Maturation of Bacteriophage T4 Lagging Strand Fragments Depends on Interaction of T4 RNase H with T4 32 Protein Rather than the T4 Gene 45 Clamp*

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In the bacteriophage T4 DNA replication system, T4 RNase H removes the RNA primers and some adjacent DNA before the lagging strand fragments are ligated. This 5′-nuclease has strong structural and functional similarity to the FEN1 nuclease family. We have shown previously that T4 32 protein binds DNA behind the nuclease and increases its processivity. Here we show that T4 RNase H with a C-terminal deletion (residues 278–305) retains its exonuclease activity but is no longer affected by 32 protein. T4 gene 45 replication clamp stimulates T4 RNase H on nicked or gapped substrates, where it can be loaded behind the nuclease, but does not increase its processivity. An N-terminal deletion (residues 2–10) of a conserved clamp interaction motif eliminates stimulation by the clamp. In the crystal structure of T4 RNase H, the binding sites for the clamp at the N terminus and for 32 protein at the C terminus are located close together, away from the catalytic site of the enzyme. By using mutant T4 RNase H with deletions in the binding site for either the clamp or 32 protein, we show that it is the interaction of T4 RNase H with 32 protein, rather than the clamp, that most affects the maturation of lagging strand fragments in the T4 replication system in vitro and T4 phage production in vivo.

DNA synthesis on the lagging strand of the replication fork is accomplished by the rapid repetition of a cycle in which primase makes a short RNA primer that is elongated by polymerase; the primer is removed from the previous fragment, and the two fragments are joined by DNA ligase. The efficient sealing of adjacent fragments is essential to maintain the accuracy of DNA replication. The accumulation of nicks and gaps on the lagging strand ultimately results in double-stranded breaks, increased mutation frequencies, and cell lethality (reviewed in Refs. 1 and 2). The lagging strand cycle must be repeated every few seconds because the discontinuous fragments are so short, 1–2 kb in prokaryotes and less than 200 bases in eukaryotes. In each of the replication systems studied, the primers are removed by a member of a family of 5′-nuclease with conserved sequences and similar structures (Fig. 1). It is important that the process of primer removal from one fragment is coordinated with the elongation of the next fragment to ensure rapid and accurate replication. Our studies indicate that the mechanism by which lagging strand polymerization and primer removal are coordinated in bacteriophage T4 replication is different from that used in eukaryotes.

The phage T4 member of the FEN1 nuclease family was called T4 RNase H because it hydrolized the RNA strand in an RNA:DNA hybrid, as expected for an enzyme removing the RNA primers. However, it also acts as a 5′-nuclease on DNA duplexes (3). Genetic studies indicate that either T4 RNase H or the 5′- to 3′-exonuclease of the Escherichia coli host DNA polymerase I is necessary for phage production (4). A T4 mutant with a large deletion (Δ118–305) in the rnh gene gives a burst size of 50% of wild type T4 phage in a wild type host, but a burst of only a few phage per infected cell under nonpermissive conditions in E. coli PolA12, which has a conditionally lethal mutation in the host nuclease. Short DNA fragments accumulate, consistent with a defect in removing the primers from lagging strand fragments that prevents ligation of adjacent fragments. Phage production is restored by supplying T4 RNase H on a plasmid. The T4 rnh deletion mutant is also hypersensitive to UV irradiation and to antitumor agents that induce T4 topoisomerase cleavage products (5) and is defective in DNA homing (6). Processing of the RNA transcript that serves as the primer for leading strand synthesis at the T4 uvsY origin appears to be impaired in the T4 rnh mutant (7).

The 5′-nucleases in this family have both a 5′-exonuclease activity that degrades RNA:DNA and DNA:DNA duplexes, giving short oligonucleotide products, and a flap endonuclease activity that cuts close to the junction of single- and double-stranded DNA on fork and flap substrates (reviewed in Ref. 1). The relative strength of these two activities differs within the family, with the exonuclease stronger in the phage enzymes (T4 RNase H (8) and T5 5′ to 3′-exonuclease (9)) and the flap endonuclease stronger in the FEN proteins (10). The 5′-exonuclease activity of T4 RNase H is nonprocessive, removing a single oligonucleotide (predominantly dimers and trimers) each time it binds its substrate. On substrates where the T4 gene 32 ssDNA1-binding protein can bind behind the nuclease, its processivity is increased, so that a total of about 10 short oligonucleotides are hydrolyzed at each binding. However, the flap endonuclease of T4 RNase H is inhibited when 32 protein binds to the single-stranded flap (8). Similarly, FEN1 cutting of flaps long enough to bind RPA, the eukaryotic counterpart of 32

