Mechanism of Action of RNase T*

I. IDENTIFICATION OF RESIDUES REQUIRED FOR CATALYSIS, SUBSTRATE BINDING, AND DIMERIZATION*

Escherichia coli RNase T, an RNA-processing enzyme and a member of the DEDD exonuclease superfamily, was examined using sequence analysis and site-directed mutagenesis. Like other DEDD exonucleases, RNase T was found to contain three conserved Exo motifs that included four invariant acidic residues. Mutagenesis of these motifs revealed that they are essential for RNase T activity, indicating that they probably form the RNase T catalytic center in a manner similar to that found in other DEDD exonucleases. We also identified by sequence analysis three short, but highly conserved, sequence segments rich in positively charged residues. Site-directed mutagenesis of these regions indicated that they are involved in substrate binding. Additional analysis revealed that residues within the C-terminal region of RNase T are essential for RNase T dimerization and, consequently, for RNase T activity. These data define the domains necessary for RNase T action, and together with information in the accompanying article, have led to the formulation of a detailed model for the structure and mechanism of action of RNase T.

RNase T is one of eight exoribonucleases present in Escherichia coli (1). It belongs to the DEDD exonuclease superfamily, characterized by common motifs containing four invariant acidic residues, which in DNA polymerases were shown to form the exonuclease active site (2). RNase T plays an important role in stable RNA metabolism in E. coli, including tRNA end turnover (3) and 3’ maturation of many stable RNAs (4–7). RNase T proteins are closely related to the proofreading domains/subunits of bacterial DNA polymerases (2, 8), and, interestingly, E. coli RNase T also displays strong DNA exonuclease activity (9, 10).

Although the substrates of RNase T in vivo share a common sequence feature consisting of a stable, double-stranded (ds) stem followed by a few unpaired 3’ nucleotides, RNase T actually is a single-strand-specific exoribonuclease that acts in the 3’-to-5’ direction in a non-processive manner (11). However, whereas other E. coli exonuclease B stop several nucleotides downstream of an RNA duplex, RNase T can digest RNA up to the first base pair, although it slows as it approaches the duplex structure. The presence of a free 3’-hydroxyl group is required for the enzyme to initiate digestion (11).

Homogeneous RNase T has been prepared from both normal and overexpressing cells (12, 13). The enzyme forms a homodimer in vitro and in vivo, and formation of the dimer seems to be required for it to function (14). Some residues needed for dimerization have been identified, as well as the importance of hydrophobicity in the dimerization process (13, 14).

In this study, we investigate in detail structure-function relationships in RNase T using a combination of sequence analysis and site-directed mutagenesis. Our data indicate that the conserved acidic residues, as well as several other residues at the DEDD signature motifs, are important for RNase T catalytic activity, consistent with a common catalytic mechanism for all DEDD members. In addition, we also identified three other conserved sequence segments (the nucleic acid binding sequence (NBS)1 segments), containing a high level of positively charged residues in RNase T orthologs. Kinetic analyses of the corresponding mutants suggest that the basic residues of the NBS segments are involved in nucleic acid binding.

Finally, we have shown that the C-terminal region of RNase T is important for RNase T dimerization and, consequently, for activity. These data identify residues needed for RNase T action and provide essential information for the development of a detailed model of RNase T action presented in the accompanying article (15).

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, T4 polynucleotide kinase, and T4 DNA ligase were obtained from New England Biolabs. Calf intestine alkaline phosphatase was purchased from Promega. [gamma-32P]ATP (6000 Ci/mmol) was from PerkinElmer Life Sciences. Sequagel, for single nucleotide resolution analysis, was purchased from National Diagnostics. The QIAEX II gel extraction kit was purchased from Qiagen Inc. The RNase T substrate, tRNA-C-C-[3H]IA, was prepared by [3H]IA incorporation into tRNA-C-C using tRNA nucleotidyltransferase and [3H]ATP as described previously (12). DNA oligonucleotides were synthesized by the DNA Core Facility of our department. Ultragel AcA44 was from Amersham Biosciences. The gel filtration calibration kit was from Pharmacia. Peroxidase-labeled anti-rabbit IgG and the ECL substrate were from Amersham Biosciences. All other chemicals were reagent grade.

