Unexpected Electrophoretic Migration of RNA with Different 3′ Termini Causes a RNA Sizing Ambiguity That Can Be Resolved Using Nuclease P1-Generated Sequencing Ladders†

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ABSTRACT: It has been widely believed that the electrophoretic migration difference of otherwise identical RNAs with a P versus OH terminus would be the same as occurs for DNA, a fairly reproducible ∼1/2 nucleotide (nt) offset. RNA with a 5′-OH indeed migrates ≤1 nt slower than if it had a 5′-P. Surprisingly, however, RNA with a 3′-OH terminus (generated by many cellular RNases of interest) migrates anywhere from ∼1/4 to ∼2 nts slower than the otherwise identical molecule with a 3′-P or 2′,3′-cyclic-P terminus (as present on standard RNase-generated sequencing ladders). This previously unrecognized variability in electrophoretic migration offset causes a 1−2 nt ambiguity in a commonly used method of RNA size determination. We also show two ways to overcome this problem and enable rigorous sizing of 3′-OH terminating RNAs. Most convenient is to use sequencing standards generated by nuclease P1, which is generally sequence-nonspecific but we show becomes G-specific or A-preferential under certain reaction conditions.

Accurate electrophoretic sizing of nucleic acid molecules using sequencing standards prepared from the same nucleic acid is a fundamental technique of molecular biology. Molecules frequently sized in this manner include the products from numerous RNA processing enzymes, structure-sensitive nucleases, transcriptional termination, reverse transcriptase, S1 nuclease, and chemical cleavage reagents (e.g., *1*−*5*). For DNA sizing, end-labeled fragments and homologous dideoxy or chemical sequencing ladders (*6*, *7*) precisely comigrate if they have the same terminal phosphate (P) or hydroxide (OH) character, while an extra P on either end causes a reproducible ∼1/2 nt faster migration (*2*). Thus, 1/2 nt migration correction enables faithful sizing of DNA fragments using sequencing standards that have a different P or OH terminus.

For RNA sizing, sequencing ladders from the homologous end-labeled molecule are commonly prepared using RNases T1, A or *B. cereus*, U2, CL3, and *Phy M*, as well as alkali, which all initially generate 2′,3′-cyclic phosphate (2′,3′-cyclic-P) and 5′-OH termini (*8*−*10*). (These enzymes cleave 3′ of G, C + U, A, C, U + A, and any residue, respectively.) The RNases then more slowly convert the cyclic phosphate to a 3′-monophosphate (*10*−*12*), although it remains unclear which terminus predominates in the partial digests used for sequencing ladders. In contrast to these sequencing RNases, many cellular nucleolytic events of interest generate 3′-OH and 5′-P termini (*10*). These include cleavages at the 3′ and 5′ ends of tRNAs, rRNAs, and snoRNAs, at the 3′ end of pre-mRNAs, at trypanosome RNA editing sites, and by several structure-specific enzymes including mung bean, S1, and cobra venom nucleases. When electrophoretically compared to their homologous, enzymatically generated sequencing ladders, these cleavage products generally migrate out of phase (e.g., *4*, *5*, *13*−*18*) due to the different P or OH termini. It has been widely assumed that RNA molecules with an extra terminal P, like DNA, will migrate ∼1/2 nt faster than the equivalent molecule with a terminal OH, and that this will be applicable whether the difference is 5′ or 3′.

Alternatively, RNA sequencing ladders can be prepared by chemical reactions, involving a specific base modification and cleavage, generally by aniline (*19*). Because the downstream cleavage product has a normal 5′-P terminus, these chemically generated markers are often used for 3′-labeled RNA. However, the upstream chemical cleavage product does not end with a normal nucleotide, but rather unphosphorylated, abasic, cleaved ribose derivatives (*3*; Peattie, personal communication). Hence, such chemical reactions do not produce equally clear sequencing ladders from 5′-labeled RNA and generally are not used for its analysis (*19*; D. Peattie, personal communication).