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1 The abbreviations used are: ssDNA, single-stranded DNA; PCNA, proliferating cell nuclear antigen; RPA, replication protein A; pol, polymerase; b, base; gp, gene product.
protein, is inhibited when RPA is present (1). In the bacteriophage T4 replication system, the polymerase is held on the primer by the gene 45 clamp, which is loaded by the 44/62 clamp loader complex. T4 32 protein plays a major role in orchestrating the lagging strand cycle by increasing primer synthesis, promoting loading of the clamp by the clamp loader, and increasing the processivity of both polymerase and RNase H (11, 12). We have previously shown that T4 RNase H removes the RNA primers and about 30 nucleotides of adjacent DNA from each lagging strand fragment during DNA replication in vitro, and that it is the 5′-exonuclease activity, rather than the flap endonuclease, that is responsible for most of this digestion (13). Our studies indicated that, on most molecules, polymerase filled in the gap between adjacent fragments before the nuclease could bind for a second round of degradation. The amount of DNA removed along with the primers was similar to the DNA removed during a single binding by T4 RNase H, when 32 protein was behind it. Thus our studies were consistent with a model in which the extent of degradation was controlled by the difference in rates of digestion by T4 RNase H and synthesis by polymerase, when 32 protein covered the single-stranded DNA between them.

In eukaryotic DNA replication, both FEN1 nuclease and the nuclease activity of the Dna2 helicase-nuclease have roles in removing primers from eukaryotic lagging strand fragments (reviewed in Refs. 1 and 14). Recent studies (15–18) indicate that, in contrast to T4 RNase H, FEN1 uses its flap endonuclease to remove the primer and adjacent DNA, after a flap is created by the polymerase extending the upstream fragment. The rate of strand displacement synthesis by Saccharomyces cerevisiae pol δ with the PCNA clamp and replication factor C clamp loader is increased by FEN1, so that these four proteins together catalyze efficient nick translation. The interaction between FEN1 and the PCNA replication clamp is clearly important, because there was less strand displacement synthesis with a FEN1 protein with a mutation in the C-terminal PCNA interaction site (16).

In this paper we show that T4 RNase H, like FEN1, is stimulated by its replication clamp, the T4 gene 45 protein. However, in contrast to FEN1, T4 RNase H interaction with the 45 clamp is not required for the normal processing of lagging strand fragments. Instead, it is the T4 RNase H interaction with 32 protein that is essential. T4 RNase H interacts with the clamp through a conserved clamp-binding motif at the N terminus of the nuclease. A C-terminal helical bundle at the C terminus of the nuclease is needed for its stimulation by 32 protein. An N-terminal deletion in T4 RNase H that prevents stimulation by the clamp does not decrease fragment sealing in the T4 replication system in vitro. Plasmid encoding this mutant T4 RNase H can replace the wild type in restoring production of T4 phage with a disrupted rnh gene. C-terminal deletions that abolish T4 RNase H interaction with 32 protein strongly impair fragment maturation in vitro and fail to restore T4 Δrnh mutant phage production in vivo.

EXPERIMENTAL PROCEDURES

DNA Substrates—Oligonucleotides were made and reverse phase-purified by Sigma-Genocea, except that oligonucleotides longer than 50 bases were gel-purified. The 3′ or 5′ end-labeled partial duplexes were made by annealing an 84-mer DNA complementary to nucleotides 6196–6281 of M13mp19 to the viral single-stranded DNA, as described previously (13). Nicked or gapped molecules were made as described (13) by annealing oligonucleotides complementary to the following sequences of M13mp19 behind the labeled 56-mer: nicked, 41-mer, 6282–6322; gap of 7, 61-mer, 6289–6350; 14, 61-mer, 6296–6357; 28, 43-mer, 6309–6351; 100, 44-mer, 6382–6426; 200, 44-mer, 6482–6526; and 1479, 42-mer, 510–551. The construction and nicking of the 2.7-kb pUCNICK circular plasmid, a pUC19 derivative with a single recognition site for the N.BbvC I nicking enzyme (New England Biolabs), have been described (19).

