The Physiological Role of RNase T Can Be Explained by Its Unusual Substrate Specificity*

Yuhong Zuo and Murray P. Deutscher‡

From the Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, Florida 33101-6129

RNase T, one of eight exoribonucleases present in Escherichia coli (1), was originally identified as an activity involved in the end-turnover of tRNA and for the 3’ maturation of 5 S and 23 S rRNAs and many other small, stable RNAs, was examined in detail with respect to its substrate specificity. The enzyme was found to be a single-strand-specific exoribonuclease that acts in the 3’ to 5’ direction in a non-processive manner. However, although other Escherichia coli exoribonucleases stop several nucleotides downstream of an RNA duplex, RNase T can digest RNA up to the first base pair. The presence of a free 3’-hydroxyl group is required for the enzyme to initiate digestion. Studies with RNA homopolymers and a variety of oligoribonucleotides revealed that RNase T displays an unusual base specificity, discriminating against pyrimidine and, particularly, C residues. Although RNase T appears to bind up to 10 nucleotides in its active site, its specificity is defined largely by the last 4 residues. A single 3’-terminal C residue can reduce RNase T action by >100-fold, and 2-terminal C residues essentially stop the enzyme. In vivo, the substrates of RNase T are similar in that they all contain a double-stranded stem followed by a single-stranded 3’ overhang; yet, the action of RNase T on these substrates differs. The substrate specificity described here helps to explain why the different substrates yield different products, and why certain RNA molecules are not substrates at all.

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‡ To whom correspondence should be addressed. Tel.: 305-243-3150; Fax: 305-243-3953; E-mail: mdeutsch@med.miami.edu.
nucleotides were 5'-labeled using [γ-32P]ATP and T4 polynucleotide kinase. Duplex-containing substrates were prepared by heating mixtures of a 5'-32P-labeled oligonucleotide and its complementary oligonucleotide (molar ratios, ~1:1.2) to 100 °C for 5 min followed by slow cooling to room temperature to anneal the two strands. RNase T reactions were carried out in mixtures containing 20 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 50 mM KCl, 5 mM dithiothreitol. For qualitative analysis, oligonucleotide substrates at 25 μM were present in each reaction with either 1× (1.1 ng/μl) or 10× (11 ng/μl) RNase T. Reaction mixtures were incubated at 37 °C for the times indicated. Reactions were stopped with 2 volumes of loading buffer containing 96% formamide and 1 mM EDTA. Reaction products were resolved on 22.5% denaturing polyacrylamide gels. Quantitative data were obtained using a PhosphorImager (Amerham Biosciences) to determine the amount of each band present. For kinetic analyses, the amount of RNase T used for each substrate was adjusted so that only 10–20% of the substrate molecules were acted upon. The total release of nucleotide was calculated from the percentage of radioactivity in the bands in each lane. If multiple reaction products were generated, the reaction rate was a weighted summation of all the bands. Thus, for example, if 1, 2, or 3 nt were removed, the corresponding bands would be weighted by multiplying by 1, 2, or 3, respectively. Kinetic constants were calculated using Lineweaver-Burk plots.

**Preparation of a Substrate with a 3'-Phosphate Terminus**—An oligonucleotide substrate containing a 3'-phosphate terminus (A16P) was prepared by periodate oxidation of A17 in lysine-HCl buffer.

**Preparation of a Substrate with a 3'-Dideoxy Terminus**—A DNA substrate containing a 3'-dideoxy terminus was prepared by primer extension in a typical DNA sequencing reaction using bacteriophage T7 DNA polymerase (Sequenase). The template DNA oligonucleotide used was the 22-mer, 5'-TATCCGCCGCTGCCACGCC-3', and the 5'-32P-labeled primer was the 13-mer DNA oligonucleotide, 5'-32P-GCCAAAGCGCCCA-3'. Only dTTP and ddATP were added for the elongation reaction, so that the fully extended primer product would contain a 3'-dideoxy terminus. In a second experiment, performed at the same time, dATP replaced ddATP to generate a 3'-OH-terminated substrate of the same sequence. The Sequenase-extended products were boiled for 5 min and kept on ice until use. They were used directly in an RNase T assay without further treatment.