This paper demonstrates that the migration offset of RNAs (∼20−200 nts in length) caused by an extra 5′-P is between ∼1/4 and ∼1 nt, but that the offset caused by an extra 2′,3′-cyclic-P (or 3′-P) can vary from ∼1/4 to ∼2 nts. Due to this previously unrecognized variability, it is important that the sequencing ladders have the same terminal P or OH character as the experimental RNA to prevent errors in size determination. We also demonstrate that nuclease P1,

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[1] Abbreviations: nt(s), nucleotide(s); OH, hydroxyl; P, phosphate; 2′,3′-cy′P, 2′,3′-cyclic phosphate; CYb, cytochrome oxidase subunit 3; A6, ATPase subunit 6.

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generally recognized as sequence-nonspecific, can be made to cleave in a G-specific or A-preferential manner, forming 3′-OH and 5′-P termini. Thus, nuclease P1 conveniently generates RNA sequencing ladders that enable RNAs with 3′-OH termini to be rigorously sized.

EXPERIMENTAL PROCEDURES

RNA Substrates. CYb-preedited mRNA (208 nt) and A6-preedited mRNA (73 nt) were synthesized by T3 and T7 RNA polymerases, respectively, as previously described (*5*, *20*). RNAs were 5′-end-labeled using [γ-^{32}P]ATP and polynucleotide kinase following phosphatase treatment or were 3′-end-labeled using [^{32}P]pCp and T4 RNA ligase as described (*20*). Alternatively, RNA was 5′-end-labeled with a thiophosphate using [γ-^{35}S]ATP (4500 Ci/mmol, 0.5 pmol/μL), polynucleotide kinase (0.8 unit/μL), and dephosphorylated CYb mRNA (1 pmol/μL) for 45 min at 37 °C; a second dose of enzyme was then added for an additional 20 min to boost incorporation in this very inefficient labeling reaction. All RNAs were purified from 6 to 9% polyacrylamide gels.

Enzymatic Treatments and Gel Electrophoresis. All enzymatic digestions were carried out for 5−15 min at 50 °C, except where indicated, using ~5 × 10^4 cpm of substrate RNA plus 1 μg of tRNA as carrier at the following buffer conditions in 10 μL. For the nuclease P1 reactions, the pH was found to be the primary determinant in the cleavage specificity, but the other components of the buffer, especially urea, also influenced specificity (data not shown). For G-specific ladders by nuclease P1 (Pharmacia or BRL), substrate RNAs were treated with 5−10 units of enzyme in 20 mM sodium phosphate or sodium citrate (pH ~9), 6.5 M urea, 1 mM $ZnCl_2$, and 0.04% (w/v) xylene cyanol; then, following denaturation at 95 °C for 2 min, aliquots were directly loaded on the gel. [The ability of nuclease P1 to cleave G-specifically was noted in a recent publication (*21*) which referenced the current article for the method, but it did not compare the migration effects of different 3′ termini or provide indication of their anomalous offsets.] For A-selective ladders by nuclease P1, substrate RNAs were incubated with ~0.01 unit of enzyme in 20 mM NaOAc (pH 5), 0.5 mM $Zn(OAc)_2$, and 12% glycerol at 37 °C for 15 min; these reactions were then ethanol-precipitated, resuspended, and denatured prior to gel loading. For G-specific ladders by RNase T1, substrate RNAs were incubated with 0.5−1 unit of enzyme (Pharmacia) in 33 mM sodium citrate (pH 5), 1.5 mM EDTA, 9.5 M urea, and 0.04% (w/v) xylene cyanol; then, following denaturation, aliquots were directly loaded on the gel. 2′,3′-Cyclic phosphodiesterase (2′,3′-cyclic nucleotide 3′-phosphodiesterase, P-6274; Sigma) reactions were carried out using RNase T1-cleaved substrate RNA and 1.5 units of enzyme in 50 mM Tris (pH 8) for 15 min at 25 °C. Calf intestinal phosphatase reactions were carried out using RNase T1 or nuclease P1-cleaved RNA and 1 unit of enzyme (Boehringer Mannheim) in 50 mM Tris (pH 8.0), 0.1 mM EDTA, 5 mM DTT, and 20 units of RNasin (Promega) for 15 min, first at 37 °C, and then at 50 °C. Mung bean nuclease digests were carried out as previously described (*20*). Electrophoresis was in Tris−borate−EDTA (pH 8.3) buffer, using 6−9% denaturing polyacrylamide gels (although gels between 4% and 20% have been used), as described (*22*).

