A detailed and structural functional model of E. coli RNase T was generated based on sequence analysis, homology modeling, and experimental observation. In the accompanying article, three short sequence segments (nucleic acid binding sequences (NBS)) important for RNase T substrate binding were identified. In the model, these segments cluster to form a positively charged surface patch. However, this patch is on the face of the RNase T monomer opposite the DEDD catalytic center. We propose that by dimerization, the NBS patch from one subunit is brought to the vicinity of the DEDD catalytic center of the second monomer to form a fully functional RNase T active site. In support of this model, mutagenetic studies show that one NBS1 residue, Arg**, sits at the catalytic center despite being on the opposite side of the monomer. Second, the complementarity of the RNase T subunits through the formation of homodimers was demonstrated by reconstitution of partial RNase T activity from monomers derived from two inactive mutant proteins, one defective in catalysis and one in substrate binding. These data explain why RNase T must dimerize to function. The model provides a detailed framework on which to explain the mechanism of action of RNase T.

RNase T, a DEDD exonuclease, plays an important role in many aspects of stable RNA metabolism (1–6). The enzyme forms a homodimer in vitro, and formation of the homodimer is required for it to function (7). In the companion article (8), RNase T was examined by sequence analysis and site-directed mutagenesis to identify regions of the protein important for catalysis, substrate binding, and dimerization. We showed that the DEDD signature motif probably forms the RNase T catalytic center. Second, sequence analysis identified several highly conserved, positively charged segments, termed NBS1–3, that are present in all RNase T orthologs, but absent from the closely related proofreading domains/subunits of DNA polymerases, that seem to be involved in the binding of nucleic acid substrates. Finally, we showed that residues at the C terminus of each monomer of RNase T are important for dimerization.

Based on this information, additional experiments, and homology modeling, we present here a structural and functional model for RNase T. Based on this model, we show that the NBS segments cluster in the tertiary structure to form a positively charged surface patch suitable for nucleic acid binding. We also propose that the DEDD catalytic center and the NBS are on opposite sides of an RNase T monomer and are brought together to create a complete active site through dimerization. Direct evidence for the complementarity of the two RNase T subunits in the homodimer was obtained by reconstituting partial RNase T activity from two inactive mutants, one an NBS mutant defective in substrate binding and the other a DEDD mutant defective in catalysis. These results explain the requirement for dimerization to generate functional RNase T.

**EXPERIMENTAL PROCEDURES**

Materials—Purified Escherichia coli RNase T was prepared as reported previously (9, 10). Purified mutant C168S RNase T was kindly provided by Dr. Zhongwei Li and was overexpressed and purified from E. coli cells as described previously (10). The bacterial strains and plasmid used here are as described in the companion study (8). Likewise, all the other reagents used in the experiments reported here have been described elsewhere (8).

Methods—Assays for RNase T activity and recombinant DNA procedures were all carried out as described in the accompanying article (8).

Sequence Analysis and Homology Modeling—Multiple sequence alignments were generated using ClustalX (11) and optimized by manual adjustments based on secondary structures derived from crystal structures or predicted secondary structures if no known structures were available. Secondary structure predictions were generated on the PHD (12) and 3D-PSSM (13) web servers. The homology model of E. coli RNase T was constructed using the program MODELLER (14). Crystal structures of E. coli DNA exonuclease I (Protein Data Bank structure number 1FXO) (15) and E. coli oligoribonuclease served as templates for the homology modeling.

Affi-Gel Blue Chromatography—Partially purified RNase T mutant proteins were obtained using Affi-Gel Blue chromatography. The S100 fraction (∼ 30 ml) was prepared in loading buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM diithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 100 mM KCl, and 10% glycerol. After loading the S100 fraction onto an Affi-Gel Blue column (1.0 × 60 cm), the column was washed with 2 volumes of the loading buffer and 2 volumes of washing buffer. The washing buffer was the same as loading buffer except that it contained 100 mM KCl. RNase T was eluted by increasing the KCl concentration to 1 M. Peak fractions eluted from Affi-Gel Blue are about 50% pure based on SDS-PAGE and staining with Coomassie Blue.

