

Translation toeprinting assays using fluorescently labeled primers and capillary electrophoresis

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BioTechniques 38:397-400 (March 2005)

The protocol described here is an adapted version of the toeprinting assay in which the oligonucleotide used to prime the reverse transcription step is labeled with a fluorescent dye instead of ^{32}P . By using a fluorescent dye, the results of the assay are obtained within one hour by direct electrophoresis of the samples on an automated sequencing machine. This eliminates the need to cast and run polyacrylamide gels and to wait to expose dried gels. We show that an identical toeprint was found for the chloramphenicol acetyltransferase transcript using this nonradioactive method, which is in agreement with the previously published ^{32}P -labeled method. Furthermore, in addition to being a faster and safer method, a larger region of sequence can be analyzed with one primer in a single experiment.

INTRODUCTION

The primer-extension inhibition (toeprinting) assay is an increasingly popular method used to investigate the fundamental processes that occur during translation, from factors that affect events occurring during initiation to termination (1–10). In a toeprinting assay, mRNA is translated using purified ribosomal complexes that are obtained from an appropriate source, including prokaryotes, mammals [purchased commercially as rabbit reticulocyte lysates (RRLs)], plants (commercially available as wheat germ extracts), and fungal extracts (1,7,8,10). Cycloheximide (CHX) is added to the reaction to inhibit elongation, thereby arresting the position of the ribosomes on the transcript. The mRNA complex is then copied into cDNA using a specific labeled primer and, where the reverse transcriptase meets the ribosome bound to the mRNA, polymerization is halted, and a “toeprint” fragment is generated (11–14). Typically, the position of the P site of the stalled ribosome is 15–17 nucleotides upstream of the toeprint. If the position of the ribosomes along the transcript is non-random, the corresponding fragment will be abundant. This would be seen on a gel as a band or, in the method described here, as a peak in the electropherogram when the samples are run on an automated DNA sequencing machine and analyzed

using standard software.

The utilization of fluorescently labeled oligonucleotides has been described in other applications that incorporate a PCR step but has not been used in a toeprinting assay (15–18). Here we report the use of a fluorescent oligonucleotide primer instead of a radioactive oligonucleotide in a toeprinting assay as first suggested by Sachs et al. (8).

MATERIALS AND METHODS

In Vitro Transcription

The chloramphenicol acetyltransferase (*cat*) gene was transcribed in vitro with T7 RNA polymerase (MBI Fermentas, Sunderland, UK) from pT7CAT (19) linearized with *Hind*III, according to the manufacturer's protocol. In addition, an m7G cap analogue (Promega, Southampton, UK) was incorporated. mRNA was purified using TRIzol® (Invitrogen Paisley, UK) and stored at -70°C . mRNA was quantified on a NanoDrop® (ND-1000) spectrophotometer (Labtech International Ltd., Luton, UK) and qualitatively assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Stockport, UK).

Toeprint Assay

The primers were synthesized and labeled with 5'-FAM (TAG Newcastle Ltd., Tyne & Wear, UK). The primers used were CAT1 (5'-FAM-CTCAAATGTTCTTTACGATGCC-3') and CAT2 (5'-FAM-CAAGAATGTGAATAAAGGCCGG-3'). Twenty picomoles of primer and 50–1000 ng mRNA (optimally 500 ng) were combined in 50 mM Tris-HCl, pH 7.5, and heated to 68°C for 2 min and cooled to 37°C for 8 min and immediately added to the translational mixture, comprising 50% RRL, 20 μM amino acid mixture minus methionine, 20 μM amino acid mixture minus leucine (Promega), and 1.5 U/ μL ribonuclease inhibitor (MBI Fermentas). Where appropriate, 500 $\mu\text{g}/\text{mL}$ CHX (Sigma, Poole, UK) were added before or after the reaction. The reactions were incubated at 30°C for 20 min. For the primer extension stage, 4 μL of the translation reaction were supplemented with 50 mM Tris-HCl, pH 7.5, 40 mM KCl, 3.5 mM MgCl_2 , 5 mM dithiothreitol (DTT), 0.8 mM each dNTP (MBI Fermentas), 500 $\mu\text{g}/\text{mL}$ CHX, 1.5 U/ μL ribonuclease inhibitor, and 5 U/ μL SUPERSCRIPT® II RNase H⁻ reverse transcriptase (Invitrogen) in a final volume of 20 μL and placed at 25°C for 10 min. This step was carried out at 25°C , following a method previously described (14); however, identical results were obtained when the primer extension reaction was carried out at 37°C (data not shown).