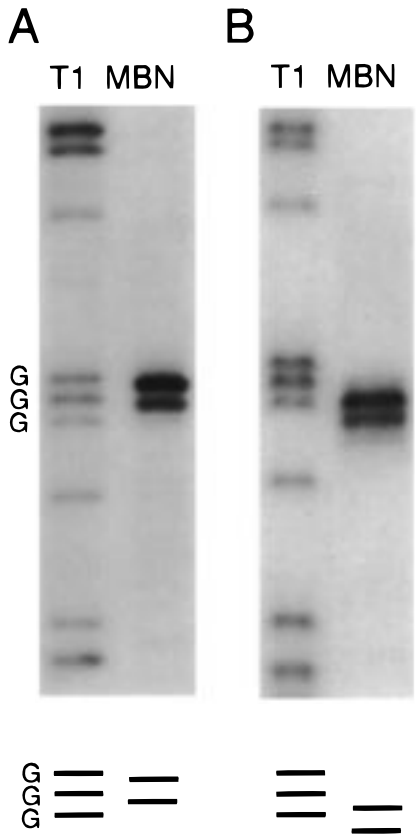

FIGURE 1: Variable electrophoretic migration offset caused by different 3′-terminal groups on RNA. 5′-^{32}P-labeled CYb pre-mRNA was treated with RNase T1 (T1) or mung bean nuclease (MBN), analyzed on 6% polyacrylamide sequencing gels, and detected by autoradiography in two separate experiments (A, B). Below, the relative band positions are diagrammed. The G triplet is ~90 nts in length. The accurater sizing of these MBN fragments is shown in Figure 3.

RESULTS

Variable Migration Effects Caused by Different 3′ Termini of RNA. In probing the structure of *Trypanosoma brucei* mitochondrial pre-mRNAs using mung bean nuclease (*16*), which generates 3′-OH termini, we noted that the experimental RNA migrated differently on different gels, relative to RNase-generated sequencing standards of the same 5′-end-labeled RNA. This is illustrated in Figure 1, where identically prepared mung bean nuclease digests of 5′-end-labeled cytochrome *b* pre-mRNA were run alongside RNase T1-generated G-ladders of the same RNA. Although it was expected that the experimental and marker fragments would migrate out of phase since they have 3′-hydroxyl and 3′-phosphorylated termini, respectively (*10*), it was unexpected that their offset would vary in different electrophoretic gels by almost 2 nts (Figure 1; diagrammed below). Control studies showed that the characteristic doublet of bands generated from this RNA in various mung bean nuclease digestion reactions coelectrophoresed, indicating the cleavage sites are reproducible (data not shown). Thus (5′-labeled) RNA with a 3′-OH cannot be faithfully sized using standard RNase-generated markers that have 3′-phosphorylated termini, since their relative migration differs in different electrophoretic gels. The magnitude of the migration offset does not correlate with readily controllable properties, such as fragment size, gel percentage, buffer, or pH (see below).

