Benchmarks

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Single-Nucleotide Resolution of RNA Strands in the Presence of their RNA Complements

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Double-stranded (ds)RNA is important for a variety of biological systems. The discovery of the dsRNA-binding motif (dsRBM), coupled with the occurrence of this motif in a wide variety of functionally diverse proteins, has led to increased interest and study of dsRNA (6,14). For example, the dsRNA-activated protein kinase (PKR), an enzyme involved in the cellular antiviral response, contains two tandem copies of the dsRBM. In addition, the dsRNA adenine deaminases (dsRADs) contain three tandem copies of this motif (7). Likewise, the study of the RNA-dependent RNA polymerase (RdRP) activity associated with RNA virus transcriptases and replicases also requires the use of dsRNA. In each of these systems,

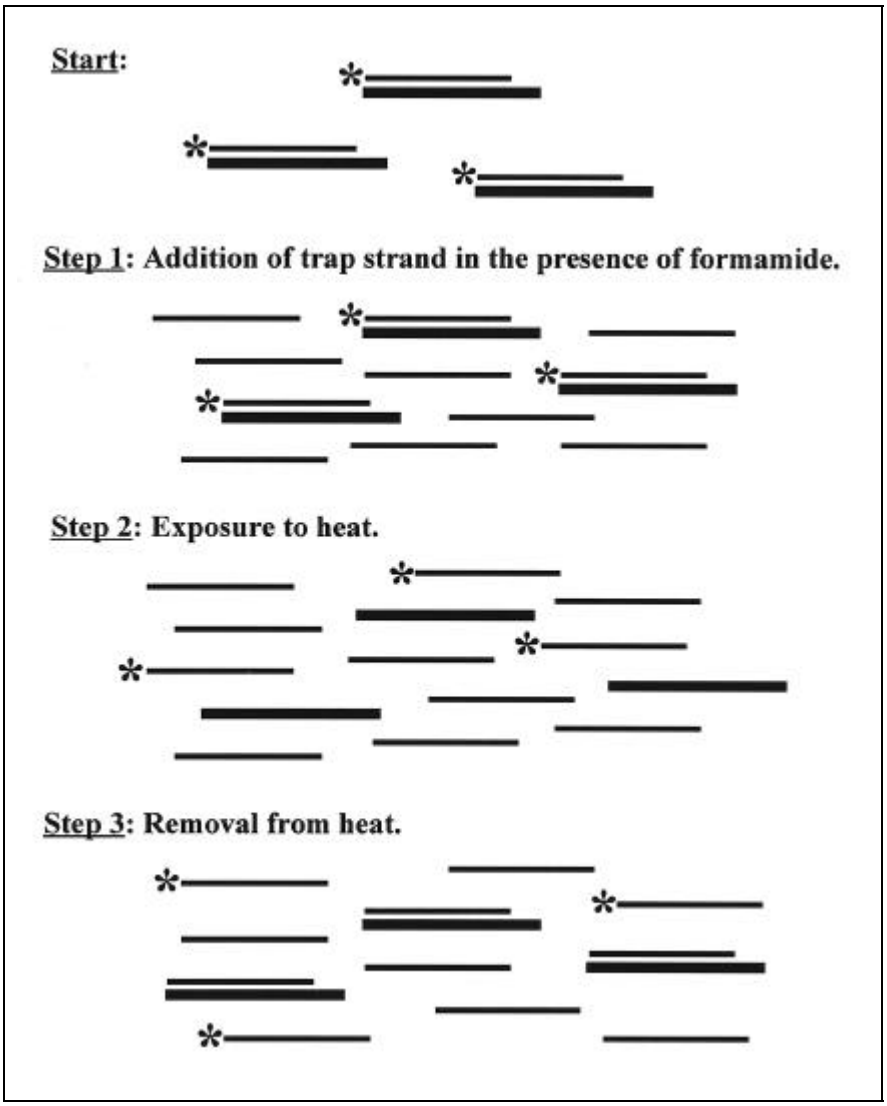

Figure 1. Schematic for the mechanism of trap strand invasion. Start: dsRNA products of the 3D^{pol} primer-extension assay. Step 1: An excess of unlabeled RNA, trap strand, which is identical to that of the end-labeled RNA under investigation, is added to the dsRNA products in the presence of formamide. Step 2: Samples are heated to 65°C for 2–5 min resulting in the denaturation of the dsRNA products. Step 3: Samples are removed from the heat and loaded on a denaturing polyacrylamide gel. The trap strand precludes re-annealing of the end-labeled RNA.

the length of the typical RNA used is in the 10–80 bp range (1,9).