RNase T Activity Assay—RNase T reactions were carried out under buffer conditions containing 20 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 50 mM KCl, and 5 mM dithiothreitol. Sonicated cell extracts were prepared as described previously (16) and were used for all activity measurements. Reaction mixtures were incubated at the indicated temperature for 5 min. For tRNA substrates, acid-soluble radioactivity was determined (12), whereas when the tetrancleotide, dA4, and the dinucleotide, dA2, were used as substrates, reactions were stopped with 2 volumes of loading buffer containing 96% formamide and 1 mM EDTA, and the reaction products were analyzed by denaturing polyacrylamide gel electrophoresis on a 22.5% gel. Quantitative data were obtained.

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Primers T5P and T3p, which contain SacI and BamHI sites, respectively, were used for cloning the rnt gene into plasmid pUC18 to generate plasmid pUT18. The primer pairs, TNN5 and TNM3, TBE5 and TBE3, and TNN5 and TNH5, were used to introduce new restriction sites, NgoMI, BspEI, and NheI, respectively, into the rnt gene of plasmid pUT18 with silent mutations. Restriction sites and mutated codons in the primer sequences are underlined. Changed nucleotides corresponding to the point mutations are shown in bold.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Mutation</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T5P</td>
<td></td>
<td>AAAGAGCTCTACGGCGGCCGCG</td>
</tr>
<tr>
<td>T3P</td>
<td></td>
<td>AAAGGATCTGGAATCTGTCG</td>
</tr>
<tr>
<td>TNN5</td>
<td></td>
<td>GAAACAGCGGGCTTTAAACGCAAAAC</td>
</tr>
<tr>
<td>TNM3</td>
<td></td>
<td>GGCCTTAAGGGCGGTATTTCGAC</td>
</tr>
<tr>
<td>TBE5</td>
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<td>ACGGCTCGATTGACGCCGCCGCCC</td>
</tr>
<tr>
<td>TBE3</td>
<td></td>
<td>TACTACGGAGCTTTAATACCTTTCAC</td>
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<tr>
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<td></td>
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<td></td>
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<tr>
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<td>R15A</td>
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<td>D15A</td>
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<td>D23A</td>
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<td>E25A</td>
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<tr>
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<td>R114A</td>
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<td>H120A</td>
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<tr>
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<tr>
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<td>W207A</td>
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</tr>
<tr>
<td>TW207Stop</td>
<td></td>
<td>AAGAGATCTCAGCGCCCGACGACGACGAC</td>
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</table>

with a PhosphorImager (Amersham Biosciences). One unit of RNase T activity is defined as the amount of enzyme that releases 1 μmol of mononucleoside/h.

**Results**

**Sequence Analysis of RNase T**—The sequence of *E. coli* RNase T is closely related to that of the proofreading domains/subunits of bacterial DNA polymerases, such as the ε subunit of DNA polymerase III (Dp3E/DnaQ protein in *E. coli*) (2, 8). Shown in Fig. 1 is a multiple sequence alignment of RNase T orthologs from a number of completed genome sequences. Also included are the Dp3E orthologs from the corresponding genomes for comparison. Like all other DEDD family members, the Exo motifs, especially the invariant acidic residues in these motifs common to all DEDD members, are also conserved in the RNase T orthologs. The three highly conserved Exo motifs are labeled ExoI, ExoII, and ExoIII in Fig. 1. RNase T orthologs are highly homologous to each other, with generally more than 50% sequence identity. Thus, besides the Exo motifs, there are many other highly conserved sequence segments among RNase T orthologs. Of these, the most prominent are three conserved, positively charged regions specific for the RNase T orthologs but absent from the bacterial DNA polymerase III As. They are labeled NBS1, NBS1′, and NBS3, because, as will be shown, they cluster to form a nucleic acid-binding site. The selection of residues for site-directed mutagenesis in this study was based on multiple sequence alignments, such as those shown in Fig. 1. Our focus has been on conserved residues at the Exo motifs, the NBS segments, and the putative dimerization interface.

