

Method for assigning double-stranded RNA structures

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Secondary structural motifs of RNA are composed of single- and double-stranded regions. Double-stranded RNA (dsRNA) participates in the antiviral functions of the interferon-induced protein kinase PKR, gene regulation, and gene silencing, or RNA interference (RNAi) (1–4). Thus, it is important to have accurate methods for identifying double-stranded segments of RNA. Structure mapping with ribonuclease V1 (RNase V1), a double-stranded RNA-specific ribonuclease that also cleaves stacked single-stranded regions, is a common approach for detecting double-stranded segments (5–9). However, it has been uncertain how to assign bands.

Standard enzymatic structure mapping involves limited digestion of an end-labeled RNA with various nucleases that are structure- or sequence-specific. Most commonly, the 5' end of the RNA is radiolabeled, and digestion reactions are fractionated by denaturing polyacrylamide gel electrophoresis (PAGE) alongside hydrolysis and sequencing ladders. The hydrolysis ladder enables numbering of bands, while a sequencing ladder, such as an RNase T1 digestion (G-specific), enables assignment of bands (Figure 1). If the RNA is 5' end labeled, then both ladders comprise labeled products with a 3'-terminal phosphate, as with many of the commonly used ribonucleases (e.g., RNases T2, A, U2, *Phy* M, and CL3). Conversely, RNase V1 leaves a 3'-terminal hydroxyl (5–9).

Assigning structured regions digested by RNase V1 has been problematic. In some instances, the lengths of labeled RNase V1 products have been estimated by comparison to fragments produced by nonsequence-specific nucleases that leave

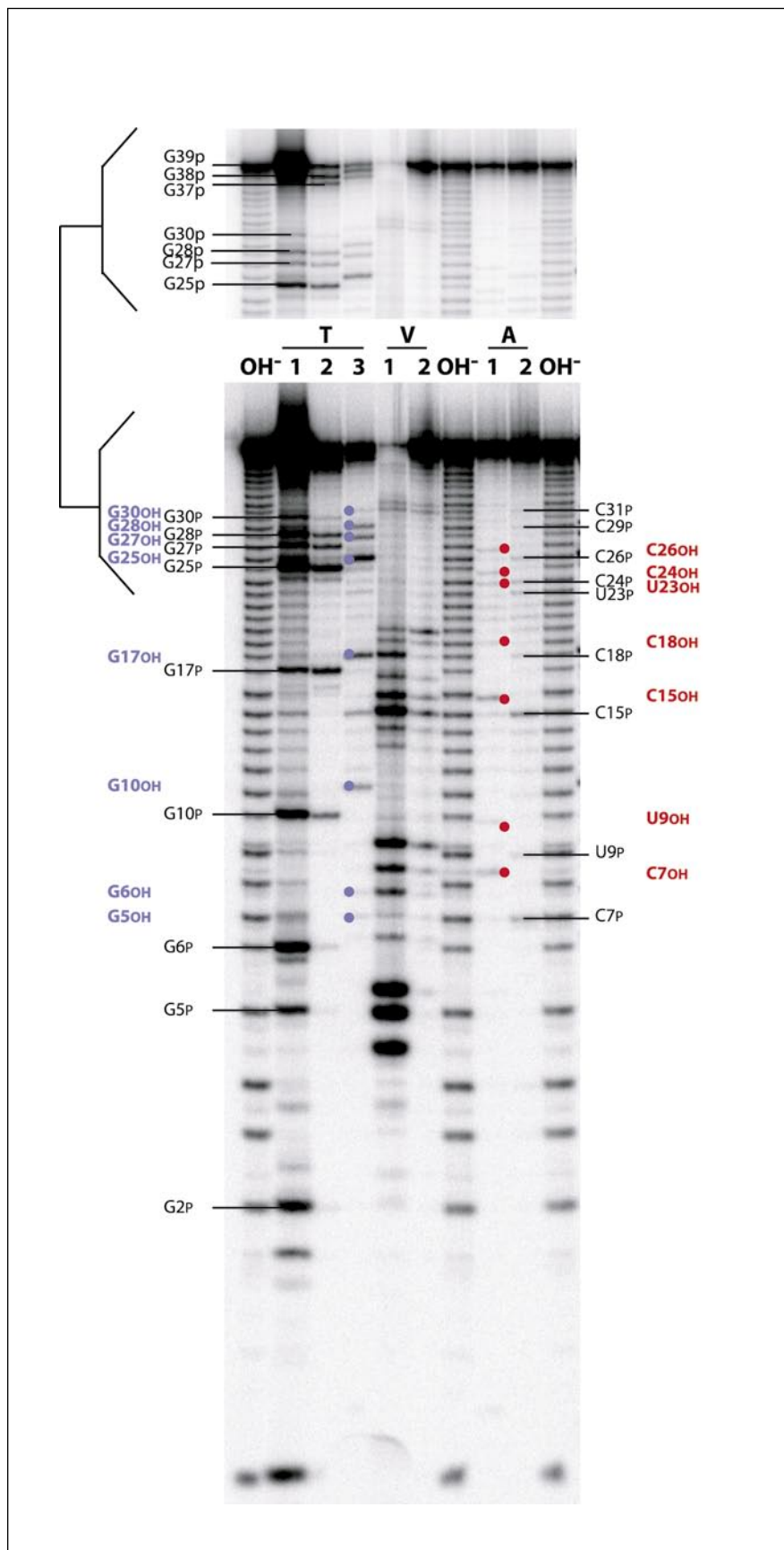
the same termini, such as nuclease S1 or Mung Bean nuclease, combined with comparison to sequence-specific ribonucleases that leave the incorrect termini, such as RNase T1 (7,10–12). However, this provides minimal confidence in assignments, since the relative electrophoretic gel mobility of various oligonucleotides with a 3'-terminal phosphate versus hydroxyl is unknown. While it has been demonstrated that short (<10 nt) RNase V1 products have reduced mobility relative to RNase T1 products (13,14), it is not obvious that the migration of these products should differ by only one nucleotide, as is commonly assumed. Moreover, since removal of the 3'-terminal phosphate has a large effect on charge but a minimal effect on size and shape, we reasoned that it might affect the mobility of even longer oligonucleotides (e.g., approximately ≥ 40 nt). Herein, we describe a simple method that produces appropriate RNase V1 sequencing ladders from T4 polynucleotide kinase (PNK) modification of RNase T1 and RNase A digests.

