Bacteriophage T4 RNase H, which removes the RNA primers that initiate lagging strand fragments, has a 5'-to-3' exonuclease activity on DNA:DNA and RNA:DNA duplexes and an endonuclease activity on flap or forked DNA structures (Bhagwat, M., Hobbs, L. J., and Nossal, N. J. (1997) J. Biol. Chem. 272, 28523–28530). It is a member of the RAD2 family of prokaryotic and eukaryotic replication and repair nucleases. The crystal structure of T4 RNase H, in the absence of DNA, shows two Mg2+ ions coordinated to the amino acids highly conserved in this family. It also shows a disordered region proposed to be involved in DNA binding (Mueser, T. C., Nossal, N. G., and Hyde, C. C. Cell (1996) 85, 1101–1112). To identify the amino acids essential for catalysis and DNA binding, we have constructed and characterized three kinds of T4 RNase H mutant proteins based on the possible roles of the amino acid residues: mutants of acidic residues coordinated to each of the two Mg2+ ions (Mg2+-1: D19N, D71N, D132N, and D155N; and Mg2+-2: D157N and D200N); mutants of conserved basic residues in or near the disordered region (K87A and R90A); and mutants of residues with hydroxyl side chains involved in the hydrogen bonding network (Y86F and S153A). Our studies show that Mg2+-1 and the residues surrounding it are important for catalysis and that Lys87 is necessary for DNA binding.

The crystal structure of T4 RNase H, in the absence of DNA, has been solved (4) (Fig. 1). The proposed active site includes the bacteriophage T5 D15 and T7 gene 6 exonucleases, the 5’-to-3’exonuclease domains of DNA polymerases from bacteria such as E. coli, Mycobacterium tuberculosis (Mtb), and Thermus aquaticus (Taq), and the eukaryotic nucleases such as murine FEN2-1 and human RAD2 analog (also called MF-1 or FEN-1) (for review, see Ref. 4). All of these 5’-to-3’exonucleases degrade DNA:DNA and RNA:DNA duplexes to short oligonucleotide products. Many of these proteins have also been shown to possess a flap endonuclease activity (5–7). These 5’-nucleases have sequence similarity to the larger repair proteins such as human XPG (bottom sequence, Fig. 1A). In this figure, the highly conserved residues are marked in red, and the moderately conserved residues are marked in green. Sequence alignments for additional members of this family can be found in Ref. 4. The crystal structure of T4 RNase H, in the absence of DNA, has been solved (4) (Fig. 1B, left panel). The proposed active site (Fig. 1B, right panel) contains 2 Mg2+ ions separated by approximately 7 Å and a number of conserved amino acids. The first Mg2+ ion (Mg2+-1) is coordinated directly to Asp132 and through water molecules to Asp139, Asp71, and Asp155. The second Mg2+ ion (Mg2+-2) is coordinated through water molecules to Asp157, Asp206, and Tyr296. The hydroxyl side chain of Tyr296 as well as that of Ser154, which is near Asp19, are involved in the hydrogen bonding network surrounding the Mg2+ ions. The crystal structure shows a disordered region from residues 89 to 97, containing a number of positively charged residues. It has been proposed that this region is involved in DNA binding (4).

### Identification of Residues of T4 RNase H Required for Catalysis and DNA Binding

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1. M. Bhagwat and N. J. Nossal, in preparation.
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**Key Points**

- T4 RNase H is a member of the RAD2 family.
- It has a 5' to 3' exonuclease activity.
- The crystal structure of T4 RNase H in the absence of DNA has been solved.
- The protein has a disordered region near residues 89 to 97.
- Mg2+ ions are crucial for catalysis and DNA binding.
- Mutants have been constructed to identify essential amino acids.

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[1] Bhagwat, M., Hobbs, L. J., and Nossal, N. J. (1997) J. Biol. Chem. 272, 28523–28530.

[2] Mueser, T. C., Nossal, N. G., and Hyde, C. C. Cell (1996) 85, 1101–1112.