**Mutating DEDD Signature Motifs Results in Loss of RNase T Activity**—To determine whether the invariant acidic and other residues present within the Exo signature motifs in RNase T actually are important for activity, several of these residues were mutated to alanines. Similar levels of RNase T overexpression were observed for the wild-type protein and for each of the mutant proteins based on Western blot analysis (data not shown). RNase T activity was assayed in cell extracts using RNA primers shown in Table I. All mutant rnt clones were generated in the pUT18 plasmid by PCR, and all the mutations were confirmed by DNA sequencing. The pUT18-based constructs were also used to overexpress RNase T in CAN20-12ET cells that lack endogenous RNase T.

**NBS Segments Are Important for RNase T Activity against tRNA**—Based on sequence analysis, the NBS sequence segments are characteristic for RNase T proteins. These segments
are rich in positively charged arginine and lysine residues, raising the possibility that they may form a nucleic acid-binding site. To investigate whether the NBS segments might participate in substrate binding, alanine substitutions were made for several of the arginine and lysine residues individually and in combination. As shown in Table III, mutations of residues within each NBS segment result in reduction of RNase T activity. Except for R13A, which is essentially inactive, individual alanine substitutions lead to 3–7-fold reductions in activity, whereas multiple substitutions lead to cumulative defects in activity under usual assay conditions. In contrast to the mutations in the DEDD motifs, the NBS mutant proteins, especially the multiple mutants, have greatly elevated $K_m$ values for tRNA, but essentially no alterations in $V_{\text{max}}$. The increased $K_m$ values probably result from weakened RNase T binding of the tRNA substrate upon alanine substitution for the basic residues in the NBS segments. Earlier work (13) showed that for RNase T, the $K_m$ value for tRNA is near its binding constant.

NBS1 residue Arg 13 seems to differ from the other single mutants in that mutant R13A displays extremely reduced RNase T activity. In fact, R13A is the most inactive of all the RNase T point mutations made. This finding suggests that Arg13 plays an important role in RNase T action and may affect not only substrate binding but also RNase T catalysis. We will return to this point in the accompanying article (15).

NBS Mutations Have Little Effect on RNase T Activity against Short Oligonucleotides—To further examine the role
of NBS segments in substrate binding and catalysis, the RNase T activity of several NBS mutants was also assayed using very short oligonucleotides as substrates. We reasoned that if the NBS residues were distant enough from the catalytic center, they might be required for action on tRNA, but not for the binding of very short substrates. The DNA oligonucleotides dA4 and dA2 were used for these studies rather than RNA oligonucleotides because DNA substrates have considerably lower Km values (10, 11). As shown in Table IV, for NBS1 mutant R15A and the NBS2/NBS3 triple mutant K108A/R112A/K139A, alanine substitutions have virtually no effect on RNase T activity against the short oligonucleotides, in contrast to the major loss in activity when tRNA was the substrate (Table III). These findings support the conclusion that the NBS basic residues contribute little to the binding of short oligonucleotides at the RNase T active site, even though they are essential for binding of long substrates, such as tRNAs. These data further support the idea that the reduced RNase T activity of NBS mutants using tRNA as substrate is most probably caused by weaker substrate binding, not reduced catalysis.

The C-terminal Region of RNase T Is Essential for RNase T Dimerization—Earlier work suggested that formation of an RNase T homodimer is required for RNase T to function (13, 14). Comparison of various protein structures of DEDD domain-containing proteins suggests that the very C termini of the DEDD domains are all directly involved in the interfacial interactions between domains or subunits. Involvement of the C terminus may also be true for dimerization of RNase T, as suggested by the structural modeling described in the companion article (15). This conclusion was also supported by a mutation near the C terminus (G206S) that was shown previously to affect RNase T dimerization (14). To further confirm the direct involvement of the C terminus of RNase T in its dimerization, we mutated the last tryptophan residue to an alanine (W207A). A second mutant, W207Stop, lacking the last nine residues at the C terminus, was also generated. These two RNase T mutant proteins were each examined for their dimerization status using gel filtration run at different temperatures.

