and IRES *trans*-acting factors (ITAFs) are conveniently contributed by rabbit reticulocyte lysate (RRL). Another advantage of this protocol is the use of a fluorescently-labeled primer and fluorescence gel-based imager, rather than a radiolabeled primer. This eliminates extra steps, including radiolabeling the primer, as well as drying the gel and exposing it to an intensifying screen. The fluorescent bands are recorded in real time, as the gel runs, allowing for greater resolution. Uncapped X-linked inhibitor of apoptosis protein (XIAP) IRES RNA is used as an example here, although capped mRNAs can also be analyzed by this technique[8].

Unlike western analysis, which measures the final output of the translation process in cell lysates, toeprinting is an *in vitro* approach to measure translation initiation complex formation on an RNA. This reductionist approach allows for the highly detailed study of substrates or factors that regulate translation initiation (*e.g.*, capped or un-capped mRNA, IRES structure, presence or absence of poly-A tail, provision of specific protein factors, *etc.*). Hence, toeprinting can be used to study different modes of translation[8] or the effects of mRNA structures, such as IRESes, on protein synthesis[9,10].

Protocol

NOTE: RNA is highly susceptible to degradation by ribonucleases (RNases). Take standard precautions to keep the RNA intact. Change gloves frequently. Use filtered pipette tips, nuclease-free plasticware, and nuclease-free chemicals in all steps of the protocol. Use nuclease-free or diethyl pyrocarbonate (DEPC)-treated water for all solutions.

1. Preparation of Solutions

1. Prepare Toeprinting buffer: 20 mM Tris-HCl (pH 7.6), 100 mM KOAc, 2.5 mM $Mg(OAc)_2$, 5% (w/v) sucrose, 2 mM dithiothreitol (DTT), and 0.5 mM spermidine.
    1. Store DTT and spermidine in single-use aliquots at -20 °C to avoid repeated freeze-thaw cycles.
    NOTE: DTT and spermidine should be added to the toeprinting buffer immediately before use. A solution lacking DTT and spermidine can be stored at -20 °C.
2. Prepare aliquots of 85 mM 5'-guanylyl imidodiphosphate (GMP-PNP) and 91 mM adenosine triphosphate (ATP). Store the aliquots at -20 °C.
3. Prepare 450 mL of 6% polyacrylamide-7M urea gel mix: 67.5 mL of 40% acrylamide:bis-acrylamide (19:1), 189 g of urea, 112.5 mL of 5x TBE (Tris/borate/thylenediaminetetraacetic acid (EDTA)), and 120 mL of water. Dissolve the urea by warming in a 37 °C water bath or on a hot plate with stirring. Filter the solution (*e.g.*, 0.2 µm nitrocellulose vacuum filter).
NOTE: The solution can be stored at 4 °C for at least one month.
Caution: Monomeric acrylamide is a neurotoxin which can be absorbed through the skin. Take great care to avoid skin contact (*i.e.*, wear gloves, a lab coat, and eye protection). Polyacrylamide should also be handled with care, as polymerization may not proceed to 100% completion.
4. Prepare formamide loading dye: 95% formamide, 18 mM EDTA, 0.025% (w/v) sodium dodecyl sulfate (SDS), 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol. Store at -20 °C.
5. Dissolve 1 nmol of primer (5' CTCGATATGTGCATCTGTA; 5' end-labeled with IRDye 800) into 100 µL of water for a working concentration of 10 pmol/µL. Store at -20 °C in single-use aliquots (approximately 10 µL), protected from light.