**RESULTS**

**Oligonucleotides Serve as Mimics for RNase T Action on tRNA**—RNase T is known to play an important role in tRNA end-turnover and 3' maturation. As a first step to examining the details of RNase T's substrate specificity, we investigated whether oligonucleotide substrates could be used to accurately mimic the enzyme's action on tRNA and on tRNA precursors, thereby simplifying identification of the specificity determinants. The oligonucleotides examined were derived from sequences at the 3'-end of tRNA Tyr and its precursors. These included the 11-mer, 5'-CCCCACCCCA-3', the 14-mer, 5'-CCCCACCCCAACAUCA-3', and the 17-mer, 5'-CCCCACCCCAACAUCAU-3'. These molecules correspond to the mature 3'-end of tRNA Tyr and to precursors with 3 or 6 extra 3' residues, respectively. In addition, the 7-mer complementary oligonucleotide, 5'-GGUGGGG-3', was also present in those experiments in which we mimicked the complete acceptor stem of the tRNA and the precursors. The use of these particular substrate analogues allowed comparison with earlier studies of the in vitro and in vivo action of RNase T on tRNA Tyr and tRNA Tyr 3' and 5' extensions (4, 14).

Fig. 1 (A–C) shows the action of RNase T on oligonucleotides that mimic the aminoacyl stems of mature tRNA Tyr and of its precursors with 3 or 6 extra 3' residues. As shown in Fig. 1A, RNase T slowly removes a single 3' terminal A residue from the mature tRNA analogue, but then essentially stops at the –CC sequence, in complete accordance with its action on full-length tRNA molecules (3). Likewise, RNase T rapidly removes the 3...
extra residues following the -CCA sequence in the precursor analogue to first generate an intermediate with 2 extra residues, followed by formation of the mature end, and eventually the /H11002 product (Fig. 1 B). Similar accumulation of the /H11001 intermediate was observed previously in studies of the maturation of tRNA1 Tyr and tRNATyrsu3 (4, 14). RNase T processing of the precursor analogue with 6 extra residues was considerably slower (Fig. 1 C), again in keeping with what was observed earlier with tRNAs (14). The data presented suggest that synthetic RNA oligonucleotides can serve as useful analogues to mimic RNase T action on tRNA precursors, thereby simplifying studies of its specificity.

**RNase T Acts on Single-stranded RNA**—Single-stranded RNA oligonucleotides are also acted on by RNase T in a manner similar to its action on the duplex-containing substrates (Fig. 1, compare D–F to A–C). However, one major difference observed between the ssRNA and dsRNA substrates was in the digestion of the 11-mer. In the presence of the 7-mer complementary strand (Fig. 1A), RNase T action on the 11-mer was significantly slower than when the 11-mer alone was used as substrate (Fig. 1D). Quantitative analysis indicated that the presence of the complementary 7-mer reduced the kcat 10-fold. These data suggest that proximity to a ds stem may slow RNase T action. It should be noted that even with ssRNA, RNase T does not digest through the CC sequence left at the 3'-end of the 11-mer once the terminal A residue had been removed. Interestingly, RNase T appears to act on the ss and dsRNA substrates in a stepwise manner, with each major intermediate containing a 3' terminal CC, UC, or CU doublet. The reason for this stepwise phenomenon will be examined in the following sections. Additional evidence for RNase T action on ssRNA comes from studies of RNA homopolymers (see below).

**RNase T Action Is Single Strand-specific**—RNase T is a major contributor to the final 3'-exonucleolytic trimming during the maturation of most stable RNAs (4–7). The deduced secondary structures of these RNAs share a common feature in that their 5'- and 3'-ends pair with each other to form stable, ds stems followed by a few unpaired 3'-molecules (6, 7). To understand how such duplex structures close to the 3' terminus affect RNase T action, we used the 17-mer RNA oligonucleotide, alone or paired with complementary strands of various lengths, as substrates for RNase T (Fig. 2). Inasmuch as the 17-mer RNA oligonucleotide is relatively resistant to RNase T digestion (see Fig. 1), a 10-fold higher level of RNase T (molar ratio RNA:RNase T of 50:1) was used in these analyses so that differences could be observed more readily. Under these assay conditions the 17-mer ssRNA is digested quickly in a stepwise fashion (Fig. 2 A). As noted above, each intermediate accumulating during the digestion process is one with either a terminal CC, UC, or CU sequence, suggesting that RNase T has difficulty in removing such sequences.