[3] The abbreviations used are: FEN, flap endonuclease; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride.
Two different mechanisms have been proposed for E. coli RNase H, which also acts on RNA-DNA duplexes: a one-metal mechanism based on that for DNase I (8, 9) and a two-metal mechanism based on that for the 3'→5'-exonuclease of polymerase I (10). In the one-metal mechanism, an active site acidic amino acid acts as a nucleophile and abstracts a proton from a water molecule to create a hydroxyl ion that attacks the phosphodiester bond. The pentavalent species formed during the transition state is stabilized by the Mg$^{2+}$ ion. In the two-metal mechanism, one of the bound Mg$^{2+}$ ions promotes the formation of the hydroxyl ion that attacks the scissile phosphodiester bond. The second Mg$^{2+}$ ion serves to stabilize the oxyanion leaving group and also to stabilize the pentavalent species formed during the transition state. In 3'→5'-exonuclease of polymerase I, the divalent metal ions are 3.9 Å apart (11). T4 RNase H does contain two Mg$^{2+}$ ions, but they are separated by 7 Å, which is far apart for the two-metal mechanism. However, the crystal structure was solved in the absence of DNA, and it is possible that when DNA is bound, there may be a substantial rearrangement bringing the ions closer.

What is the mechanism of the nuclease activity of T4 RNase H? To address this question, we have made three kinds of mutants of T4 RNase H based on the possible roles of the amino acid residues (Fig. 1B): mutants of the acidic residues surrounding the Mg$^{2+}$ ions, mutants of the residues with hydroxyl side chains that are involved in the hydrogen bonding network, and mutants of the residues that may be involved in DNA binding. In this paper, we report the effects of these mutations on the exonuclease activity, the flap endonuclease activity, and on binding to DNA. The implications of these results on the possible roles of the two Mg$^{2+}$ ions and the active site residues are discussed.
EXPERIMENTAL PROCEDURES

Unless otherwise indicated, the materials and methods are those described in the accompanying paper (3).

Site-directed Mutagenesis—The mutants D71N, K87A, Y86F, and R90A were prepared by the overlap extension method (12) using the polymerase chain reaction. The plasmid pNN2202 (encoding the wild type T4 RNase H) (1) was amplified by pfu DNA polymerase from the PstI site to the NcoI site using two end primers, GGATAAAGATATA-CAAAGAAGAATCTGC and CCCCATTTCTTATGCTACGGAACAT-TGC, and two complementary mutagenic primers (mutant codon in bold), D71N (CTGTGTTTAATACCCGCAAATCT and CAGATTTTGCGTTATTAAACAG), K87A (GCTTTATTATTGTCAAAACC- GTGGAAAGAGCAG and GTGCCCTTCCACGGTTTTTAGAATAATT-AACG), Y86F (CCTTTATTATTTAAGAAAAAGCTGG and GCCAC-GGGTTTTTCGATTTTTAATGAGG), and R90A (GCTTTATTATTTAAGAAAAGCTGG and CTTGCTTTTTACGTTTTT-TCTTATAATATTACAGG).

Each amplification reaction was carried out using 30 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min, followed by a final elongation phase at 72 °C for 7 min. The amplified products were purified from an agarose gel using the Qiaquick kit (QIAGEN). The final amplified product was cut with AI and NsiI and then ligated into pNN2202 that had been cut with the same enzymes. The DNA was sequenced using Sequenase 2.0 to confirm the mutation and to make sure that no other mistakes were introduced by the pfu DNA polymerase.

The mutants D19N, D155N, D157N, D200A, and D200N were made from pLysS (15) for expression of the mutant T4 RNase H proteins, as described for the wild type protein (16). Because the plasmid with D71N mutation could not be transformed into E. coli BL21(DE3)pLysS (15), the mutant sequences were confirmed by sequencing the DNA between the appropriate sites.

To determine the amino acids important for the cleavage of the phosphodiester bond by T4 RNase H, we selected residues for mutation based on the crystal structure of T4 RNase H (4) and its sequence similarity to other prokaryotic and eukaryotic nucleases (Fig. 1A). As indicated in the Introduction, the proposed active site contains two Mg2+ ions surrounded by a number of conserved acidic amino acids (Mg2+–1: Asp19, Asp71, Glu30, and Glu31; Mg2+–2: Asp157 and Asp200). We mutated each one of these aspartates individually to aspargine and in addition, Asp157 to alanine. We also mutated two residues with hydroxyl side chains, Tyr46 and Ser112, to phenylalanine and alanine, respectively. In addition, we made mutants of residues close to (K87A) or within (R90A) the disordered region to study the possible roles of these amino acids in binding to DNA.