In addition to phosphorylating the 5' hydroxyl terminus of DNA or RNA, T4 PNK (New England Biolabs, Beverly, MA, USA) has a nonsequence-specific 3' phosphatase activity that can remove a 2', 3', or 2',3'-cyclic phosphate (15–17). We found T4 PNK useful for converting RNase T1 and RNase A sequencing ladders into ladders with 3'-terminal hydroxyls. A synthetic 39-nt RNA (Dharmacon, Lafayette, CO, USA), 5'-GGCCG-GCAUGCUGCCAGCCUCCUCGCG-GCGCCGGCUGGG-3', was chosen for study since it contains double- and single-stranded segments and is long enough to allow terminal phosphate effects on electrophoretic mobility

of short (<10 nt) and long (>20 nt) products to be determined. This oligonucleotide (39-mer) mimics a folding intermediate of the hepatitis delta virus (HDV) ribozyme (18). The 39-mer was 5' end-labeled and subjected to limited digestions under native conditions by RNases T1, V1, and A and denaturing conditions by RNase T1 and alkaline hydrolysis (Figure 1). In certain instances, the RNase T1 and A digestions were followed by T4 PNK treatment.

The 39-mer was deblocked according to the manufacturer's instructions and desalted on a C₁₈ Sep-Pak[®] Cartridge (Waters, Milford, MA, USA). The resulting RNA pellet was suspended in 1× TE (10 mM Tris, pH 8.0, 1 mM EDTA) and 5' end-labeled by T4 PNK using [γ -³²P]ATP (PerkinElmer Life and Analytical Sciences, Boston, MA, USA). The labeled 39-mer was PAGE-purified [10% polyacrylamide (29:1)/7.0 M urea/1× Tris-borate EDTA (TBE)] and structure-mapped with RNases T1 (0.01 U/ μ L), V1 (0.002 and 0.0001 U/ μ L), and A (1 ng/mL). The RNA was heated to 90°C for 2 min and cooled on bench top for 10 min, followed by addition of RNA Structure buffer (Ambion, Austin, TX, USA). Titrations of nucleases (Ambion) were carried out to find the optimal single-digestion conditions. Digestion reactions were stopped using Inactivation/Precipitation buffer (Ambion), precipitated, and dissolved in sterile water. To afford complete removal of the 3'-terminal phosphate, digestion products (2.4 nM) were incubated with T4 PNK (1 U/ μ L; T4 PNK buffer) at 37°C for 30 min. T4 PNK treatment was also performed *in situ* with RNase T1, which allowed for partial elimination of the 3' phosphate-terminated species (data not shown). Other conditions for driving complete removal of the terminal phosphate have been described (14,15,17). All samples were fractionated on a 20% polyacrylamide (29:1)/7 M urea/1× TBE gel (Figure 1).