As shown in Fig. 2 (B–D), addition of complementary oligonucleotides dramatically affects RNase T action. Thus, formation of a 7-base pair (bp) duplex leads to accumulation of the 10-nt intermediate, whereas less of the 7-nt intermediate is generated compared with ssRNA (Fig. 2B). Extending the duplex length to 9 bp (Fig. 2C) further stabilizes the 10-nt intermediate, and, at the same time, 11- and 12-nt intermediates accumulate to a higher level, supporting the conclusion that RNase T action slows down as it gets close to a double-stranded stem. Finally, when a completely double-stranded 17-mer is
used as substrate (Fig. 2D), RNase T action essentially ceases. The very slow removal of a single nucleotide from this substrate is probably due to the presence of two AU base pairs at the 3’-end, which may lead to some terminal “breathing.” These observations indicate that RNase T is a single strand-specific exoribonuclease that can act only on the single-stranded portion at the 3’-end of an RNA substrate. The presence of a stable RNA duplex also will slow RNase T action as the duplex is approached.

Non-processivity of RNase T Action—During its action on the substrates examined in Figs. 1 and 2, RNase T seemed to slow down at certain nucleotide sequences. However, this stepwise digestion was not observed when RNA homopolymers were used as substrates (Fig. 3). Rather, in its action on homopolymers, RNase T generated a ladder of products with a random distribution, indicating that RNase T action is non-processive. Given sufficient enzyme and time, RNase T can digest ssRNA to dinucleotides, and even mononucleotides, although the final step from dinucleotide to mononucleotide proceeds extremely slowly (see below).

Effect of Substrate Length—To determine the effects of substrate length on RNase T action, a series of RNA homopolymers, A2, A4, A6, A8, A10, and A17, were evaluated as substrates. Based on the $K_m$ measurements shown in Table I, length does not appear to be a factor affecting RNase T action once a substrate is at least 10 nt long. However, for oligonucleotides shorter than about 10 nt, RNase T action displays considerable sequence length dependence. The $K_m$ values increase gradually from ~20 μM for a substrate 10 nt or longer to about 150 μM for the tetranucleotide, A4. These data suggest that RNase T has an extended RNA-binding site that can interact with as many as 10 nt of a ssRNA segment. Likewise, below 6 nt there is a dramatic effect on the $k_{cat}$ of the reaction. The dinucleotide, A2, is a particularly poor substrate with a $K_m$ of ~1 mM and a $k_{cat}$ only about 2% that of the longer substrates. These data explain why digestion of oligonucleotides essentially stops at the dinucleotide level.

Base Specificity of RNase T Action—In contrast to DNA substrates, on which RNase T action shows very little base specificity (11), RNase T action on RNA substrates is strongly influenced by the nucleotide to be removed. Thus, although A17 could be completely converted to A2 over a period of 30 min (Fig. 3A), 10 times as much RNase T was required to shorten U17 to a dinucleotide in the same period of time (Fig. 3, C and D). RNase T action on C17 was even slower; this substrate could only be partially shortened even after 90 min of treatment in the presence of the high level of RNase T (Fig. 3B). Kinetic analyses (Table I) showed that, for the 17-nt homopolymers, A17 ($k_{cat}$ ~ 2000 min$^{-1}$) is a much better substrate than either U17 ($k_{cat}$ ~ 700 min$^{-1}$) or C17 ($k_{cat}$ ~ 60 min$^{-1}$). $k_{cat}/K_m$ values for these substrates differ by as much as 100-fold. Poly(G) homopolymers were not included in this analysis, because they tend to form complicated structures that interfere with both RNase T action and with the electrophoretic procedure. However, qualitative analyses with other G-containing oligonucleotides (e.g. the complementary 7-mer, GGUGGGG,
used above) suggested that the rate of RNase T action on G residues may be similar to that on A residues (data not shown). These data show that RNase T displays base specificity strongly favoring the removal of purine nucleotides over pyrimidine nucleotides. This strong base specificity probably accounts for the stepwise digestion described above in RNase T action on certain substrates (Figs. 1 and 2).

Sequence of 3′-Terminal Residues Defines RNase T Specificity—The action of RNase T on the homopolymer substrates demonstrated that the enzyme is strongly affected by the nucleotides present in a single-stranded RNA substrate. To examine this phenomenon in more detail, a variety of single and multiple nucleotide substitutions were made in the 11-mer and 14-mer substrates studied in Fig. 1 (B and C), and these new oligonucleotides were evaluated both for their ability to be shortened by RNase T and for the intermediates that accumulated during the digestion. Based on a large number of experiments of this type (data not shown), it appeared that the ability of RNase T to digest a single-stranded RNA substrate was determined almost exclusively by the actual sequence of the four 3′-terminal residues and not just the base composition of the substrate. A hint of the importance of the 3′-terminal sequence was already suggested by the stepwise digestions shown in Figs. 1 and 2, which indicated that RNase T has difficulty removing pyrimidine doublets.