RESULTS

To determine the amino acids important for the cleavage of the phosphodiester bond by T4 RNase H, we selected residues for mutation based on the crystal structure of T4 RNase H (4) and its sequence similarity to other prokaryotic and eukaryotic nucleases (Fig. 1A). As indicated in the Introduction, the proposed active site contains two Mg2+ ions surrounded by a number of conserved acidic amino acids (Mg2+–1: Asp19, Asp71, Glu30, and Glu31; Mg2+–2: Asp157 and Asp200). We mutated each one of these aspartates individually to aspargine and in addition, Asp157 to alanine. We also mutated two residues with hydroxyl side chains, Tyr46 and Ser112, to phenylalanine and alanine, respectively. In addition, we made mutants of residues close to (K87A) or within (R90A) the disordered region to study the possible roles of these amino acids in binding to DNA.

Purification of the Mutant Proteins—All of the plasmids, except the one with D71N mutation, were transformed into E. coli BL21(DE3)-pLysS (15) for expression of the mutant T4 RNase H proteins, as described for the wild type protein (16). Because the plasmid with D71N mutation could not be transformed into E. coli BL21(DE3)-pLysS, the D71N mutant protein was purified from E. coli MV1190 carrying the appropriate plasmid by infecting with M13mGp1–2, encoding T7 RNA polymerase, as described (1). All of the mutant proteins except D155N and D200A were expressed well and were soluble. There was enough soluble protein to purify the D155N but not the D200A mutant protein. The mutant proteins were partially purified by Whatman P11 phase-velocity column chromatography on phosphocellulose. The 0.5M KCl step elution fraction, which contained fewer contaminating proteins than the 0.3M KCl fraction, was used for the nuclease and DNA binding experiments.

DNA Binding—Binding of the flap DNA structure by the wild type and mutant proteins was measured with a gel mobility shift assay, as described (3).

Purification of Mutant T4 RNase H—We partially purified all of the mutant proteins and characterized the effects of each mutation on the nuclease activities of T4 RNase H and on its binding to DNA. The mutants were constructed by site-directed mutagenesis and the proteins expressed, as described under “Experimental Procedures.” They were partially purified by chromatography on phosphocellulose. The 0.5 M KCl step elution fraction, which contained fewer contaminating proteins than the 0.3 M KCl fraction, was used for the nuclease and DNA binding assays. Fig. 2 is an SDS-polyacrylamide gel of fractions of the Y86F and K87A mutant proteins. Similar purification was achieved with all other mutant proteins (data not shown). The activities of the partially purified wild type and mutant T4 RNase H were compared with corresponding fractions from cells with the vector plasmid and with highly purified wild type protein. The partially purified wild type enzyme showed activity similar to that of homogeneous T4 RNase H, except for some contaminating 3'- to 5'-exonuclease activity (see Fig. 4).
**Exonuclease Activity of the Mutant Proteins—T4 RNase H**

The substrate (5′-end-labeled 34-mer DNA annealed to M13, 1 nM) was treated with wild type or mutant T4 RNase H proteins at 30 °C for the indicated time periods. The approximate concentrations of the proteins, represented by large and small rectangles, respectively, were 35 and 7 nM in the left and the middle panels. In the right panel, the large and small rectangles represent protein concentrations of approximately 35 and 3.5 nM, respectively. The products were analyzed on 20% polyacrylamide, 7 M urea gels.

**Binding of the Mutant Proteins to the Flap Substrate—**We used a gel mobility shift assay to study the effects of the mutations on binding to the flap substrate. Because T4 RNase H requires Mg$^{2+}$ for its activity, the binding studies were performed in the absence of Mg$^{2+}$ to prevent degradation of the DNA substrate. Fig. 5 shows the mobility shift caused by the binding of wild type, Y86F, R90A, and K87A to the flap substrate. The binding of K87A mutant protein was reduced by approximately 50% compared with that of wild type, and the binding of R90A was reduced slightly. All other mutant proteins had DNA binding affinity similar to that of wild type T4 RNase H (data for remaining mutant proteins not shown).