Comparing T4 PNK-treated (Figure 1, lanes T-3 and A-1) to T4 PNK-untreated (Figure 1, lanes T-2 and A-2) digestions revealed that the migrations of products are retarded in treated lanes. Next, we compared the RNase V1 digestions (Figure 1, lanes V-1 and V-2)



to the T4 PNK-treated digestions. In all cases where a comparison was possible, RNase V1 products co-migrated with bands in treated lanes. Moreover, it is worth noting that, in many instances, bands in the hydrolysis ladder do not align with bands in the RNase V1 or T4 PNK-treated lanes. This effect is particularly noticeable for G1, C3, C4, G6, C7, A8, and G10 (Figure 1).

The aforementioned assumption that short RNase V1 products migrate one nucleotide slower than RNase T1 and hydrolysis products would have led to incorrect labeling of several bands (e.g., G10 as C11 and G6 as C7 in Figure 1, lane T-3). Also, slower electrophoretic mobility of the T4 PNK-treated samples is found not only for shorter digestion products, but also for products longer than 30 nt (Figure 1).

In summary, T4 PNK-modified RNase T1 and RNase A ladders enable definitive assignment of the products of an RNase V1 digestion. Modification of RNases T1 and A digestion products is a simple and efficient method that should be extendable to other ribonucleases such as RNase U2 (A-specific). This approach should lead to increased accuracy in the identification of biologically significant dsRNA structures.

Figure 1. Sequencing of RNase V1 structure mapping products. Lanes labeled OH⁻ are an alkaline hydrolysis under denaturing conditions. Remaining lanes show limited digestions of 5' end-labeled 39-mer under native conditions using RNases T1 (G-specific), V1 (dsRNA-specific), and A (C, U-specific). Lanes T-1 and T-2 are standard denaturing and native RNase T1 digestions, respectively. Lane T-3 is an ethanol-precipitated fraction of T-2 that was subsequently treated with T4 polynucleotide kinase (PNK) to remove the 3'-terminal phosphate. Lanes V-1 and V-2 are native digestions using 0.002 and 0.0001 U/ μ L RNase V1, respectively, showing a range of bands with a 3'-terminal hydroxyl. Lanes A-1 and A-2 are native digestions, whereas A-1 is an ethanol-precipitated fraction of A-2 that was subsequently treated with T4 PNK to remove the 3'-terminal phosphate. Sequence assignments of bands are shown along the sides of the gel. The left side provides assignments for the RNase T1 lanes (PNK-treated band assignments are indicated by a blue dot "•"), while the right side provides assignments for the RNase A lanes (PNK-treated band assignments are indicated by a red dot "•"). A "P" following a nucleotide indicates that it has a 3'-terminal phosphate, while an "OH" indicates absence of a 3'-terminal phosphate. The upper portion of the gel is reproduced at the top of the figure using a higher maximal intensity scale to allow details of this region to be discerned.

Indeed, very recently a PNK-treated RNase T1 ladder was used to align Dicer and RNase III-digested RNAs (19).

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Extending AFLP sequences by long template PCR amplification of a fungal genomic library

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In this research, amplified fragment-length polymorphism (AFLP)-based differential display was used to identify gene sequences that were differentially expressed in a UV mutant and a wild-type isolate of the filamentous fungus *Sclerotinia sclerotiorum*. One known gene (*S. sclerotiorum* hexose transporter, GenBank® accession no. AAT74609.1) and several novel gene sequences were identified by this method. We sought to obtain full sequences of these genes from a genomic library of *S. sclerotiorum*. The conventional method for retrieving sequences from a genomic library is repeated rounds of library plating, plaque lifts, and hybridization to a labeled probe in order to eventually isolate and amplify individual phage clones containing the desired gene

sequence (1,2). This method is quite time-consuming and requires access to resources for Southern blot analysis. A PCR-based method, inverse PCR (3), can also be used to extend short sequences directly from genomic DNA, however, we have not found that method to be very rapid or to work consistently. Here we describe a simple, rapid, and reliable method that we have used repeatedly to extend gene sequences from a genomic library.

An *S. sclerotiorum* genomic library was created as follows: *S. sclerotiorum* genomic DNA was partially digested with the restriction enzyme *Sau3AI* (Roche Diagnostics, Indianapolis, IN, USA). The DNA fragments were size-fractionated on an agarose gel, and 5–12 kb fragments were excised and purified with a QIAEX® II gel