Reconstitution of RNase T Activity from Inactive Mutants—Sonicated cell extracts were prepared as described previously (16) from CAN20-12ET cells carrying the corresponding pUT18 plasmid. For reconstitution, equal amounts of proteins from the two extracts were mixed, incubated at 37°C for 30 min, and then cooled at room temperature for 30 min. The mixtures were then used directly for activity measurement. Activity assays were carried out in 50-μl reaction mixtures containing 20 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 50 mM KCl, 5 mM diithiothreitol, and 20 μg of rRNA-CC3-[3H]A substrate. For comparison, individual mutant cell extracts and the parental strain extract were treated and assayed in parallel.

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§ The abbreviations used are: NBS, nucleic acid binding sequence; RNT, RNase T; DP3E, DNA polymerase III ε subunit.
Mechanism of Action of RNase T

RESULTS

Homology Modeling of E. coli RNase T—The DEDD exonuclease superfamily can be divided into two groups, DEDDy and DEDDh, depending on whether a conserved Y-x (3)-D sequence or a H-x (4)-D sequence is present in the ExoIII motif (17). RNase T is a member of the DEDDh group. Many protein structures from the DEDDy group, primarily homologous domains of DNA polymerases, have been available for some time. However, DEDDh protein structures have appeared only recently. In this study, we used the crystal structures of two DEDDh members, that of E. coli exonuclease I (Protein Data Bank structure number 1FXX) (15) and that of E. coli oligoribonuclease, solved recently in our department,2 as templates to model the E. coli RNase T structure. E. coli exonuclease I exists as a monomer, whereas oligoribonuclease forms a homodimer. It was known that RNase T must form a homodimer to function (7). Therefore, a monomer structure of RNase T was built first and optimized before it was modeled into a homodimer based on the relative orientation of the two subunits in the oligoribonuclease homodimer. Because of the lack of sequence homology, twelve amino acids each from the N and C termini of RNase T were omitted in the structural models shown here (Fig. 1). Some internal loop regions were modeled with predicted secondary structures as additional spatial restraints. The results of the homology modeling follow.

Putative RNase T Catalytic Center—The active sites of DEDD superfamily members share four invariant acidic residues embedded within the three Exo motifs. These residues, together with the substrate, are known to bind two divalent metal ions (18). In E. coli RNase T, the four acidic residues are Asp23, Glu25, Asp125, and Asp186 (Fig. 1). Also important is residue His181, which, by analogy with the structure of the Klenow fragment DNA polymerase exonuclease domain (18), binds to a water molecule coordinated with one of the metal ions and also hydrogen bonds to a non-bridging oxygen in the penultimate phosphate group of the substrate. Residue His181 is highly conserved in both RNT and DP3E proteins. All of these residues were shown to be important for catalysis by RNase T (8).

In the presumed mechanism of action of the exonuclease domain of Klenow fragment DNA polymerase, an additional residue, Tyr497, helps to align the hydroxide ion for hydrolysis (19). In E. coli RNase T, as well as all other DEDDh members, such as DP3E, DNA exonuclease I and oligoribonuclease, a conserved His(His181) is present at the adjacent position in the sequence. This residue also is required for RNase T catalysis (8). In the tertiary structure modeled here, His181 sits at a position similar to that of Tyr497 in the Klenow fragment (Fig. 1) and therefore may play a role in catalysis similar to that proposed for Tyr497.

Similar to other DEDD structures, the four catalytically important acidic residues in the model of RNase T sit at the bottom of a narrow pocket that allows only single-stranded terminal residues to enter. Surrounding this narrow pocket are several conserved phenylalanine residues, Phe29, Phe77, and Phe124 (Fig. 1), which may be in direct contact with the substrate, and are thought to contribute to substrate recognition (21). Residues Phe29 and Phe77 occupy positions corresponding to residues Leu361 and Phe473, respectively, in the Klenow fragment exonuclease domain. These residues are important for catalysis by the Klenow fragment. Similarly, in our model, it is likely that the 3′-terminal base of the substrate is sandwiched between the side chains of residues Phe29 and Phe77. It has been suggested that phenylalanine side chains strongly prefer to make van der Waals contacts with A, U, or G residues but not with C residues of an RNA molecule (20). Therefore, this Phe-base interaction may account for the known RNase T discrimination against removal of C residues (21). Similar to the role of Tyr497 in the Klenow fragment, the third Phe residue, highly conserved Phe124, might stack against the penultimate base of the substrate.