**Effect of Temperature on the Exonuclease Activities of the Mutant Proteins**—The mutants of the residues coordinated directly or through water molecules to Mg$^{2+}$-1 completely lost their nuclease activities (D19N, D71N, D132N, and D155N) but still bound to flap DNA. Hence, Mg$^{2+}$-1 has a catalytic role. In contrast, the mutants of the residues surrounding Mg$^{2+}$-2 either maintained their activity (D200N and Y86F) or had reduced activity (D157N). To determine whether mutations in residues surrounding Mg$^{2+}$-2 changed the stability of the enzyme, we first compared the exonuclease activity of the active mutant protein D200N with that of the wild type at various temperatures (Fig. 6). The activity of the pure wild type enzyme increased 10 times, and that of D200N increased about 2 times as the reaction temperature was increased from room temperature to 45 °C. Partially purified wild type enzyme showed a similar increase in activity (data not shown). For the wild type and the D200N mutant proteins, the ratio of the trinucleotides to dinucleotides released increased with increasing temperature.

Further, we incubated both the wild type and the mutant proteins at 45 °C in the absence of the DNA substrate for various time periods, followed by a 1-min reaction at 45 °C. The results indicate that the D200N and D157N mutant proteins were not more thermolabile than the pure wild type T4 RNase H protein (Fig. 7) or the partially purified wild type T4 RNase H protein (data not shown).

**Effects of Mg$^{2+}$ and Mn$^{2+}$ Ion Concentrations on Exonuclease Activity—**To determine whether Mg$^{2+}$-2 is loosely bound in the mutant proteins, we studied the effect of altering the Mg$^{2+}$ ion concentration on the activities of the D157N and D200N mu-
tant proteins. Similar to the pure wild type, D200N mutant protein showed maximal activity in 10 mM magnesium acetate (Fig. 8). The partially purified wild type T4 RNase H also showed maximal activity in 10 mM magnesium acetate (data not shown). The low activity of D157N did not increase with the elevated magnesium acetate concentration. However, the D157N mutant had higher activity when magnesium chloride was replaced by manganese chloride (Table I). In addition, the ratio of trinucleotide to dinucleotide products released by both D157N and D200N increased when magnesium chloride was replaced by manganese chloride (Table I).
TABLE I

| Enzyme and product | MgCl₂ | MnCl₂ |
|--------------------|-------|-------|
|                    | 1 min | 10 min | 1 min | 10 min |
| Wild type          |       |        |       |        |
| Trinucleotide (fmol) | 1.24  | 0.98   | 0.21  | 1.26   |
| Dinucleotide (fmol) | 0.64  | 0.73   | 0.12  | 0.71   |
| TriDi              | 1.94  | 1.34   | 1.75  | 1.77   |
| D157N              |       |        |       |        |
| Trinucleotide (fmol) | 0.01  | 0.05   | 0.40  | 0.39   |
| Dinucleotide (fmol) | 0.01  | 0.12   | 0.35  | 0.38   |
| TriDi              | 1.0   | 0.42   | 1.14  | 1.03   |
| D200N              |       |        |       |        |
| Trinucleotide (fmol) | 0.62  | 0.59   | 0.39  | 2.01   |
| Dinucleotide (fmol) | 0.88  | 1.38   | 0.31  | 1.27   |
| TriDi              | 0.70  | 0.43   | 1.26  | 1.58   |

DISCUSSION

Bacteriophage T4 RNase H is a 5'-nuclease that is required to remove the RNA primers from lagging strand fragments during DNA replication and has significant amino acid sequence similarity to other prokaryotic and eukaryotic nucleases with the same function (for review, see the Introduction and Fig. 1A). In the recent crystal structure of the enzyme, solved in the absence of a substrate, the highly conserved acidic residues are clustered together surrounding two Mg²⁺ in a cleft that appears to be wide enough for single- or double-stranded DNA (4) (see Fig. 1B). At the top of the cleft there is a short basic disordered region that has been proposed to play a role in binding the substrate. As a step toward understanding the mechanism of this important enzyme, we have constructed and characterized T4 RNase H mutants altered in active site residues that are coordinated to the two Mg²⁺, are in the disordered region, or possess hydroxyl side chains.