2. Preparation of mRNA

1. Amplify DNA templates for the synthesis of mRNA by polymerase chain reaction (PCR) from appropriate templates (*i.e.*, genomic DNA or plasmid DNA, as appropriate). Use a high-fidelity DNA polymerase according to the manufacturer's instructions, with the following reaction conditions (35 cycles): melt, 98 °C, 10 s; anneal, 53 °C, 20 s; extend, 72 °C, 30 s.
NOTE: The forward primer (5' AAGCTTAATACGACTCACTATAG) incorporates the T7 promoter sequence to allow for RNA transcription; the reverse primer (5' T_{51}GAATTCGGATCCGACCGTGG) includes 51 thymine residues to provide a poly-A tail for the transcribed RNA. Note that RNA can also be *in vitro* transcribed from plasmid DNA.
2. Use an appropriate transcription kit to *in vitro* transcribe IRES-containing RNA or capped RNA from the DNA template. Follow the manufacturer's instructions. Prepare the RNA sample in standard 20 µL reaction volumes. Treat the newly-synthesized RNA with 2 units of RNase-free DNase for 30 min at 37 °C.
3. Dilute the DNase-treated RNA to 110 µL with nuclease-free water add 110 µL acid phenol, vortex 5 s and centrifuge for 3 min at 20,000 x g at room temperature. Remove 100 µL of the aqueous phase to a new 1.5 mL microfuge tube, add 10 µL of 3 M sodium acetate, and vortex 2 s. Add, 3 volumes of 100% ethanol, vortex 5 s, and precipitate the RNA at -20 °C overnight.
4. Centrifuge at >20,000 x g for 30 min at 4 °C and discard the supernatant. Wash the pellet with 500 µL of ice-cold 70% (v/v) ethanol and repeat the centrifugation. Aspirate as much of the supernatant as possible and air-dry the pellet for 5 - 10 min. Be careful not to dislodge the pellet.
5. Resuspend the RNA in the appropriate volume of nuclease-free water to yield a working concentration of 0.5 µg/µL. This can be stored at -80 °C or used immediately.

3. Toeprinting Reaction

1. Mix 15 µL of Rabbit Reticulocyte Lysate (RRL, not nuclease-treated), 1 µL (40 units) of RNase Inhibitor, 1 µL of 91 mM ATP (1.82 mM final) and 1 µL of 85 mM GMP-PNP (1.7 mM final) in a 1.5 mL microfuge tube. Incubate at 30 °C for 5 min.
NOTE: The RRL should be aliquoted for single use to avoid repetitive freeze-thawing. A critical control is a reaction lacking RRL, in order to elucidate the natural pauses of reverse transcription due to the secondary structure of the RNA. Add toeprinting buffer to replace RRL for this control.
2. Add 0.5 µg (1 µL) of RNA and incubate at 30 °C for 5 min.

3. Add 22 µL of Toeprinting buffer and incubate at 30 °C for 3 min.
4. Add 0.5 µL (5 pmol) of IRDye-labeled primer and incubate on ice for 10 min.
5. Add 2 µL of 25 mM dNTPs (final concentration: 1 mM each), 2 µL of 100 mM $Mg(OAc)_2$, 1 µL of Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) and 3.5 µL of Toeprinting buffer. The final volume is 50 µL.
6. Incubate the reaction at 30 °C for 45 min.
7. Add 200 µL of nuclease-free water and immediately extract with 250 µL of 25:24:1 phenol:chloroform:isoamyl alcohol (pH approximately 8.0). Vortex 5 s and centrifuge at 20,000 x g for 3 min at room temperature. Remove the aqueous phase to a new 1.5 mL microfuge tube, add 3 volumes of 100% ethanol, vortex 5 s, and precipitate at -20 °C overnight.
8. Centrifuge at >20,000 x g for 30 min at 4 °C and discard the supernatant. Wash the DNA pellet with 500 µL of ice-cold 70% (v/v) ethanol. Centrifuge at >20,000 x g for 15 min at 4 °C. Aspirate as much supernatant as possible and air dry the pellet for 5 - 10 min. Be careful not to dislodge the pellet.
9. Dissolve the pellet in 6 µL of nuclease-free water and add 3 µL of formamide loading dye.

4. Sequencing Reactions

1. Use the DNA template from 2.1 and the IRDye-labeled primer from step 3.4 for standard sequencing reactions, using dideoxynucleotides (ddNTPs) as chain terminators. Use an appropriate DNA sequencing kit and follow the manufacturer's instructions.
2. Mix 6 µL of each sequencing reaction with 3 µL of formamide loading dye.