Sequencing

Sequencing ladders were generated using the Sequenase™ Version 2.0 DNA Sequencing kit (USB; Amersham, Buckinghamshire, UK) with the same labeled primer/mRNA amounts that are described above. Reactions were carried out according to the manufacturer's protocol with the following modifications: avian myeloblastosis virus (AMV) reverse transcriptase and supplied buffer (RT reaction buffer, pH 8.3; Promega) replaced the DNA-dependent polymerase, and 7.5 μM dATP (MBI Fermentas) were added to the 1 \times labeling mixture. It was necessary to use this method of sequencing rather than

using labeled ddNTPs because the same fluorescent label must be used to ensure equal mobility of all the fragments, including the toeprint.

Analysis

Primer extension and sequencing products were extracted with phenol, and the ethanol-precipitated pellets were resuspended in 4 μ L of Hi-Di™ formamide (Applied Biosystems, Warrington, UK). A 2- μ L aliquot was brought to a total volume of 12 μ L with Hi-Di formamide, which included 0.5 μ L ROX 500 size standard (Applied Biosystems). Alternatively, a QIAquick® nucleotide removal kit (Qiagen, Crawley, UK) can be used prior to precipitation and resuspension in formamide.

The products were separated by electrophoresis using standard GeneScan® conditions on an ABI PRISM® Genetic Analyser 3100 (Applied Biosystems). Briefly, the instrument was set up with POP4 (performance-optimized polymer 4) and a 36-cm capillary and operated with 3100 Data Collection Software v1.1 (Applied Biosystems) using the standard 36 cm/POP4 run module, comprising 1500 s run time at 15 kV, 60°C, with 22 s injection. Resulting fragment files (*.fsa) were analyzed using GeneScan Analysis v3.7.1 software. A new project was created containing the relevant *.fsa files. Each file was analyzed with default analysis parameters and the size standard file GS500-250.szs. A view in which all the electropherograms could be overlaid was selected, and the samples were aligned using the ROX size standard in each lane. The scales for the x-axis and y-axis were adjusted to make the peak appearance similar to that of a sequencing electropherogram. Next to the name of each sample in the legend below the electropherogram, two buttons appear with which the appearance of the electropherogram was further modified. Using the left button, the height of each peak was adjusted to make the signal strength of each sequencing ladder appear equal. The right button was used to change the color of each sequencing ladder, allowing each base to be distinguished. Additional colors can then be used to distinguish the ROX

size standard and the actual toeprint peaks. Thus, the position of the major toeprint peak was determined directly from the surrounding sequence. The electropherograms were captured from GeneScan using the program SnagIt 7.0 (TechSmith, Okemos, MI, USA).