Consequently, to systematically examine the importance of the sequence of the four 3′-terminal residues, a series of RNA tetranucleotides, containing only A and/or C residues, were prepared that were evaluated for their ability to act as substrates of RNase T. The data in Fig. 4 and Table II show profound differences among the various tetranucleotides tested, even ones with identical base compositions. Interestingly, the most effective substrate is CCAA, whereas another with the same base composition, ACCA, is the poorest. The two substrates differ in \( k_{\text{cat}}/K_m \) values by a factor of 10⁻⁴. Digestion of the various substrates (Fig. 4) conforms with the previous observations that RNase T action essentially ceases at −CC sequences. Thus, ACCA and CCCA are relatively inactive as substrates (Fig. 4, C and F); a single terminal A residue is efficiently removed from ACCA and CCCA, at which point digestion stops (Fig. 4, B and E); and two A residues are removed from AAAA and CCAA (Fig. 4, A and D).

Based on these studies with tetranucleotides, it appears that RNase T action strongly disfavors C residues in the two 3′-terminal positions, especially the last one. A single substitution of C for A at the last residue of the 3′-terminus (CCCA compared with CCCC) leads to over a 100-fold reduction in \( k_{\text{cat}}/K_m \). In the penultimate position (CCCC compared with CCAA), an A to C change reduced \( k_{\text{cat}}/K_m \) only about 10-fold. Surprisingly, RNase T seems to favor C residues at the −3 and −4 position of the RNA substrate. Thus, ACCA is a much better substrate than AAAA, and even CCCA is a more effective substrate than ACCA (Table II). These data demonstrate that the four terminal residues combine to define RNase T substrate specificity. Although the studies with RNA homopolymers suggested that RNase T might bind up to about 10 nt of the substrate, the data suggest that residues beyond the last four seem to contribute only to the stability of binding, not to the specificity.

RNase T Action Requires a Free 3′-PO₄ Terminus and a DNA Substrate with a 3′-PO₄ Terminus and a DNA Substrate with a 3′-PO₄ terminus and a DNA substrate with a 3′-deoxyterminus.

It was already known that RNase T does not act on tRNA-CCP, a 3′-phosphate-containing macromolecular substrate (2). To confirm that this lack of action on a 3′-phosphate-terminated molecule was not due to RNA structure, the oligonucleotide, A16P, was tested as an RNase T substrate. As shown in Fig. 5A, using the same assay conditions, A17 was almost completely digested to the dinucleotide after about 2 min of RNase T treatment, whereas A16P was completely inactive, even after 30 min. These data indicate that the presence of a 3′-phosphate protects against RNase T action.

In a second experiment, RNase T activity on a DNA substrate containing a 3′-deoxyterminus was compared with its action on a 3′-OH-containing substrate of the same sequence. As shown in Fig. 5B, after 2 min of RNase T treatment, the original DNA substrate with a 3′-OH terminus was completely removed. However, the substrate with the deoxyterminus was quite stable, even after 30 min of RNase T treatment. These data suggest that a free 3′-OH group is necessary for RNase T action.

DISCUSSION

In this report we used synthetic RNA oligonucleotides to study in detail the substrate specificity of RNase T. Our data show that RNase T is a single strand-specific exoribonuclease that digests non-processively in a 3′ to 5′ direction. The enzyme slows as it approaches a duplex structure, and it stops completely at the RNA duplex itself. Most interestingly, RNase T action on ssRNA is sequence-specific, and this sequence-specificity is determined largely by the last four nucleotides at the 3′ terminus. If we define the subsites on the enzyme at which these last 4 residues bind as subsites 1–4, then subsites 1 and 2 strongly favor an A residue over a C residue for RNase T cleavage, whereas C residues are favored at subsites 3 and 4.

Of these four positions, the one binding the 3′-terminal residue shows the most dramatic effect. Thus, a single A to C change at the 3′ terminus of an RNA substrate (CCCA to CCCC) makes it greater than 100-fold more resistant to RNase T cleavage. One obvious possibility for this strong discrimination against a terminal C residue is that, at the catalytic center of RNase T, interactions with a terminal C residue are disfavored. A recent, detailed computational analysis of protein-RNA complexes may explain the basis for this discrimination. In those studies it was found that phenylalanine side chains strongly prefer to make van der Waals contacts with A, U, or G residues, but they do not interact with C residues (16). Based on structural modeling of RNase T using E. coli exonuclease I...
as the template (17), it is likely that the 3'-terminal base of the RNA substrate is sandwiched between two phenylalanine side chains, those of residues 29 and 77. Moreover, both of these phenylalanine residues are highly conserved in RNase T orthologues. The computational analysis also showed that, in contrast to phenylalanine, tyrosine-base interactions show a similar level of preference for all four bases in RNA (16). Thus, if phenylalanine-RNA interactions are responsible for RNase T substrate specificity, as suggested, mutating residues Phe-29 and Phe-77 to tyrosines should convert RNase T to a more nonspecific ribonuclease. It will be interesting to see whether further work bears out this prediction.