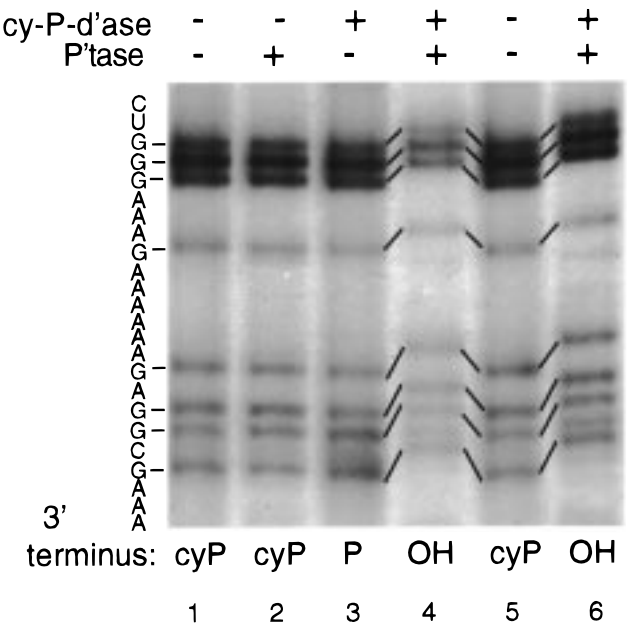

FIGURE 2: Relative mobility of RNAs with 2′,3′-cyclic-P, or 3′-P, or 3′-OH termini. After 5′-labeling with phosphatase-resistant [^{35}S]-thiophosphate, CYb pre-mRNA was treated with RNase T1 (lanes 1, 5) and additionally with phosphatase (lane 2), 2′,3′-cyclic phosphodiesterase (lane 3), or 2′,3′-cyclic phosphodiesterase followed by phosphatase (lanes 4, 6). RNAs were analyzed as in Figure 1. Corresponding bands are indicated by lines. This gel shows from ~70 to ~90 nts in length.

We next examined whether conversion of RNase T1-generated ends from their initial 2′,3′-cy-P to a 3′-P could affect electrophoretic migration and possibly explain the variable migration offset of Figure 1. From the literature, it was unclear which of these ends predominate in standard sequencing ladders. To this end, we used RNA with a phosphatase-resistant 5′-[^{35}S]thiophosphate label, that can be retained while enzymatically altering the 3′ terminus of the RNA (L.N.R. and B.S.-W., unpublished). A G-ladder was prepared using RNase T1 (lanes 1, 5), and aliquots were then treated with 2′,3′ cyclic phosphodiesterase which converts 2′,3′-cyclic-P to 3′-P termini, with phosphatase which removes 3′-P but not 2′,3′-cyclic-P termini, or with both enzymes. Phosphatase treatment does not perceptibly affect the electrophoretic migration (lanes 1, 2), and 2′,3′-cyclic phosphodiesterase causes the fragments to migrate only minimally faster (lane 3), but their combined action causes the RNA to migrate substantially slower (lanes 4, 6). Thus, the sequencing fragments generated by RNase T1 retain their 2′,3′-cyclic P termini, and they migrate very similarly to otherwise identical RNA with 3′-P termini (lanes 1−3), but considerably faster than the same RNA with 3′-OH termini (lane 4). This migration offset is ~1.25 nts in Figure 2 but can range from ~1/4 to ~2 nts in different experiments (see ahead to Figure 3, and data not shown). These results underscore that assuming a standard migration offset would cause an ambiguity of 1−2 nts in RNA sizing. They also demonstrate that the variable migration offsets observed in Figure 1 could not be accounted for by a differential conversion of the markers to 3′-P termini.

Diagnostic Sequencing Ladders Can Be Generated Using Nuclease P1. The above data demonstrate that accurate mapping of the 3′ end of a (5′-labeled) RNA fragment should be performed with sequencing ladders bearing the same 3′ terminus. While 5′-[^{35}S]thiophosphate-labeled RNA could be used, preparing the ladders as in Figure 2, lane 4, the initial ^{35}S labeling reaction is very inefficient. Using chemically generated sequencing ladders for 5′-labeled RNAs would also present a problem, for they do not end with normal 3′-OH nucleotides but with abasic, cleaved ribose derivatives (*3*; *19*; D. Peattie, personal communication). Seeking to prepare 3′-OH-bearing sequencing standards, we turned to nuclease P1, which is known to generate 3′-OH and 5′-P termini but has traditionally been used as a sequence-nonspecific RNase (e.g., *23*, *24*). However, we found that under certain reaction conditions nuclease P1 is strikingly sequence-specific. At pH ~9, especially in urea, nuclease P1 is G-specific (Figure 3A, lanes 1, 2). Its partial digest appears very similar, but offset, compared to a RNase T1 digest (which ends in 2′,3′-cyclic P). As expected, nuclease P1-generated G-ladders migrate in phase with the mung bean nuclease-generated fragments (lanes 3, 4), which also end in 3′-OH. Notably, the migration offsets of the ladders prepared with RNase T1 and nuclease P1 differ between ~1/4 and ~2 nts, and can span virtually this entire range over the length of a single gel (lanes 1, 2). While in some gels the shorter fragments have a bigger offset, other gels show the reverse (data not shown), reinforcing the unpredictable magnitude of the migration offset. Thus, nuclease P1-generated G-specific ladders permit convenient and accurate mapping of 3′-OH termini of 5′-labeled RNA.