T4 RNase H Interaction with the Clamp and 32 Protein

T4 Replication Proteins—Wild type T4 RNase H, T4 DNA polymerase, T4 gene 45 clamp, genes 44/62 clamp loader, gene 41 helicase, gene 59 helicase loading protein, and gene 61 primase were purified to apparent homogeneity as described by Novell et al. (20). Wild type 32 protein was purified as described (21). The truncated 32 proteins, 32-A and 32-B, were the generous gift of David Geidroc (22, 23). T4 DNA ligase was obtained from U. S. biochemical Corp.

Nuclease and Polymerase Assays—Unless otherwise indicated, reaction mixtures (10 μl) contained 1.0 μM substrate, 25 mM Tris acetic, pH 7.5, 63 mM potassium acetate, 6 mM magnesium acetate, 20 mM dithiothreitol, 10 μM DNA polymerase, 2 μM primer, 20 μg/ml bovine serum albumin, ATP present at 1 mM and each DNTP at 250 μM. Unless otherwise indicated, reaction mixtures without T4 RNase H, ligase, or DNA polymerase were incubated for 2 min at 30 °C, and the reaction was begun by the addition of the nuclease, or a mixture of the nuclease, ligase, and polymerase, as noted in the figures. Aliquots were taken at the times indicated, and the reaction was stopped by addition of 10 μl of a solution containing 10% (v/v) formamide, 1 mM EDTA, and bromphenol blue, and 33 mM EDTA. Products were heated for 3 min at 95 °C before electrophoresis on polyacrylamide (19.1), 7 μm gels of the percentage indicated in the figure legends. Gels were exposed to Kodak Biomax MR film or were scanned and quantified with a Fujifilm FLA 3000 Phosphorimagerr and Fuji Multigaage software.

In the experiments shown in Figs. 2B and 10, 5-μl aliquots of the reaction mixtures were removed at the indicated times and then heated for 20 min at 60 °C to inactivate the enzymes. BsNI endonuclease (2 units) (New England Biolabs) was then added, and the incubation at 60 °C continued for 30 min, before adding 7 μl of the formamide stop solution.

Coupled Leading and Lagging Strand Synthesis—The reaction mixtures (10 μl) contained 1.6 μM singly nicked pUCNICK plasmid (2.7 kb), 250 μM each of the four deoxynucleoside triphosphates, 100 μM each of the four deoxynucleoside diphosphates, 25 μM Tris acetic, pH 7.5, 60 mM potassium acetate, 6 mM magnesium acetate, 10 mM dithiothreitol, and 20 μg/ml bovine serum albumin. The protein concentrations were 2 μM wild type or mutant T4 RNase H, 328 nM 41 helicase, 30 μM wild type DNA polymerase, 242 μM 44/62 clamp loader, 162 μM 45 clamp, 95 μM 59 helicase loading protein, and 64 nM 61 primase. When indicated, RNase H was 100 μM, and DNA ligase was 200 Weiss units/ml. Reaction mixtures without polymerase, primase, helicase, RNase H, and DNA ligase were incubated for 2 min at 37 °C, and synthesis was begun by the addition of polymerase, primase, and helicase. RNase H and DNA ligase were added 1 min later. At the times indicated, aliquots of the reaction mixtures were mixed with an equal volume of 0.2 μM EDTA to stop the synthesis, and the products were analyzed by 0.6% alkaline agarose gel electrophoresis (24) and trichloroacetic acid precipitation (25).