In earlier work, it was shown that RNase T also is a DNA exonuclease (10, 11). However, in contrast to the major difference in the rate of removal of A and C residues observed when RNA oligonucleotides are the substrates, digestion of DNA oligonucleotides displays much less sequence dependence (11). This difference can be explained based on the preferences of phenylalanine residues for their interactions with nucleic acid bases in various contexts (16, 19). Thus, whereas there is poor interaction with C residues in RNA molecules, the van der Waals contacts between Phe and A and between Phe and C in ssDNA are essentially the same. These observations support the conclusion that interactions between the 3'-terminal residue of the nucleic acid substrate and phenylalanine side chains in the active site are important determinants of RNase T specificity.

The specificity of RNase T in the in vitro reactions goes a long way in explaining the various functions of the enzyme in vivo. Thus, RNase T has not been implicated in either mRNA degradation or in removing long 3'-trailer sequences from RNA precursors. In both of these situations, the probability of encountering multiple pyrimidine residues, and even multiple C residues, is quite high, precluding RNase T from acting effectively on such substrates. On the other hand, RNase T is a major contributor to the final 3' trimming of a variety of stable RNA precursors (4–7), and to the end-turnover of tRNA (3). Other RNases generally are not able to approach closer than within 4 nt of a duplex with any degree of effectiveness. In these reactions, RNase T needs to remove only a small number of residues, making it less likely that random sequences would cause RNase T to stop.

All the in vivo substrates of RNase T share the common structural feature of having their 5'- and 3'-ends pair with each other to form a stable RNA duplex followed by several unpaired nucleotides. It was suggested previously (7) that the double-stranded stem might play an important role in the action of RNase T on these substrates. However, it is now clear that in addition to the inability of RNase T to digest an RNA duplex, the sequence of the unpaired residues also affects RNase T digestion, providing one explanation for why processing of precursors by RNase T can leave different lengths of 3' overhang that varies with the substrate. Thus, the combination of the double-stranded stem, the actual sequence of the single-
stranded residues to be removed, and the assembly of RNAs into RNP structures (5, 6) all affect how close to the ds stem RNase T will proceed. The mature 3'-H11032-ends of tRNA and tmRNA are –CCA, and each has 4 unpaired terminal residues. Inasmuch as RNase T action is non-processive, the mature 3'-H11032-ends of these molecules can be protected by rapid aminoacylation. If, by chance, RNase T does remove the 3'-terminal A residue, the resulting –CC sequence greatly slows any additional digestion, allowing tRNA nucleotidyltransferase to repair the –CCA sequence (3). During tRNA maturation, the most active substrates for RNase T appear to be the 3'-precursors (–CCAN), because these usually are not observed in cells or extracts containing RNase T (4, 14). On the other hand, for those tRNA precursors that contain multiple C and U residues immediately after the –CCA sequence, RNase T has been found not to be involved in their maturation. Rather, other enzymes, such as RNase PH, became more important. For example, the 3' maturation of tRNAval (precursor 3' trailing sequence –CUUC or –CUUUCUC) and tRNA2B (precursor 3' trailing sequence –UCCU) is unaffected in RNase T- strains, whereas precursors to these tRNAs do accumulate in RNase PH- strains (4).

The maturation of several other RNAs by RNase T also leaves 4 unpaired 3'-terminal residues (7). Both 4.5 S RNA and 6 S RNA have –CC residues at their mature 3'-ends, explaining why RNase T digestion stops. M1 RNA terminates with a –CCU sequence, which also would be expected to be resistant to RNase T. The mature 3'-ends of 5 S and 23 S rRNAs terminate with either 1 or 2 unpaired U residues, respectively. Although these ends may afford some protection against further RNase T action, previous studies showed that assembly into the ribosome structure is a major determinant protecting the RNA (5, 6). Based on these and other examples, it is clear that the unusual substrate specificity of RNase T is an important aspect of its function in vivo.

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