Residues Required for Catalysis—Our studies on the mutagenesis of the active site residues of T4 RNase H show that mutation of any of the residues coordinated directly or through water molecules to Mg²⁺-1 (D19N, D71N, D155N, or D132N) causes the complete loss of both the exonuclease and flap endonuclease activities but does not affect binding to DNA. These results indicate that residues Asp¹⁹, Asp¹⁵⁵, Asp¹³², Asp⁷¹, and Mg²⁺-1 have a role in catalysis. At present it is not clear whether any of these aspartates has a specific function beyond being an essential part of the binding site for Mg²⁺. A divalent cation may increase the rate of the hydrolysis of a phosphodiester bond in a number of ways. It may neutralize the substrate, the pentavalent transition state, or the product (17), or it may be involved in the formation of the hydroxyl ion that attacks the scissile phosphodiester bond. The inactive mutant proteins of T4 RNase H are currently being used for co-crystallization with DNA to define the DNA binding site and the actual role of Mg²⁺-1 and the aspartates coordinated to it.³

Our finding that T4 RNase H proteins with mutations in the residues coordinated through water to Mg²⁺-2 either retain their activity (D200N and Y86F) or have reduced activity (D157N) (Figs. 3 and 4) suggests either that Mg²⁺-2 can remain bound in the absence of any one of these residues or that this metal ion is not required for catalysis. We were unable to test the possibility that the carbonyl oxygen of D200N is important for activity because the D200A protein was expressed poorly (not shown).

Mg²⁺-2 could play a less direct role by stabilizing the protein or by forming a part of the binding site for the substrate, as initially proposed by Mueser et al. (4). However, our studies indicate that the D157N and D200N mutant proteins are not less thermostable than the wild type (Fig. 7). In addition, Mg²⁺-2 is not loosely bound in the D157N mutant protein, as increasing the Mg²⁺ ion concentration did not increase the activity of the D157N mutant protein markedly (Fig. 8). However, the exonuclease activity of the D157N mutant is increased when magnesium chloride is replaced by manganese chloride. A similar increase in activity when Mn²⁺ replaced Mg²⁺ has been reported for the active site mutants of EcoRV (18) and BamHI (19) restriction endonucleases. For both the D200N and D157N mutant proteins, the ratio of trinucleotides (di- and tri-nucleotides) released is less than that of the wild type protein in Mg²⁺ (Fig. 6 and Table I) and increases in Mn²⁺ (Table I). It is possible that there is a distortion in the metal or DNA binding site on mutating Asp⁷¹ or Asp¹³² to asparagine, which is partially corrected by the Mn²⁺ substitution. Thus, at this time the role of Mg²⁺-2 is still ambiguous, but it should be clarified when a structure of the enzyme with its substrate is available. As already noted in the Introduction, in the absence of substrate, the two Mg²⁺ in the T4 RNase H cleft are more widely separated (7 Å) (4) than those in the 3'- to 5'-exonuclease domain of E. coli polymerase I (3.9 Å), for which a two-metal hydrolytic mechanism has been proposed (11).

³ T. C. Mueser and C. C. Hyde, personal communication.

FIG. 7. The D157N and D200N mutant proteins are not thermostable compared with the wild type (WT) protein. T4 RNase H (mutant or highly purified wild type) was diluted and preincubated at 45 °C for various time periods. The reaction mixtures containing the substrate (5'-end-labeled 34-mer annealed to M13, 1 nM) were preincubated at 45 °C for 2 min. The preincubated enzymes were reacted with the preincubated reaction mixtures at 45 °C for 1 min. Only the products of the reactions are displayed. The concentration of the D157N mutant protein was approximately 100 times that of the wild type (1.4 nM) because of the reduced activity of the mutant protein. Similar results were obtained with the partially purified wild type protein (not shown).

FIG. 8. Effect of Mg²⁺ ion concentrations on the activities of the D157N and D200N mutant proteins. The exonuclease activity of the wild type (solid square), D157N (crossed square), and D200N (solid circle) proteins was measured with 5'-end-labeled 34-mer annealed to M13mp7 (1 nM) in varying concentrations of magnesium acetate at 30 °C for 1 min. In this experiment, about 100 times more D157N protein was used than the highly purified wild type T4 RNase H (1.4 nM) because of the reduced activity of the D157N mutant protein. The total product (trinucleotides plus dinucleotides, fmol) released is plotted against the Mg²⁺ concentration.
Our studies suggest that the tyrosine closest to Mg\(^{2+}\)-2 (Tyr\(^{86}\)) and the serine closest to Mg\(^{2+}\)-1 (Ser\(^{153}\)) in the T4 RNase H cleft are not essential for the stabilization of the transition state or the product during the exonuclease reaction. The mutant Y86F maintains its activity, whereas that of the S153A protein is moderately reduced (Figs. 3 and 4).