Plasmids Encoding T4 RNase H Mutant Proteins—The C-terminal deletion of T4 RNase H ΔC 278–305 (ΔC) was made by cloning plasmid pNN2202 (3) (wild type T4 RNase H under the control of the T7 promoter) partially with Sapl and totally with EcoRI restriction nucleases, and ligating the duplex made by annealing 5′-AAAGAAGGAATCTGCTTAATTGACTT and 3′-PshAI nucleases and inserting the duplex formed from 5′-TGGCAAAATTTATTCATATTTTGTAAAAGCGGGTCTTTAG-3′ and 3′-32P[dTTP (20)). Wild type 32 template was provided by site-directed mutagenesis of the wild type gene in the plasmid pV/H9004 (26) using the method of Kunkel et al. (25), modified by using T4 DNA polymerase, T4 44/64 clamp loader, and 45 clamp to copy the ssDNA template. The oligonucleotide primers (the sequence complementary to the mutation codon is underlined) used are as follows: L3A, 5′-CCACAAT-TCATTCCTGCTCAATCATATATCATC-3′; M6A, 5′-GTAATTCCTATCACAACGGACCTTCAAAATCC-3′; L4A3M6A, 5′-GTAATTCCTATCACAACGGACCTTCAAAATCC-3′; and 3′-ACATGGTTTCTTCCATTGACAGCAGATTTAACGTA-3′. Point mutations in the N-terminal region of T4 RNase H were made by site-directed mutagenesis of the wild type gene in the plasmid pV/H11032 (20). Using the method of Kunkel et al. (25), modified by using T4 DNA polymerase, T4 44/64 clamp loader, and 45 clamp to copy the ssDNA template. The oligonucleotide primers (the sequence complementary to the mutation codon is underlined) used are as follows: L3A, 5′-CCACAAT-TCATTCCTGCTCAATCATATATCATC-3′; M6A, 5′-GTAATTCCTATCACAACGGACCTTCAAAATCC-3′; L4A3M6A, 5′-GTAATTCCTATCACAACGGACCTTCAAAATCC-3′; and 3′-ACATGGTTTCTTCCATTGACAGCAGATTTAACGTA-3′.
Temperature controlled PL promoter, as well as a compatible plasmid pGP1.2 (29), a plasmid with the gene for T7 RNA polymerase under the interruption in the gene for RNase HI and a temperature-sensitive fomyl fluoride hydrochloride) and PC buffer B (PC, buffer A containing residues 118–305 in the gene encoding T4 RNase H. The host ethyl)-benzenesulfonyl fluoride hydrochloride, and broken by sonication-S (Perspective Biosystems) by using linear gradients formed from raphy first on SP-Sepharose (Amersham Biosciences) and then on Po- at 4 °C. T4 RNase H was purified from the supernatant by chromatog-raphy.

Scientific BioFlo 3000 fermentor, and protein synthesis was induced by addition of 1 mM isopropyl thiogalactoside for 2 h at 37 °C, as described for the wild type protein (20), and partially purified by the small scale procedure described in Ref. 27. For ΔC-(278–305), plasmid pMB5002 in BL21(DE3)plpS was grown in Luria Broth with 50 μg/ml of carbeni-cillin (Invitrogen) at 24 °C to A600 = 0.4 in a 20-liter New Brunswick Scientific BioFlo 3000 fermentor, and protein synthesis was induced by addition of 1 mM isopropyl thiogalactoside. The cells (45 g) were harvested after overnight induction, resuspended in 50 mM Tris-Cl, pH 7.5, 500 mM NH₄Cl, 10 mM MgCl₂, 2 mM dithiothreitol, and 0.1 mM 12-amin-ethyl)-benzenesulfonyl fluoride hydrochloride, and broken by sonication, and the cell lysate was centrifuged at 100,000 × g at 4 °C. T4 RNase H was purified from the supernatant by chromatography first on SP-Sepharose (Amersham Biosciences) and then on Por-roS-5 (Perspective Biosystems) by using linear gradients formed from PC buffer A (50 mM Tris-Cl, pH 8.0, 100 mM NH₄Cl, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, and 25 μg/ml 4-(2-aminooxybenzyl)-benzenesul-fonyl fluoride hydrochloride) and PC buffer B (PC, buffer A containing 750 mM NaCl).