NBS Segments Cluster Together in RNase T Tertiary Structure—We identified, based on sequence analysis (8), three highly conserved sequence segments (NBS1–3) that are present in RNase T protein but absent from the closely related DNA polymerase III. The structural modeling, described here, indicates that these three NBS sequence segments cluster in the tertiary structure of RNase T (Fig. 1). The NBS segments are rich in basic residues. E. coli RNase T contains 18 Arg/Lys residues, and nine of them are present in the three NBS seg-

Fig. 1. Structural model of E. coli RNase T (residues 13–203). Twelve amino acids each from the N and C termini of RNase T were omitted in these models because of the lack of sequence homology with the template proteins. A, ribbons (25) diagram of an RNase T monomer showing the conserved DEDD residues (red), the NBS basic residues (blue), the interfacial hydrophobic residues (orange), and the three highly conserved phenylalanine residues lining the DEDD center (yellow) as ball-and-stick illustrations. B, ribbons diagram of an RNase T homodimer with the DEDD residues from one subunit shown in red and the NBS basic residues from the other subunit shown in blue as ball-and-stick illustrations. Also shown as ball-and-stick residues are the interfacial hydrophobic residues (orange) and the three conserved phenylalanine residues lining the DEDD center (yellow). C, molecular surface representation of an RNase T homodimer calculated using GRASP (26), shown in the same orientation as in B. Regions of negative electrostatic surface potential are illustrated in red, whereas positive potential is represented in blue. The DEDD center (red) and the NBS patch (blue) are marked with colored ovals.
ments. In addition, several other Arg/Lys residues are near the NBS segments in the tertiary structure. Therefore, on the surface of RNase T, the basic residues form a well defined, positively charged patch (see Fig. 1C) that serves, we propose, as the substrate binding site (8). Surprisingly, however, this positively charged patch is quite distant from the putative catalytic center in an RNase T monomer. The next section will explain how the separated substrate-binding site and the catalytic center combine to allow RNase T to function.

**RNase T Dimerization Interface—**E. coli RNase T exists as a homodimer in vitro and in vivo, and the formation of the homodimer is essential for RNase T to function (7, 8, 10). As with the tertiary structures of other known DEDD exonuclease domains, the modeled RNase T monomer has a relatively flat shape (Fig. 1A). Using the oligoribonuclease homodimer as the template, RNase T was modeled into a homodimer (Fig. 1B). The resulting homodimer model has a number of features that make the structure attractive. Thus, it buries many highly hydrophobic residues at the interface, and the dimerization interface is located at a region similar to those of the inter-domain interfaces of other DEDD proteins, such as Klonef fragment (18) and **E. coli** exonuclease I (15). Formation of the homodimer of RNase T leads to a much more globular-shaped protein (Fig. 1B). The buried interfacial hydrophobic residues include Phe14, Phe17, Met46, Phe149, Leu154, Leu157, Phe194, Ile197, Val198, Trp201, and probably Trp207; the latter residue had to be omitted from the models shown in Fig. 1 because the corresponding residue is lacking in the template structures. All of these residues cluster in the tertiary structure and form a hydrophobic core. The missing N-terminal and C-terminal residues would be expected to interact with the other subunit and to help bury the hydrophobic core within the dimerization interface.

Upon formation of an RNase T homodimer, the positive surface patch formed by the NBS segments on one subunit is brought to the vicinity of the DEDD catalytic center present on the other subunit (Figs. 1B and 2). In fact, one residue, Arg13, from one subunit is directly adjacent to the catalytic center of the other subunit. The complementarity of the two RNase T subunits to generate a complete active site would explain the dimerization requirement for RNase T to function. Based on the RNase T homodimer modeled here, the distance from the DEDD catalytic center to the far edge of the positively charged NBS patch measures about 40 Å, which could accommodate 8–10 nucleotides of a single-stranded RNA segment, in excellent agreement with experimental observations (21). Additional experiments supporting the proposed model are presented below.