We also noted that at pH 5, especially in the absence of urea, nuclease P1 preferentially cleaves RNA 3′ of A residues, again generating 3′-OH and 5′-P termini (Figure 3B). Under these conditions, there is also cleavage within oligo(U) tracks but to a lesser extent than at the nearest A residues. The strongest bands of such a nuclease P1 digest appear much like an offset version of the A-specific ladder generated by RNase U2, which has 3′-phosphorylated termini (lanes 2, 3). Thus, in combination with a standard RNase sequence determination, these two kinds of nuclease P1-generated markers permit precise mapping of the 3′-OH end of any RNA fragment, not only ones that terminate in G residues.

Electrophoretic Effects of Different 5′ Termini on RNA. To determine whether OH versus P differences at the 5′ end of RNAs cause as aberrant migration as they do at the 3′ end, we repeated the analysis on 3′-labeled RNA. Comparison of G-ladders generated by nuclease P1 (that have 5′-P termini) and by RNase T1 (that have 5′-OH termini) shows a more modest but still significantly variable migration offset. In different gels, analyzing RNAs from ~20 to ~200 nts, molecules with a 5′-P migrate ~1/4 to ~1 nt faster than an otherwise identical RNA with a 5′-OH terminus (Figure 4 and data not shown). As expected, phosphatase treatment of the nuclease P1- and RNase T1-generated ladders causes them to precisely comigrate (data not shown). Thus, to rigorously size (3′-labeled) RNAs that end in a 5′-P, one could use sequencing ladders prepared chemically or by nuclease P1, or one could phosphatase-treat standard enzymatically generated marker RNAs and the experimental RNA.

DISCUSSION

Many cellular events of interest generate RNA ends that are 3′-OH, yet sequencing ladders standardly used to map