**Results**

**T4 RNase H Exonuclease Activity Is Stimulated When the T4 Gene 45 Clamp Protein Is Loaded Behind It**—The T4 gene 45 replication clamp protein is loaded preferentially at the 3′ end of a junction between single- and double-stranded DNA and at the 3′ side of a nick (11, 12), whereas T4 RNase H is loaded at the 5′ end (3) (see Fig. 1C and diagrams at the top of Fig. 2A). The clamp protein can move in both directions on the duplex DNA. It can also track for short distances on single-stranded DNA but falls off single-stranded DNA more rapidly than double-stranded DNA (30). In Fig. 2, the nicked, gapped, and partial duplex substrates were made by annealing oligonucleo-tides to single-stranded circular DNA and were labeled at the 3′ end of the downstream 86-base fragment. Addition of the T4 clamp, clamp loader, and ATP stimulates the 5′-to-3′ exonuclease on the nicked substrate (Fig. 2A, lane 9) and on the substrate with the 28-base gap (lane 14), where the clamp can be loaded behind the nuclease but does not stimulate on the partial duplex (lane 4). The nuclease, by itself, has similar activity on the partial duplex and the gapped molecule (Fig. 2A, lanes 2 and 12) but has very little activity on the nicked sub-strate (lane 7), where there is no single-stranded DNA behind the 3′-labeled duplex. As we have shown previously (8), 32 protein increases the processivity of the nuclease when there is single-stranded DNA behind the nuclease (Fig. 2A, lanes 3 and 13). However, it does not increase the nuclease activity on the nicked substrate (Fig. 2A, lane 8). There will be more clamp loaded in front of the nuclease in reactions with 32 protein because 32 protein increases the loading of the clamp by the clamp loader (31, 32). Interference by this increased clamp in front of the nuclease is the likely reason that there is less activity on the nicked substrate when 32 protein is present in addition to the clamp, clamp loader, and ATP (compare Fig. 2A, lanes 9 and 10, and Fig. 2B, reactions 4 and 5). When polymerase is present to extend the duplex available to the clamp ahead of the nuclease, there is no longer inhibition by 32 protein (Fig. 2B, compare reactions 12 and 13). Note that in Fig. 2B, the reaction products were cut with BstNI restriction nuclease to allow determination of the extent of hydrolysis at the 5′ end.

ATP and the T4 gene 44/62 clamp loader are needed in addition to the clamp for stimulation of T4 RNase H activity on nicked substrates. This is shown, using 5′ end-labeled sub-strates, in Fig. 3. Addition of the clamp did not change the size of the oligonucleotides removed by the nuclease, which are predominantly dimers and trimers (8). The same size distribution is observed when a 5′-labeled partial duplex is cut by the nuclease alone (Fig. 3, reaction 2), and when the nicked sub-strate is cut in the presence of the clamp, clamp loader, and ATP (reaction 10).

The T4 clamp can move along single-stranded DNA, but it falls off more rapidly than on a duplex (30, 33). We detected some stimulation of RNase H when the gap between the nucleotides was increased from 28 to 100 bases, but none with a gap of 200 bases (Fig. 4).

**The Clamp Does Not Increase the Processivity of T4 RNase H**—Circular replication clamps surrounding the DNA tether their respective polymerases on the template, thus increasing their processivity (number of nucleotides added per polymerase binding). We have shown previously that T4 RNase H by itself is nonprocessive, hydrolyzing a single small oligonucleotide with each binding, but that the nuclease becomes processive when the T4 gene 32 protein is added (8). In that study, hydrolysis of a partial duplex like that in Fig. 2A (left) by T4 RNase H alone was halted by addition of a fork DNA trap, unless 32 protein was present (8). Likewise, the limited hydrolysis of the nicked substrate by RNase H alone was stopped by addition of fork DNA after 20 s (Fig. 5, compare reactions 4 and 6). The increased rate of hydrolysis of the nicked substrate with the clamp (Fig. 5, reaction 7) is not the result of an increase in processivity. This hydrolysis stopped completely as soon as the fork DNA trap was added (Fig. 5, reaction 8).

**Mutations in a Conserved Clamp Interaction Motif at the N terminus of RNase H Eliminate Stimulation by the Clamp**—A short conserved motif essential for interaction with replication clamps has been identified in bacteria, archaea, and eu-karyotes (reviewed in Refs. 34 and 35). The N terminus of T4 RNase H has a sequence similar to the C-terminal clamp interaction motifs identified previously in T4 DNA polymerase (36), and the T4 gene 33 and 55 late transcription activators (37) (Fig. 6A). Shamoo and Steitz (38) have determined the crystal structure of the clamp from the closely related phase RB69, complexed with a C-terminal peptide from RB69 DNA polymerase. The most highly conserved RB69 polymerase residues (Fig. 6A, shaded letters) directly contacted the clamp in their structure. The analogous shaded residues in p21 contact human PCNA clamp (39), and those from Archaeoglobus ful-gids FEN1 contact the archaeal clamp (40).