**NBS1 Residue Arg13 Affects RNase T Activity against Short Oligonucleotides—**In the proposed model, the three NBS sequence segments cluster in the RNase T tertiary structure to generate a substrate-binding domain that is, however, distant from the DEDD catalytic center in an RNase T monomer. Through dimerization, this NBS cluster is brought to the vicinity of the DEDD catalytic center of the other subunit. As was shown in the accompanying article (8), mutation of several basic residues in the NBS domain affects RNase T binding of tRNA substrates. However, there is essentially no effect on activity against the short oligonucleotides dA4 and dA2. These data suggest that these residues, although important for substrate binding, are at least four nucleotides away from the catalytic site. However, this is not true for one NBS basic residue, Arg13. Alanine substitution of Arg13 not only completely abolished RNase T activity against tRNA (8) but also, as shown in Table I, dramatically affects activity when short oligonucleotides are used as substrates. The R13A mutation leads to a major reduction in Vmax, an increase in Km, and a 105–106-fold reduction in the Vmax/Km value for each substrate. This observation provides direct evidence that Arg13 from one subunit must be very near the DEDD catalytic center of the other subunit and that it is involved in both catalysis and binding (Table I). Thus, these data suggest that the NBS patch extends from about 8–10 nucleotides away, as noted earlier, to the immediate vicinity of the DEDD catalytic center.

**Reconstitution of RNase T Activity from Two Inactive Mutant Monomers—**Based on the structural model, a fully functional RNase T active site is composed of an NBS substrate-binding site and a DEDD catalytic center coming from two different subunits but brought together through dimerization (Fig. 2). According to this model, RNase T activity should be abolished by mutations at the catalytic center, at the substrate-binding site, or at the dimerization interface, and this was shown experimentally (8). A direct demonstration of the model would be the reconstitution of RNase T activity by the formation of heterodimers from subunits derived from two homodimers mutated at either the catalytic site or the substrate-binding site, as diagrammed in Fig. 2. In the accompanying article (8), we showed that the W207Stop mutant is defective in dimerization but displays temperature-sensitive activity such that at 25 °C, its activity is virtually the same against a tRNA substrate as the wild-type enzyme. This mutant protein provided an ideal platform to test this prediction because it could be used to easily generate heterodimers by first incubating at higher temperature to generate monomers, and then cooling to re-form the dimers. The protocol is described under “Experimental Procedures.”

Using the pUT-W207Stop plasmid as starting material, the double-mutants D23A/W207Stop, E25A/W207Stop, and R13A/W207Stop were constructed and expressed in the exonuclease-deficient strain, CAN20-12ET (Table II). The D23A/W207Stop and E25A/W207Stop constructs each contain a mutation at the DEDD catalytic center, whereas the R13A/W207Stop construct contains an additional mutation at R13. As shown in Table II, RNase T activity in extracts is very low.

### Table I

| RNase T Mutation | dA4 | dA2 |
|------------------|-----|-----|
|                  | Km  | Vmax/Km | Vmax/Km | Km  | Vmax | Vmax/Km |
| Wild type        | μM | unit/mg | 850     | 27  | 25   | 0.25 |
| R13A             | 105| 1700     | 0.24    | 140 | 0.25 | 0.0018 |
for each of the individual double mutants. However, by incubating together equal amounts of the complementary mutant extracts, substantially increased RNase T activities were observed. The activity corresponds to ~10% of that of the parent strain W207Stop under the same conditions. As shown in Fig. 2, the maximum possible activity expected would be 25%, because only 50% of the dimers formed would be heterodimers, and only one of the two active sites on the heterodimer would be active. Thus, only a maximum of 25% of control activity should be reconstituted.

**DISCUSSION**

DEDD exonucleases are involved in many aspects of nucleic acid metabolism (17). In many instances, the DEDD domain is associated with various other functional domains, which may account for the variety of functions carried out by these enzymes. Although DEDD domains by themselves may display limited or no exonuclease activity, other domains or protein subunits can stimulate such activity. For example, removal of the polymerase domain from Klenow fragment leaves a DEDD exonuclease domain with no detectable activity (22). In *E. coli* DNA polymerase III, although the DEDD exonuclease subunit (ε subunit) is independently active as an exonuclease, its activity is enhanced by association with the polymerase subunit (α subunit) and the θ subunit (23, 24). Likewise, *E. coli* RNase T requires formation of a homodimer to function (7, 8, 10).