5. Preparation of Sequencing Gel and Electrophoresis

NOTE: This protocol uses a fluorescence gel-based imager and a 21 cm x 23 cm x 0.2 mm gel, but can be adapted for other sequencers or gel-sizes, if required.

1. Thoroughly clean the 0.2 mm spacers as well as the short (23 × 25 cm) and long (30 × 25 cm) glass plates with 100% ethanol. Air dry.
2. Mix 30 mL of 6% polyacrylamide-7M urea gel mix with 200 µL of 10% (w/v) ammonium persulfate (APS) and 20 µL of tetramethylethylenediamine (TEMED). Pour the gel, taking care to avoid bubbles, and insert the 'shark-tooth' gel comb. Allow the gel to polymerize for 1 h at room temperature.
3. Assemble the sequencing apparatus and fill the reservoirs with 1x TBE.
4. Pre-run for 15 min at 1500 V until the optimal temperature of 55 °C is achieved.
5. Heat the sample/loading dye mix (from steps 3.9 or 4.2) to 85 °C for 5 min. Load 1 µL onto the sequencing gel.
6. Run the gel at 1500 V for 8 h. The machine will read the bands in real time.
7. Disassemble the apparatus, and dispose the acrylamide gel and running buffer.

Representative Results

We have previously described the ability of the XIAP IRES to support cap-independent translation initiation *in vitro*[8,10]. Toeprinting was the key technique to interrogate the mechanistic details of the XIAP IRES initiation complex. A DNA construct encoding an mRNA containing the XIAP IRES (**Figure 1A**) was *in vitro* transcribed and subjected to toeprinting analysis. The mutant variants of the XIAP IRES mRNA used here are represented in **Figure 1B**. Reverse transcription of the XIAP mRNA-ribosome complex yielded typical toeprints +17 to +19 nt downstream of AUG (**Figure 1C, lane 1**). This is indicative of ribosome recruitment to the start codon and the formation of stable ribosome-RNA complexes[5,6,7]. Ribosome recruitment was strongly impaired in the absence of a poly-A tail (**Figure 1C, lane 9**), as previously reported[11]. Toeprint formation was also strongly impaired in the absence of RRL and GMP-PNP (**Figure 1C, lane 8**) and for the start codon mutant (**Figure 1C, lane 7**), confirming that the observed toeprint is not a structure-induced pause of reverse transcription but is, in fact, specific to initiation complex formation. Toeprint formation was impaired for the 5' polypyrimidine tract (PPT) mutant (**Figure 1C, lane 2**) and the 3' PPT mutant (**Figure 1C, lane 4**), which disrupt IRES structure (**Figure 1B**)[8,10,12]. Toeprint formation was restored when the PPT mutants were transcribed with a 5' cap (**Figure 1C, lanes 3 and 5**). Toeprint formation was also restored for the PPT double mutant (**Figure 1C, lane 6**), which restores the secondary structure (**Figure 1B**)[10]. Together, these data indicate that the secondary structure of the XIAP IRES is critical for translation initiation of un-capped RNA and that IRES structure is dispensable for cap-dependent translation initiation. To further demonstrate the specific requirement for a 5' cap in the absence of an IRES structure, human β-globin (HBB) mRNA was subjected to toeprinting analysis (**Figure 1D**). No toeprint was observed in the absence of a 5' cap (**Figure 1D, lane 1**) but ribosomes were successfully recruited to capped HBB RNA (**Figure 1D, lane 2**).