The N-terminal region of RNase H was disordered in the crystal structure of Mueser et al. (41) (Fig. 1A) and is distant from the essential residues that surround the two magnesiums in the active site of the enzyme. To evaluate the importance of the motif in T4 RNase H, we have constructed a protein with a deletion of this disordered region (Δ2–10, hereafter ΔN) and several point mutants (Fig. 6B). The mutant RNase H proteins
were expressed and purified as described under “Experimental Procedures.” Each of the mutant proteins had exonuclease activity similar to the wild type on the partial duplex substrate (Fig. 6B, right panel). Addition of the clamp and clamp loader did not stimulate hydrolysis by the deletion mutant H9004N or the proteins with mutations of two residues, L3A and M6A, corresponding to RB69 DNA polymerase residues contacting the clamp (Fig. 6B, center panel). Mutations in two other residues, M5A and D8A, which correspond to RB69 DNA polymerase residues that did not contact the clamp, were stimulated by the clamp like the wild type nuclease.

**A C-terminal Domain of T4 RNase H Is Required for Its Interaction with 32 Protein—**T4 gene 32 ssDNA-binding protein increases the processivity of T4 RNase H and controls how much DNA is removed from lagging strand fragments by the nuclease (see Introduction) (8, 13). The C terminus of RNase H has a pair of helices (H12 and H13) that protrude away from the catalytic site in the direction where we expected 32 protein should be located (Fig. 1A) (see “Discussion”). We find that T4 RNase H with a deletion of the last helix (H9004N295–305), both helices (H9004N286–305), or the entire region from 278–305 (H9004NC) retain exonuclease activity on the partial duplex substrate but are not stimulated by 32 protein (Fig. 7). The C-terminal region is not required for stimulation by the clamp protein (Fig. 6B, left panel). Conversely, the N-terminal mutant (ΔN), not affected by the clamp (Fig. 6B), is stimulated normally by 32 protein (Fig. 7A).
32 protein has a central core (residues 22–253) that contains the DNA-binding cleft, a short N-terminal “B-domain” (residues 1–21) that is thought to bind to the adjacent 32 protein when it cooperatively bound to DNA, and an acidic C-terminal “A-domain” (residues 254–302) that has profound effects on the T4 replication system (reviewed in Ref. 42). Both helicase-dependent leading strand synthesis (43, 44) and the elongation of primers made by the primase-helicase (43) are greatly decreased when 32 protein is replaced by 32 protein without the A-domain (32-A). We find that the A-domain is not required to increase the processivity of T4 RNase H. 32-A protein stimulated the nuclease to the same extent as full-length 32 protein (Fig. 8). 32 protein missing the B-domain (32-B) failed to stimulate when added either with (Fig. 8, lanes 15–18) or without (lanes 21–24) the nuclease. The lack of stimulation by 32-B may result from its greatly reduced affinity for ssDNA.

T4 RNase H Interaction with the Clamp and 32 Protein

**FIG. 2.** T4 gene 45 clamp protein stimulates T4 RNase H when loaded behind the nuclease. A, exonuclease activity of T4 RNase H in the presence of the T4 44/62 clamp loader, 45 clamp, and 32 ssDNA-binding protein. The clamp stimulates when loaded behind the nuclease on the nicked (center) or 28-base (b) gapped DNA (right) but not on the partial duplex (left), where it can only be loaded in front. 32 protein increases hydrolysis on the partial duplex and gapped substrates, where it binds on the ssDNA behind the nuclease (8). On the nicked substrate, where 32 protein can only bind ahead of the labeled downstream oligonucleotide, it increases the loading of clamps in front of the nuclease (31, 32) and reduces nuclease activity. B, when polymerase is present to extend the duplex available to the clamp ahead of the nuclease, there is no longer inhibition by 32 protein on the nicked substrate (compare reactions 12 and 13). In panel B, the products were cut with BstNI restriction nuclease after the reaction, to allow determination of the extent of hydrolysis at the 5’ end. The construction of the partial duplex, nicked, and gapped substrates, and the reaction conditions are described under “Experimental Procedures.” * marks the position of 3’ end 32P, and arrows indicate 3’ strand ends. Reactions were carried out with 3.4 nM T4 RNase H for 30 s in A and for the time indicated in B. Products are displayed on 8% polyacrylamide gels.