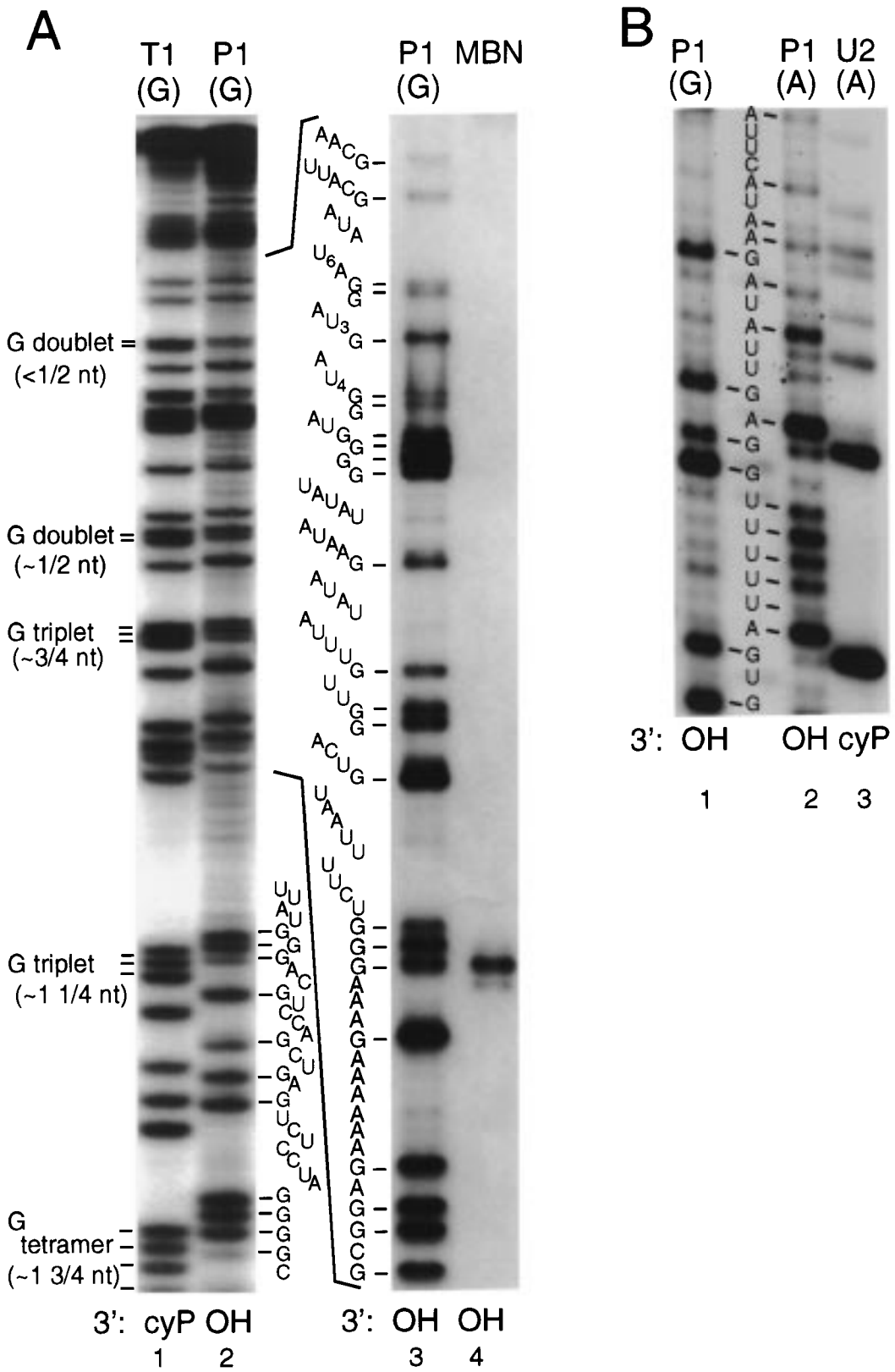

FIGURE 3: Nuclease P1 can generate G-specific or A-preferential sequencing ladders terminating in a 3′-OH. 5′-^{32}P-labeled CYb pre-mRNA (panel A) or ATPase6 pre-mRNA (panel B) was treated with nuclease P1, RNase T1, mung bean nuclease, or RNase U2 and analyzed as in Figure 2. The nuclease P1 reactions were conducted under the G-specific conditions (panel A, and panel B, lane 1) or the A-selective conditions (panel B, lane 2) (see Experimental Procedures). These gels show from 15 to >150, ~70 to ~165, and ~40 to ~65 nts, respectively. The varying migration offset is shown in panel A in parentheses.

them are 3′-phosphorylated, and it is known that nucleic acids with different termini exhibit electrophoretic migration offsets. With DNA, an extra P at either the 5′ or the 3′ terminus causes ~1/2 nt faster migration than a OH (*2*), allowing accurate mapping using homologous sequencing ladders that have a different P/OH character by applying a 1/2 nt migration correction (Table 1). It has been assumed that this same migration offset would apply to RNA, but

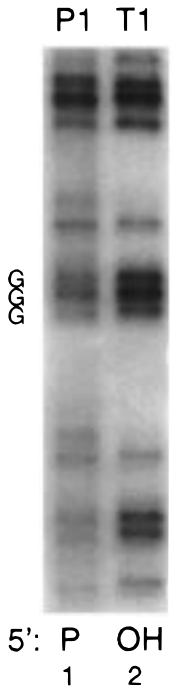

FIGURE 4: Relative mobility of RNAs with a 5′-P or 5′-OH termini. After 3′-labeling with ^{32}P-pCp, CYb pre-mRNA was treated with nuclease P1, RNase T1, or mung bean nuclease and analyzed as in Figure 2. These gels show from ~100 to ~135 nts.

Table 1: Electrophoretic Migration Offset Caused by Different Termini on RNA or DNA

terminus	charge difference	migration offset (nts) in RNA[a]	migration offset (nts) in DNA[b]
5′−P vs 5′-OH	−2	~1/4−1	~1/2 nt[c]
3′−P vs 3′-OH	−2	~1/4−2	~1/2 nt[c]
2′,3′-cy-P vs 3′-OH	−1	~1/4−2	NA
3′−P vs 2′,3′-cy-P	−1	~0	NA

[a] Faster migration of an RNA bearing the indicated phosphorylated terminus, relative to an otherwise identical RNA bearing the second noted terminus. [b] Faster migration of a DNA bearing the indicated phosphorylated terminus, relative to an otherwise identical DNA bearing the indicated OH terminus. [c] From ref 2.