**Substrate Binding**—Because T4 RNase H cuts RNA-DNA and DNA-DNA duplexes and fork and flap structures at similar rates, it must have a substrate binding site that can accommodate all of these substrates. Our mutagenesis studies show that these substrates must be positioned on the enzyme with the scissile phosphodiester bond (phosphodiester bond between the first few nucleotides from the 5'-end for RNA-DNA and DNA-DNA duplexes and the phosphodiester bonds near the junction between the single-stranded DNA and the duplex in the flap and fork substrates) near Mg\(^{2+}\)-1. Because there was no DNA in the crystal structure of T4 RNase H (4) or of the T5 D15 exonuclease (7) or the 5'- to 3'-nuclease domain of Taq DNA polymerase I (20), the position and the orientation of the substrates on this family of enzymes are still speculative.

There is a short sequence between the conserved N and I regions (Fig. 1A) that contains several basic residues in most members of this RAD2 nuclease family, particularly the short nucleases that are not attached to a polymerase. The exceptions are the larger repair nucleases, like human XPG, in which the N and I regions are separated by more than 600 residues (Fig. 1A). In the T4 RNase H structure, the region between N and I is located above the cleft with the two Mg\(^{2+}\) ions, and nine of its residues (89–97) are disordered (Fig. 1B). Mueser et al. (4) speculated that this disordered region might be involved in substrate binding. The crystal structure of the 5'- to 3'-nuclease domain of Taq polymerase also shows a disordered region, a small part of which overlaps the region disordered in the T4 enzyme (20). In the T5 D15 nuclease structure, there is a helical arch large enough to accommodate single-stranded but not duplex DNA at the back of the concave surface containing the two Mg\(^{2+}\) ions (7). Part of this arch is composed of residues corresponding to all of those disordered in T4 RNase H and some of those disordered in the Taq nuclease.

Ceska et al. (7) proposed that the single-stranded region of the flap threaded through this arch and that the duplex was bound on the enzyme surface outside the concave surface. A similar orientation for the duplex part of the flap substrate might be possible for the T4 enzyme, with the single strand threading under the disordered region. An alternate possibility is that the cleft of T4 RNase H, or the concave surface of T5 exonuclease, which in the absence of substrate appears to be too narrow to accommodate an RNA-DNA or DNA-DNA duplex, might open to bind a duplex in an orientation such that the scissile bond is above Mg\(^{2+}\)-1. Even with the duplex in this position, the single strand of the flap or the released oligonucleotide products might move under the arch or disordered region.

The notion that the single strand threads through the nucleases of this family is supported by evidence that the flap endonuclease of the calf thymus FEN-1 protein is inhibited by either binding a protein on the single strand or annealing a complementary strand (21) and that the T4 gene 32 single-stranded DNA-binding protein inhibits the flap endonuclease of T4 RNase H (3). Although simple duplex and flap substrates are hydrolyzed at similar rates by the T4 enzyme, only the flap and fork substrates bind tightly enough to be retarded in mobility shift assays (3). We mutagenized the two most conserved basic residues in and adjoining the disordered region (Lys\(^{87}\) and Arg\(^{90}\)), with the expectation that these changes might have more effect on the binding and degradation of flap than duplex substrates. Although the K87A mutant protein did show reduced binding to flap DNA, about 50% of the wild type, it had also lost the ability to cut both flap and duplex substrates. The nuclease activity of the R90A protein was reduced to a similar extent on flap and duplex substrates, but its binding to flap DNA was only slightly impaired relative to the wild type protein.