As shown previously (14), wild-type RNase T elutes as a single peak at the dimer position corresponding to 43 kDa over a wide range of temperatures (20 °C and 37 °C are presented here). In contrast, the W207Stop mutant protein elutes at the monomer position even at 10 °C (Fig. 2). A similar result was also observed for the W207A mutant (data not shown). Earlier work had shown that low temperature promotes dimerization (14). These data support the conclusion that the C terminus of RNase T is essential for RNase T dimerization.

C-terminal Mutations Lead to Temperature-sensitive RNase T Activity—Further evidence for the involvement of C-terminal residues in dimerization comes from the temperature-sensitive
nature of the activities of the W207A and W207Stop mutants (Table V). Thus, compared with wild type RNase T activity, the W207A mutant activity changes from about 2% at 45 °C to 34% at 25 °C, and the W207Stop mutant activity increases from 8% at 45 °C to about the same level as the wild type at 25 °C. These data initially were surprising because, as shown above, both the W207A and the W207Stop mutant proteins should exist as monomers at all of these temperatures and, as shown previously (14), there is a direct correlation between RNase T activity and its dimerization status. The most likely explanation is that the presence of tRNA substrate during the assay stabilizes the dimer form of the mutant enzymes and that this substrate-assisted dimerization is more effective as the temperature is lowered. In fact, tRNA has previously been shown to stabilize a dimerization mutant against temperature inactivation (13, 14).

**DISCUSSION**

Although RNase T is an important enzyme for various aspects of RNA metabolism, it was also found to act on single-stranded DNA substrates in vitro (9, 10). Among the DNA exonuclease activities found in E. coli, the RNase T DNase activity is most similar to the activity of the proofreading subunit of DNA polymerase III (10). Both activities act in the 3’-to-5’ direction on single-stranded DNA in a non-processive manner, releasing 5’ mononucleotides as the product. In fact, RNase T is a close homolog of the DNA polymerase III proofreading subunit (2, 8); both proteins belong to the DEDD exonuclease superfamily (2). The proteins of the DEDD superfamily contain a characteristic core composed of four invariant acidic residues embedded within three highly conserved sequence motifs, which were shown in DNA polymerases to form the exonuclease active site (18, 19). The studies described here, in which mutations in residues of the DEDD signature regions of RNase T dramatically reduce its activity, suggest that RNase T exploits the same chemistry for nucleic acid degradation as other DEDD exonucleases.

RNase T orthologs share a high degree of sequence identity. Besides the highly conserved DEDD signature motifs, RNase T orthologs contain other common sequence features, namely, the highly positively charged NBS sequence segments shown in Fig. 1. These regions distinguish RNase T from DNA polymerase III. Alanine substitution of basic residues within the NBS segments led to reduced RNase T activity against tRNA substrates and increased $K_m$ values. These results lead to the suggestion that the NBS segments are required for binding of tRNA substrate. In contrast, mutation of the NBS regions generally does not affect RNase T activity against short oligonucleotide substrates, suggesting that most NBS residues are at some distance from the catalytic center. However, one NBS1 mutation, R13A, strongly affects RNase T activity against both long and, as will be shown in the accompanying article, short substrates. Thus, NBS residues seem to extend from the vicinity of the catalytic center, where they would bind the 3’ end of the substrate, to regions in which they would stabilize binding of long substrates, such as tRNA. Early studies found that purified RNase T was strongly inhibited by elevated ionic strength (12), suggesting that charge interactions probably play an important role in RNase T action. This would be expected if basic residues within the NBS regions were involved in nucleic acid binding.

In addition to the catalytic center and substrate-binding sites, we have also shown that the C terminus of RNase T is important for RNase T dimerization and, consequently, for its activity. It is not yet clear how the C-terminal residues that affect dimerization are related to residue Cys168, which was shown previously to participate in RNase T dimerization (13, 14). Earlier work showed that formation of a homodimer is required for RNase T to function (14). One possibility is that the NBS substrate binding sites and the DEDD catalytic centers from two different subunits complement each other to form a fully functional active site through dimerization. Consistent with this explanation, the dimerization-defective mutant C168S has essentially unaffected tRNA binding, even at a temperature at which it is a monomer (13). Further evidence supporting the conclusion that functional RNase T arises from catalytic and substrate-binding residues contributed by the two separate monomers will be presented in the accompanying article (15).

**REFERENCES**

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