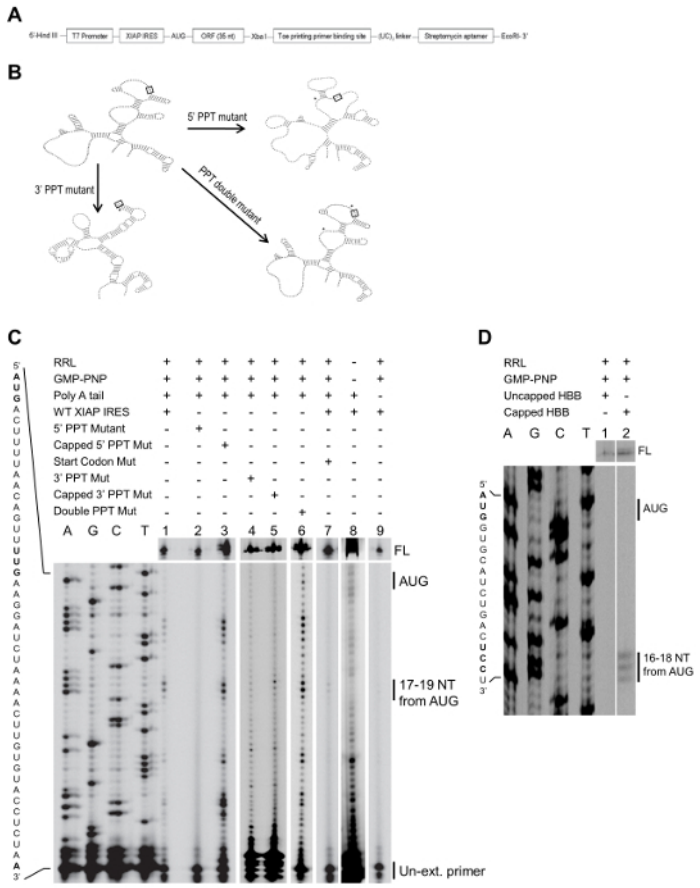

Figure 1. Toeprinting analysis confirms the importance of IRES secondary structure for the formation of translation initiation complexes on an uncapped XIAP IRES RNA. (A) Schematic diagram of the DNA construct encoding the XIAP IRES RNA used in this analysis. The 3' end of the toeprinting primer is 42 bp downstream of the start codon. **(B)** Secondary structures of the WT XIAP IRES (upper left), the 5' PPT mutant (upper right), the 3' PPT mutant (lower left), and the PPT double mutant (lower right). 'PPT Mutant' refers to a point mutation (UU to AA) in a critical polypyrimidine tract, which disrupts the secondary structure of the IRES[8,10,12]. The start codon is boxed; the point mutations are indicated with asterisks (*). **(C)** Toeprinting analysis of the ability of XIAP IRES variants to form translation initiation complexes in RRL treated with GMP-PNP and ATP. The start codon AUG was replaced with AAC in the Start Codon Mutant. To make RNA lacking a poly-A tail, the reverse primer used to make the *in vitro* transcription template simply lacked a poly-T tract. **(D)** Initiation complex was formed on the capped (but not the un-capped) 5' UTR of human β-globin (HBB). In panels (C) and (D), the left-most 4 lanes represent sequencing reactions with the dideoxy nucleotides indicated above each lane; the sequence is indicated to the left of each panel. FL, Full-length. All RNAs were un-capped unless otherwise specified. This figure has been modified from previous publications[8,10]. Please click here to view a larger version of this figure.

Discussion

Toeprinting is a powerful technique to directly measure the ability of an RNA of interest to support the formation of translation initiation complexes under highly controlled circumstances. This protocol describes a simplified technique for toeprinting mammalian RNAs. Rabbit reticulocyte lysate (RRL) is used as a convenient source of ribosomes, eIFs, initiator tRNA, and IRES *trans*-acting factors (ITAFs). The experimenter provides their RNA of choice, and can also supplement the toeprinting reaction with specific cofactors of their choosing. For instance, 48S *versus* 80S translation initiation complexes can be differentiated based on the distribution of fluorescence intensities of the toeprints[7]. The initiation complex observed will depend on the type of guanine nucleotide used. In the case of the XIAP IRES discussed here, GMP-PNP plus ATP stabilizes 48S pre-initiation complexes, characterized by a toeprint distribution +17≥+18>+19. GMP-PNP is a nonhydrolyzable GTP analog that inhibits ribosomal subunit joining, thus blocking translation initiation at the 48S step[13]. In contrast, GTP, ATP, or ATP plus GTP stabilizes 80S initiation complexes, characterized by toeprint distribution +17<+18> +19[8].