Denaturing polyacrylamide gel electrophoresis (PAGE) of dsRNA fragments longer than 10 bp is prone to a number of problems, most of which stem from the high thermal stability of RNA-RNA duplexes. While typical conditions for denaturing PAGE (8 M urea and gel temperatures of 50°–60°C) are sufficient to denature and provide single-nucleotide resolution for dsDNA and RNA-DNA hybrids, these conditions are not sufficient for comparable resolution of dsRNA. Temperatures higher than 60°C induce hydrolysis of RNA, and many dsRNA fragments melt well outside the working range for denaturing PAGE.

Indeed, mechanistic studies of RdRPs have been limited by the aforementioned problems. For example, a 15-nucleotide (nt) RNA primer strand migrates as a single, discrete band in the absence of its complement (Figure 2B, lane 1). However, this same 15-nt RNA primer strand migrates as a smear in the presence of a complementary 21-nt RNA template strand (Figure 2B, lane 2). As a result, most studies reported to date for this class of nucleic acid polymerase have completely avoided gel analysis and relied primarily on filter-binding methods to follow RdRP activity (2,4,5,8,10,11,16). In those instances in which primer extension has been followed by PAGE, glyoxal treatment of RNA has been required to resolve extended products (12). As it is essential to achieve single-nucleotide resolution of extended primers to quantify accurately product formation and to address specifically issues related to polymerase mechanism (1), an alternative approach is warranted.

We have developed a simple, effective method for analyzing RNA by denaturing PAGE in the presence of its complement. This method permits single-nucleotide resolution of dsRNA under the same conditions used for analysis of dsDNA and RNA-DNA hybrids

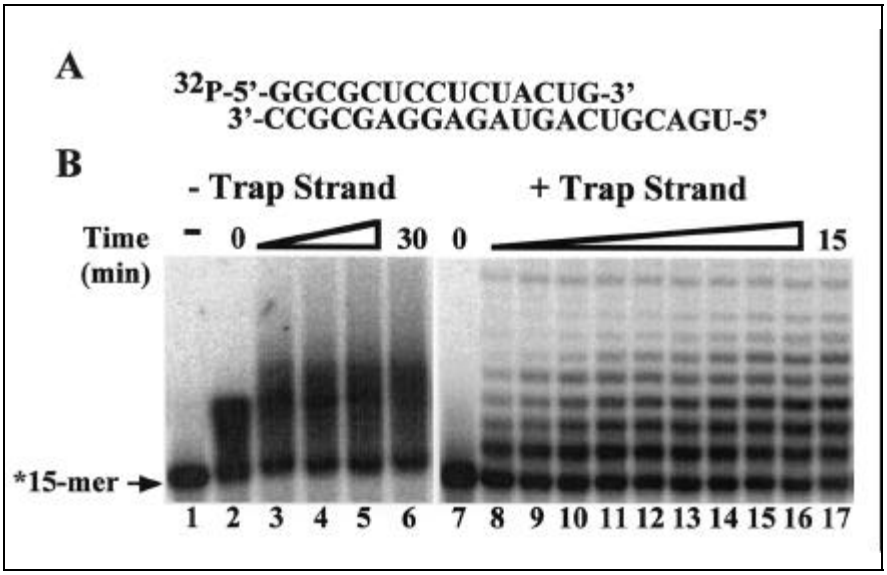

Figure 2. Primer extension by poliovirus RdRP, 3D^{pol}. (A) 15/21-mer primer/template used in this study. (B) Time course for poliovirus RdRP-catalyzed incorporation of AMP into 15/21-mer using $MnCl_2$ as the divalent cation. Reactions contained HEPES, pH 7.5 (50 mM), 2-mercaptoethanol (10 mM), $MnCl_2$ (5 mM), $ZnCl_2$ (60 μM), 3D^{pol} (5 μM), an end-labeled [^{32}P]-15/21-mer primer/template (1 μM) and ATP (500 μM). Reactions were initiated by addition of 3D^{pol} and incubated at 30°C after which the reactions were quenched at the indicated times by addition of EDTA to a final concentration of 50 mM. One microliter of the quenched reaction mixtures was added to 9 μL of loading buffer: 90% formamide, 50 mM Tris-borate, 0.025% bromophenol blue, 0.025% xylene cyanol and either no unlabeled 15-mer RNA (- Trap Strand) or 1 μM unlabeled 15-mer RNA (+ Trap Strand). Samples were heated to 65°C for 2–5 min before loading 5 μL on a 20% polyacrylamide gel containing 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) and 7 M urea. Electrophoresis was performed in 1× TBE at 75 W. Gels were visualized by a Model 445SI PhosphorImager® (Molecular Dynamics, Sunnyvale, CA, USA). The lane indicated by [-] is the labeled 15-mer primer strand alone. A detailed description of the methods used can be found in Reference 1. The RNA used in this study was synthesized by Dharmacon Research (Boulder, CO, USA).