when comparing (5′-labeled) RNA with different 3′ termini, we found a surprising variability. Fragments with 3′-P or 2′,3′-cyclic-P termini (15−300 nts in length) migrate extremely similarly, but anywhere from ~1/4 to ~2 nts faster than otherwise identical RNAs with 3′-OH termini (Figures 2 and 3). RNA fragments (20−200 nts) with different 5′ termini also exhibit a variable migration offset, a 5′-P causing ~1/4 to ~1 nt faster migration than a 5′-OH termini (Table 1; Figure 4). While a few publications show migration offsets of up to ~3 nucleotides for shorter RNA oligomers bearing P vs OH termini, it appears to have been assumed that such migration offsets will not occur for RNAs over ~10 nucleotides in length (see *13*, *25*−*27*). Thus, for the frequent cases where RNA molecules >10 nucleotides in length are sized, this variable migration offset is a previously undocumented significant concern. It is especially an issue for fragments with 3′-OH termini where there is no corresponding downstream fragment whose 5′ terminus could be independently mapped, such as one formed by an exonuclease.

The large and variable migration offset we observed for RNA with 3′-OH versus 3′-phosphorylated termini was unexpected. DNAs with 3′-P versus OH termini differ in charge by two electron units and exhibit ~1/2 nt migration offsets, so one might expect RNAs with 3′-P versus OH termini to behave similar by (Table 1). Furthermore, RNase T1-generated G-ladders end with 2′,3′-cyclic-P not 3′-P groups (Figure 2), and it could be expected that these ends which differ in charge by only one electron unit from a 3′-OH terminus would cause a still smaller migration offset. However, much larger RNA migration offsets are generally observed (Figures 2 and 3; Table 1).

The potential error in mapping 3′-OH termini of RNA when using homologous RNase-generated ladders (with 3′-phosphorylated termini) is not merely a theoretical consideration. A cursory review of the literature revealed over 100 studies, published in this and other major journals, in which 5′-labeled RNAs with a 3′-OH terminus were sized to the nucleotide using such RNase-generated sequencing ladders. In general, either no migration offset was noted and the experimental fragment was deduced to be the size of a similarly migrating or spliced sequencing marker, or a migration offset was noted and a ~1/2 nt correction was applied. Since our determinations frequently show migration offsets of ≥1 nt, we submit that these previous determinations could be in error by 1−2 nts. Indeed, there are several examples where multiple groups performed similar structural analyses on the same 5′-labeled RNA but concluded different cleavage, likely due to their gels exhibiting different migration offsets. While a few reports do note that migration offset could be an issue for RNA (e.g., *15*), no direct method of resolving it had been presented. And while nuclease P1 had been used previously under sequence-nonspecific cleavage conditions to yield a ladder of bands (e.g., *13*, *24*, and references cited therein), examination of these gels generally reveals that these bands appear to change register by 1−2 nts relative to RNase-generated ladders across the length of the gel. So the prior use of nuclease P1 did not serve to size RNA with different 3′ termini.

Despite appreciable effort, we have not identified electrophoretic conditions where RNAs ending in 3′-OH versus 3′-phosphorylated termini migrate offset by a small or reproducible amount (data not shown). We analyzed RNAs 15−300 nts in length and found similarly large and variable migration offsets when the gel and running buffers were Tris−phosphate−EDTA, Tris−acetate−EDTA, or Tris−borate−EDTA and when the pH was varied from ~8.4 to ~7. The migration offset was also not reproducibly affected by the percentage of the gel or the time of polymerization. Notably, the migration offset frequently differs by more than a nucleotide between the top and bottom portions of a single gel (Figure 3). While the bigger offset is sometimes observed for the shorter fragments, other gels show the reverse, further confounding any simple prediction of the migration offset. Thus, accurate direct RNA sizing requires that the experimental and the marker fragments have the same 3′-terminal character. To conveniently resolve this problem, we have found that nuclease P1 under certain cleavage conditions is markedly sequence-specific, cleaving in a G-specific or A-preferential manner (Figure 3). This facilitates preparation of RNA sequencing markers to unambiguously size RNAs that end in a 3′-OH.

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