The user will have to consider the type of RNA they wish to toeprint. If the aim is to study a cap-independent mechanism, such as an IRES element, the RNA can be *in vitro* transcribed with any commercially available kit. However, any mammalian RNA can be subjected to this toeprinting method, provided that it has a 5' cap structure. Capped mRNA must be generated using an appropriate kit. In any case, the manufacturer's protocols should be closely followed, as the preparation of high-quality RNA represents a key step in the procedure. Another critical point is that the phenol extraction in step 3.7 must be carried out at or above neutral pH; if acid phenol is used at this stage, the newly synthesized cDNA will be lost. It is worth noting that the concentration of magnesium acetate used in step 3.5 can influence the efficiency of reverse transcription, and may have to be optimized for each mRNA.

A few key controls are necessary to ensure the specificity of the toeprint. First, no robust toeprint should be observed in the absence of RRL or nucleotide. This ensures that the observed reverse transcription pause is due to a ribosome-RNA complex rather than a stable RNA secondary

structure or some defect with the reverse transcription reaction itself. Second, no toeprint should be observed if the start codon is mutated. This ensures that the ribosome has formed a complex specifically with the start codon of the RNA and that the user has correctly identified the 3' edge of the ribosome in complex with the start codon. This is particularly important for IRES elements, which might recruit the ribosome to an alternative start codon[14]. Similarly, the toeprint might be impaired in the absence of a poly-A tail, as was the case for the XIAP IRES[8]. However, some IRES elements form toeprints in the absence of a poly-A tail, such as the CrPV IRES[10]. Finally, in addition to the toeprint proper, other reverse transcription pauses might be observed. These can simply represent pauses due to stable secondary structures in the RNA, or they could be due to a phenomenon dubbed "ribosome jumping", wherein the ribosome slides from the start codon to other locations on the RNA[15]. To differentiate between these possibilities, the toeprinting reaction can be performed in the presence of cycloheximide[8], which effectively immobilizes the ribosome at the start codon and should reduce alternative toeprints due to ribosome jumping. Notably, different mRNAs will have different secondary structures, meaning that the number and intensity of reverse transcription pauses (*i.e.*, background bands) will also vary.

A possible limitation of this technique is that it measures ribosome recruitment to an mRNA *in vitro*, but the formation of a translation initiation complex does not necessarily mean that translation will occur *in vivo*. Therefore, toeprinting is particularly powerful when combined with *in vivo* techniques to measure translation (*e.g.*, polysome profiling[16]) and the resulting protein levels (*e.g.*, western analysis).

A more recent technique is "ribosome profiling" (also called "ribosome footprinting"), wherein high-throughput RNA sequencing is used to measure the presence of ribosomes on total cellular mRNA. This is a powerful technique to measure ribosome recruitment on a transcriptome-wide scale. Inherent to ribosome profiling is the use of reagents, such as cycloheximide, to stabilize ribosomes on mRNAs. This potentially represents a drawback, as a disproportionate number of ribosomes can be non-specifically stalled in the 5' untranslated region (UTR), which can be misinterpreted as non-canonical translation initiation[17]. In toeprinting, cycloheximide is not an integral part of the procedure, negating the possibility of such false positives. Moreover, any single mRNA can be studied in greater detail by toeprinting, as it is facile to conduct many control experiments and iterations (for instance, testing the effect of several point mutations, or the absence of a poly-A tail, on ribosome recruitment). It would be time-consuming and cost-prohibitive to carry out an equivalent array of control experiments in the context of a ribosome profiling experiment. Toeprinting is thus a complementary approach to high-throughput sequencing-based techniques, and is still commonly used for studies aiming to elucidate mechanisms of translation regulation[8,9,10,18].

Disclosures

The authors have no conflict-of-interest to disclose.

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