In this work, we have built a structural and functional model of *E. coli* RNase T based on homology modeling with crystal structures of other DEDD exonucleases as templates. According to this model, the catalytic center, consisting of the DEDD signature motifs, sits inside a narrow pocket, which is wide enough to hold only 1 to 2 single-stranded terminal nucleotides. This may account for the single-strand specificity of RNase T action (21). On the surface of RNase T, the highly conserved NBS segments cluster to form a nucleic acid-binding patch. In an RNase T monomer, the NBS patch and the DEDD catalytic center are on opposite sides of the protein. Through the formation of a homodimer, the NBS patch is brought close to the vicinity of the DEDD catalytic center of the other subunit to generate a complete active site.

Our data indicate that most NBS basic residues are at least four nucleotides away from the catalytic center. In contrast, we propose that Arg¹³ sits very near the catalytic center. Thus, mutation of this residue leads to a dramatic loss of RNase T activity and a significant increase in the $K_m$ value, with tRNA as the substrate (8) and a major reduction in activity against substrates as small as the dinucleotide, dA₂. These data are most consistent with the conclusion that Arg¹³ is very close to the DEDD catalytic center. This could only be accomplished through the formation of an RNase T homodimer, because in the monomers, these residues are on opposite faces. Upon formation of the homodimer, the NBS patch and the DEDD center...
would generate a continuous nucleic acid-binding site. The distance from the DEDD center to the edge of the NBS patch should be sufficient to bind a single-stranded nucleic acid segment of about 8–10 nucleotides, in excellent agreement with that suggested in a study of RNase T substrate specificity using a different approach (21).

The structural modeling, mutagenic analyses, and experimental observations all suggested that the two subunits of RNase T complement each other through dimerization, and this could be demonstrated directly by mixing an NBS mutant protein and a DEDD mutant protein and incubating under conditions to allow formation of heterodimer. RNase T activity was reconstituted, amounting to about half of that expected. Considering that the efficiency of monomer formation and of heterodimer formation are not known, and have not been optimized for this experiment, the level of reconstitution of activity is impressive. The functional complementarity of the RNase T subunits clearly explains the requirement of dimerization for RNase T to function.

The model also explains several other observations with RNase T mutants. For example, in an earlier study (7), a dominant-negative mutant, HA2, was isolated. HA2 contains two point mutations, R15H and G28R. Arg15 is a conserved residue in the NBS1 segment, whereas Gly28 is part of the highly conserved ExoI motif, suggesting that the HA2 mutant protein probably was defective in both substrate binding and catalysis. However, because neither mutation affects RNase T dimerization, heterodimers can form between HA2 and wild-type monomers. Because no wild-type active site could form in the heterodimer, they would be expected to be inactive; hence, this accounts for the dominant-negative phenotype. Another mutant, HA2-1, which contains the additional mutation, G206S, displays a much-reduced dominant-negative phenotype (7). Based on the model, we now know that the C terminus of RNase T is important for its dimerization. The highly conserved Gly205 and Gly206 residues may form a tight turn in RNase T. Mutation of Gly205 to Ser would disrupt folding of the C terminus and, thus, the ability of this mutant to dimerize. Consequently, it would not act as a dominant-negative mutation.

Mutations at Cys168 and residues at the C terminus lead to temperature-sensitive RNase T mutants (7, 8). However, comparison of gel filtration analyses and activity assays showed that the C-terminal mutant, W207Stop, is more deficient in dimerization yet displays higher RNase T activity than the C168S mutant. This apparent paradox also can be explained by the RNase T structural model. According to this model, the C terminus is directly involved only in dimerization, whereas Cys168 is a buried residue on a short helix formed by residues 164–172. This short helix sits very close to the DEDD catalytic center; although it contributes to dimerization through interactions with the N and/or C termini of the complementary subunit, it probably also affects local folding near the catalytic center.

The structural and functional model presented here provides a detailed framework with which to analyze the mechanism of action of RNase T and, perhaps, other members of the DEDD exonuclease family (17). So far, this model serves to explain the known properties of E. coli RNase T. It will be extremely interesting to compare this model with that of an x-ray structure of RNase T once the latter becomes available. Studies along these lines are now in progress.

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