RESULTS AND DISCUSSION

In order to evaluate whether ribosomal toeprinting could be performed using a fluorescently labeled primer, we tested the system using CAT, a transcript that had been previously examined using the traditional radiolabeled primer approach (14). In these experiments, CHX was added to the reaction before translation commenced so that ribosomes paused on the start codon. Figure 1 shows the relevant portion of the CAT transcript; the highlighted G nucleotide corresponds to the center of the observed toeprint (14); that is, the point where the AUG-positioned ribosome blocks CAT1-primed reverse transcriptase synthesis of the complementary strand. To evaluate the performance of the nonradioactive method, we used the same CAT1 primer sequence in fluorescently labeled form. The results from this primer were not

readable, probably because the 3' end of the primer is within 28 nucleotides of the predicted toeprint, and the initial primer flare seen in all fluorescent techniques interferes with the reading (data not shown). Therefore, a second primer, CAT2, was used for the rest of the study. CAT2 anneals 5' to 3' from nucleotides 227 to 206 (Figure 1). By annealing a primer further downstream, a larger region of sequence can be analyzed for potential ribosome stalling positions, up to 500 nucleotides from the primer (P.S. Gould and A.J. Easton, unpublished data).

Figure 2A shows the results obtained with the CAT2 primer. The predominant peak in the no RRL control, representing the full-length transcript (green trace), was sized at 225 based on the ROX size standards. This was two nucleotides smaller than the expected full-length product; similar discrepancies were observed for other peaks. This phenomenon has already been noted where the ABI size standards undersized the fragments by 2 to 3 nucleotides on average compared to the true size (16,18). Therefore, we recommend that accurate mapping of the size of the peaks requires that a sequencing ladder should be overlaid with the trace of interest (examples are shown in Figure 2, B

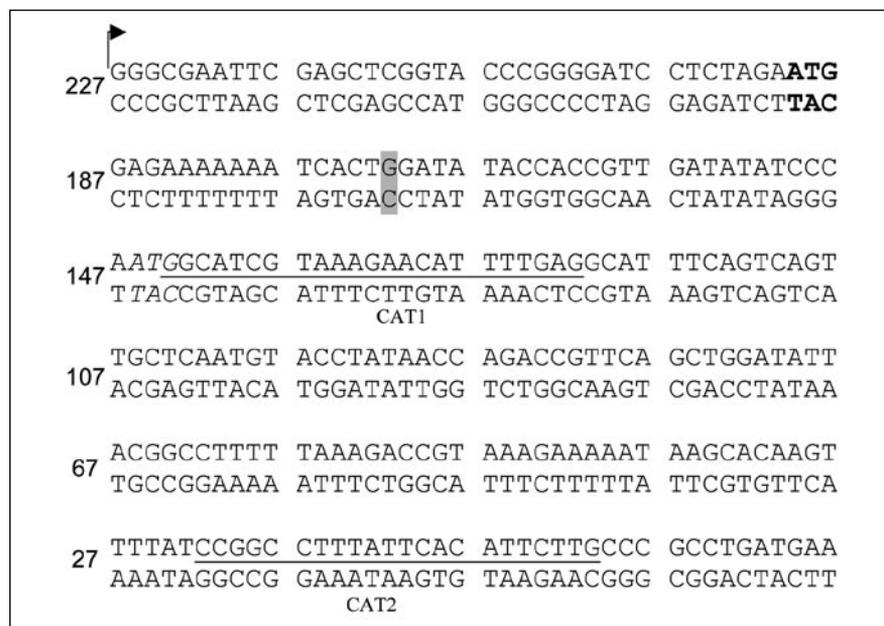


Figure 1. Sequence of the first 240 nucleotides from the transcription start of the CAT gene. The start codon (bold), the position where the CAT1 and CAT2 primers anneal (underlined), and the center of the toeprint identified by Kozak (14) when CHX was added to the RRL (highlighted in gray) are shown. An internal AUG that was later mutated to GUG is shown in italics. Numbering is from the 5' end of CAT2. CAT, chloramphenicol acetyltransferase; RRL, rabbit reticulocyte lysate.