On flap and forked substrates, wild type T4 RNase H and the active mutant proteins cut on each side of the junction between the single- and double-stranded DNA, whereas on duplex DNA they removed a mixture of short oligonucleotides (1–5 nucleotides) from the 5'-end. The average size of the products from duplex substrates was smaller with the D157N and D200N mutant proteins and increased with increasing reaction temperature with both the mutant and wild type enzymes. These observations are consistent with a need for the 5'-end to fray to reach the catalytic site, in a fashion similar to the fraying of the 3'-end to reach the active site of the proofreading 3'- to 5'-nuclease domains of polymerases (22, 23). However this fraying does not correlate simply with the melting temperature of the duplex. There was a similar distribution of product sizes with increasing temperature on the 34-mer annealed to M13 DNA (Fig. 6) and on (dT)\(^{30}\)-(poly(dA)) (not shown).

**Comparison with Related Nucleases**—Mutagenesis studies have been performed for three proteins homologous to T4 RNase H: the 5'-nuclease domains of E. coli (24) and Mtb (25) polymerase I and human FEN-1 (26). The mutants of amino acids corresponding to T4 RNase H Asp\(^{157}\), Asp\(^{71}\), Asp\(^{132}\), and Asp\(^{155}\) were inactive in E. coli polymerase I (D13N, D63A, D115A, and D138N) and in Mtb polymerase I (D21N, D73N, D125N, and D148N). For human FEN-1, the mutants of the residues corresponding to Asp\(^{19}\) and Asp\(^{71}\) (D34A and D86A) lost activity, but that analogous to Asp\(^{155}\) (D179A) was active. (The mutant corresponding to D132N was not reported.) Thus, with the exception of the mutant of human FEN-1 corresponding to D155N, the mutants of the residues surrounding the Mg\(^{2+}\)-1 were inactive in all of these homologous proteins.

T4 RNase H differs from the other proteins in the importance of residues surrounding Mg\(^{2+}\)-2. In each of the three other homologous proteins where mutagenesis studies have been reported, the mutants corresponding to D157N and D200N lost activity. In contrast, for T4 RNase H, D157N had activity, albeit reduced, and D200N had activity comparable to the wild type. However, as indicated above, the size distribution of the products released by these T4 mutant proteins was different from that of the wild type. One possible reason for the activity of T4 D200N is that Asp\(^{200}\) may not have an important role in the active site of T4 RNase H because it is neutralized by the adjacent Lys\(^{199}\). None of these other homologous proteins has lysine or arginine in the corresponding position. Finally, although the T4 mutant K87A was inactive and had reduced binding to DNA, the analogous mutant in human FEN-1 (R103A) was active.

The comparison of the mutant activities of T4 RNase H and these three homologous proteins indicates that the role of Mg\(^{2+}\)-1 is catalytic in all of these proteins, but the role of Mg\(^{2+}\)-2 varies. Differences in the structure surrounding Mg\(^{2+}\)-2 may contribute to the different biochemical characteristics of the 5'- to 3'-exonuclease and flap endonuclease activities of these proteins. E. coli polymerase I releases more monomers than oligomers as the major product of the exonuclease activity (27), whereas T4 RNase H releases dimers and trimers (3). In addition, E. coli polymerase I makes only one cut on flap structures, between the first two paired nucleotides (24), whereas yeast, mouse, and calf thymus FEN-1, and T4 RNase H make two cuts, one on each side of the junction between single- and double-stranded DNA (3, 21, 28). Our analysis
suggests that despite their sequence homology, these four proteins (T4 RNase H, *Mtb* nuclease, *E. coli* polymerase I, and human FEN-1) have subtle differences in their active site structures. Two mutants of the more distantly related eukaryotic nuclease, human XPG protein (Fig. 1A), have recently been reported (29). In contrast to T4 RNase H and related 5' exo-nucleases and flap endonucleases, XPG protein, which is required for nucleotide excision repair, has 3'-junction cutting activity on bubble substrates. The alanine mutants of Asp77 and Asp812 (corresponding to Asp71 and Asp157 of T4 RNase H) had, respectively, reduced and no residual 3'-junction cutting activity.

In summary, our mutagenesis studies of T4 RNase H reported here identify the importance of Lys87 in binding to DNA, and Mg\(^{2+}\)-1 and its coordinating residues (Asp 19, Asp71, Asp155, and Asp 132) in catalyzing the exonuclease and flap endonuclease activities of T4 RNase H.

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