by denaturing PAGE. The method relies on the addition of an excess of unlabeled RNA, the sequence of which is identical to that of the end-labeled RNA under investigation. We refer to this unlabeled RNA as the "trap strand." The trap RNA is added during preparation of samples for electrophoresis, thus requiring very little of this RNA. The trap strand is added in excess of the labeled RNA to displace this RNA and preclude re-annealing. The method is illustrated in Figure 1.

The utility of this method for the analysis of RdRP-catalyzed nucleotide incorporation is illustrated in Figure 2. The 15/21-mer primer/template used in this study is shown in Figure 2A, and the RdRP used was from poliovirus. Reactions were performed as described in the legend to Figure 2, and products were analyzed by phosphor imaging after electrophoresis on a denaturing 20% polyacrylamide gel. In the absence of a trap strand, smears were observed, and in no case was single-nucleotide resolution of the extended primer obtained (Figure 2B, lanes 2–6). However, in the presence of the trap strand, single-nucleotide resolution of the extended primer was observed (Figure 2B, lanes 8–17). These reactions were performed using Mn^{++} as the divalent cation cofactor. In other polymerase systems, this cofactor has been shown to promote mis-incorporation and incorporation of nucleotide analogs, such as dideoxynucleotides (15). As only the single, correct nucleotide was provided in the experiment shown in Figure 2B, it seems reasonable to conclude that the use of Mn^{++} as the divalent cation cofactor for $3D^{pol}$ also promotes mis-incorporation. Of course, this effect of Mn^{++} on $3D^{pol}$-catalyzed nucleotide incorporation would not have been uncovered without single-nucleotide resolution of the extended products.

Taken together, the data presented in Figure 2 show that the thermal stability of dsRNA causes incomplete denaturation of the duplex, which substantially limits the resolution of individual strands of RNA under standard conditions used for denaturing PAGE. However, in the presence of a trap strand, modest heating of the sample (65°C) and a reasonable concentration of formamide in the loading buffer (80%) are sufficient to achieve single-nucleotide resolution of RNA by denaturing PAGE. It is worth noting that single nucleotide resolution was also achieved by using a 20% polyacrylamide gel containing 40% formamide and 7 M urea. However, as the length of dsRNA and/or GC content are increased, we anticipate that the effectiveness of formamide-urea gels will be diminished. In contrast, the effectiveness of a trap strand should be independent of the length or sequence of dsRNA used.

Moreover, formamide-urea gels are difficult to prepare, require fixing before exposure and are toxic.

Two technical problems have limited quantitative, mechanistic analysis of RdRPs: *(i)* the limited availability of high-quality, synthetic RNA and *(ii)* the poor resolution of dsRNA fragments by denaturing PAGE. The recent development of novel synthetic routes for production of RNA oligonucleotides (13) and the application of the methodology described herein, provide a solution to these problems. In addition, this methodology can be applied to the analysis of dsRNA fragments used in studies of dsRBM-containing proteins and to the analysis of exceptionally stable, GC-rich dsDNA or RNA-DNA hybrids. For example, addition of a trap strand can be used to dissociate labeled RNA from its complement in hydroxyl radical mapping experiments in which labeled RNA is lost due to incomplete denaturation of duplex and exclusion of this RNA from the gel (3). In addition, stops or compressions in known regions of DNA sequencing gels could potentially be removed by addition of a DNA trap strand.

It is plausible that a DNA trap strand may substitute for an RNA trap strand. This might serve to reduce the cost of the trap strand. However, the concentration of a DNA trap strand might need to be substantially greater than the corresponding RNA trap strand, owing to the reduced thermal stability of a RNA-DNA duplex relative to a RNA-RNA duplex. Of course, another alternative to chemically synthesized RNA that would diminish the cost of the trap strand is enzymatically synthesized RNA. As long as the transcription reaction is deproteinized, crude RNA transcripts should work well in this particular application. In conclusion, the use of a trap strand is a cost-effective, rapid and safe approach to improve the quality of data obtained from denaturing PAGE analysis of radiolabeled, dsRNA.

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