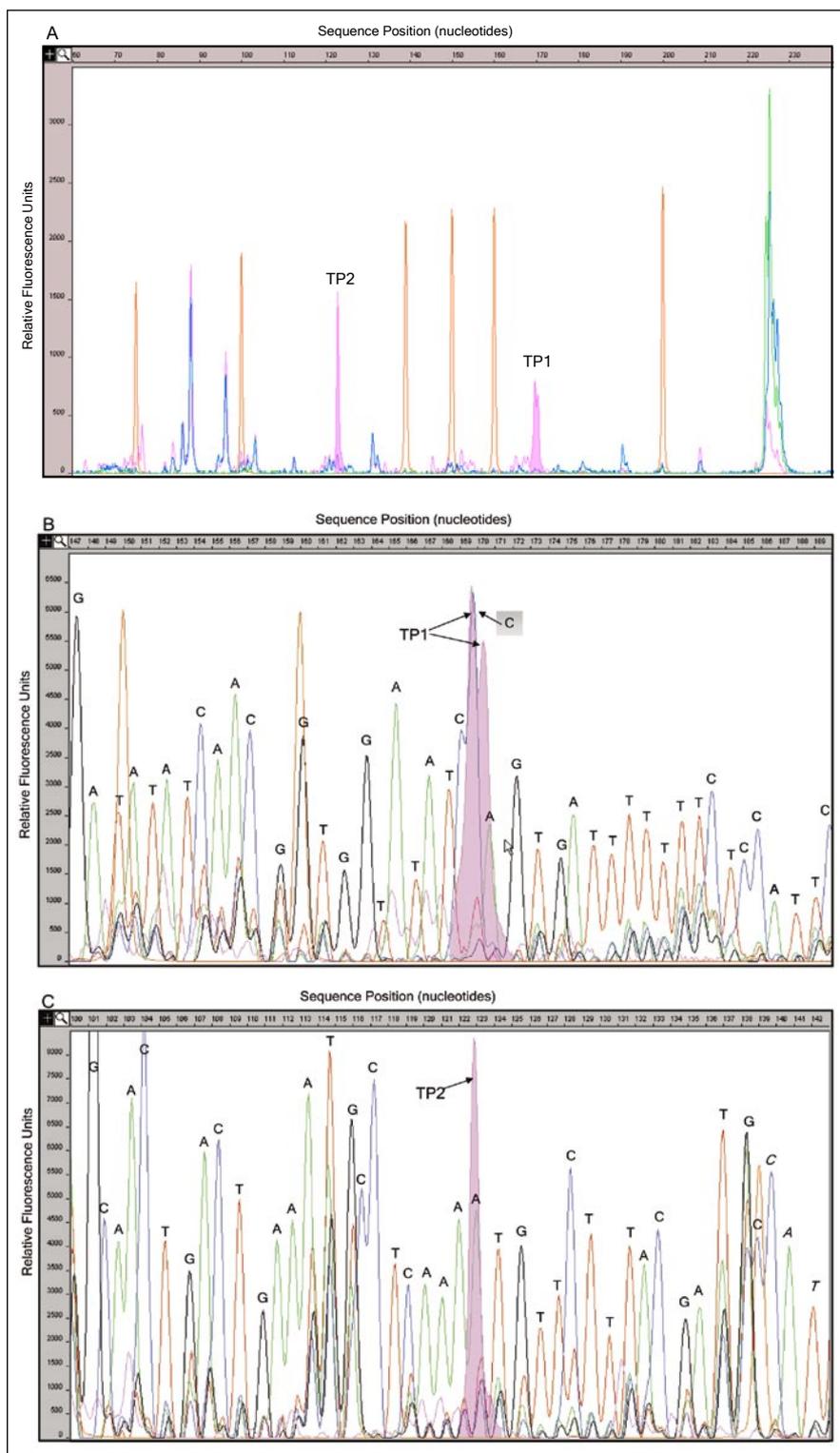


Figure 2. Electropherograms of fluorescently labeled toeprint assays. (A) Overlay of electropherograms of toeprinting reactions performed with (pink trace) and without (blue trace) the addition of CHX. The green trace corresponds to a control in which RRL was not added. ROX size standards are indicated in orange. Two major toeprints, sized at 170 and 123 nucleotides, were identified in the +RRL +CHX trace. (B) Toeprint 1 (TP1) identified in panel A and ROX 500 size standards (orange) are aligned with the sequencing ladder generated with the CAT2 primer. (C) Alignment of TP2 identified in panel A with the sequencing ladder; the internal start codon is shown in italics. CHX, cycloheximide; RRL, rabbit reticulocyte lysate.

and C). When RRLs were added to the reaction along with the full-length product (from mRNA with no bound ribosomes), a number of other peaks were detected (Figure 2A, blue trace). These peaks could represent regions in the mRNA secondary structure that naturally cause ribosomes to pause. These pauses were not seen in the previous report because this sequence region was not investigated (14). Importantly, the addition of CHX results in the appearance of an additional peak sized at 169.5 nucleotides (Figure 2A, pink trace, TP1). When this toeprint is aligned with the sequencing ladder (Figure 2B), it exactly corresponds to the position mapped previously (Figure 1; Reference 14), as much as it is possible to determine in the context of the gel distortion (14). The doublet or shoulder observed in TP1 is consistent with the “shadow” bands seen in the traditional radiolabeled toeprints (14). Thus, the technique described here gives the same result but at higher resolution than that observed with the radioactive probe. TP1 is +19 nucleotides upstream of the start codon, which indicates that translocation has not been immediately arrested by the addition of CHX but the extension of one triplet has occurred. This phenomenon has been previously noted, where the inhibition of translocation by CHX is template specific (5).

The presence of an additional toeprint (Figure 2, A and C, TP2) found in the +CHX +RRL tract was not expected. However, this can be explained by the location of an internal start codon 18 nucleotides upstream of the toeprint (Figure 2C; shown in italics). It is known that because of the artificial nature of *in vitro* translation using RRL, initiation can start internally (www.promega.com/tbs/tm232/tm232.html). Thus, if a ribosome was bound here and could not begin elongation because CHX was present, a toeprint of the observed size of TP2 would be generated. To test this, the internal start codon was removed by mutation, the experiment was repeated, and TP2 disappeared (data not shown), confirming that the presence of the AUG codon at this position is required for the toeprint. We have therefore shown that this method can be used to discover unknown regions that cause pausing, which can be subsequently investigated to determine their cause.

Aside from the general safety and

environmental advantages of not using radioactivity, the main benefit of the method described here is the ease by which data can be obtained. Here the data for 16 samples are ready in less than 1 h, with no preparation time required for pouring gels, cleaning contaminated equipment, and waste disposal. As shown in Figure 2, the output can be easily formatted into interpretable data. The raw output data from GeneScan also provide details of the height and area of each peak. Thus, if the researcher requires information on the relative abundances of different toeprints on the same transcript, this numerical data could be used. Because automated sequencing machines are now present in most research facilities, the cost of this protocol is at least equivalent if not less than the radioactive method. FAM-labeled primers are commercially available and inexpensive; once synthesized, the labeled primer is ready to use and is stable for at least 1 year. To run each sample on the capillary sequencer costs approximately \$1.50 U.S. dollars depending on such factors as maximum throughput, size standard, POP4, and 36 cm capillary, based on local pricing arrangements.

This approach has many advantages over its radioactive counterpart (15–18) including safety for the user, throughput, cost, and ease of obtaining/analyzing the results.

ACKNOWLEDGMENTS

Thanks to Mark Miller at the National Cancer Institute for his help in using GeneScan and Lesley Ward and Sue Davis for technical assistance. T7CAT was kindly provided by Anthony Marriot. This work was funded by the Biotechnology and Biological Sciences Research Council grant no. 88/P16683 (to A.J.E.) and the Joint Research Equipment Initiative grant no. 88/RE1120575 (to H.B.).

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Received 25 August 2004; accepted 5 October 2004.

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