**FIG. 3.** The T4 44/62 clamp loader, 45 clamp, and ATP are required for stimulation of T4 RNase H on a nicked substrate. Hydrolysis of 5’ end-labeled partial duplex and nicked substrates. The length of the products released from the 5’ end are not changed by addition of the clamp and clamp loader. * marks the position of 5’ end 32P. Reactions were carried out with 0.34 nM T4 RNase H for 2 min as described under “Experimental Procedure.” Products are displayed on a 20% polyacrylamide gel. b, base.

**FIG. 4.** The clamp can move across a gap of 100 bases (b) to stimulate T4 RNase H. The construction of the gapped substrates and the reaction conditions are described under “Experimental Procedures.” * marks the position of 3’ end 32P. Reactions were carried out with 6.8 nM T4 RNase H for 30 s. Products are displayed on a 6% polyacrylamide gel. b, base.
we have used the ΔN and ΔC RNase H deletion proteins in the T4 replication system (Fig. 9). The template is the 2.7-kb PUC-NICK plasmid, nicked at the single recognition site for the N.BbvC IA nicking enzyme, as described under “Experimental Procedures.” The reactions contained T4 DNA polymerase, clamp, clamp loader, 32 protein, 41 helicase, 59 helicase loader, 61 primase, and DNA ligase in addition to the RNase H indicated on the figure. In the absence of RNase H (Fig. 9, reaction 1) the short lagging strand fragments are not joined together because the RNA primer with a 5′-triphosphate at the end of each fragment prevents ligation. Lagging strand fragments are efficiently sealed with the wild type or ΔN RNase H (Fig. 9, reactions 2 and 4). Sealing adjacent fragments is impaired with the ΔC RNase H (Fig. 9, reaction 3), as shown by the products migrating with unligated fragments and intermediate length DNA migrating between the normal leading and lagging strand products. We conclude that reducing the RNase H interaction with 32 protein (ΔC), but not the clamp (ΔN), interferes with the sealing of lagging strand fragments.

The defect of T4 RNase H ΔC in preparing fragments for sealing is also evident in a model lagging strand system in which a 3′-labeled 86-base oligonucleotide and an upstream 43-mer are separated by 1479 bases, a gap length similar to the length of the Okazaki fragment (Fig. 10). During DNA synthesis, the downstream 3′-labeled fragment is extended past the BstNI site, giving a 143-base restriction product, when BstNI is added after the replication reaction (Fig. 10, reaction 7). Simultaneous elongation of the unlabeled upstream fragment creates a nicked DNA that cannot be sealed by DNA ligase without RNase H (Fig. 10, reaction 8), because there is a hydroxyl group rather than a phosphate group on the 5′ end of the downstream fragment. 5′ to 3′ digestion by T4 RNase H provides the 5′-phosphate, so that after gap filling the adjacent fragments can be sealed by ligase, giving a 191-base BstNI restriction fragment (Fig. 10, reaction 2). The number of nucleotides removed by T4 RNase H, under conditions needed for ligation, is measured by the length of products shorter than the 143-base fragment in reactions in which ligase is omitted (Fig. 10, reactions 1, 3, and 5). If digestion continues for 86 bases, the two labeled nucleotides are removed as dimers or trimers, and the 191-base ligated product will not be labeled. With the wild type and ΔN RNase H, all of the labeled fragments except the dimers and trimers are ligated (Fig. 10, compare reaction 1 with 2 and reaction 5 with 6), showing that the nuclease has made at least one cut on each fragment to provide the required 5′-phosphate.

Within 30 s, in the absence of ligase, there is extensive hydrolysis as shown by fragments shorter than 143 bases. There is little further hydrolysis at later times, because polymerase has filled the gap, creating a nick that limits access to the nuclease. When wild type RNase H is replaced by the C-terminal deletion (ΔC), there is significantly less hydrolysis. In the absence of ligase there are few products shorter than 123 bases (Fig. 10, reaction 3), and at 2 min 43% of the labeled fragments could not be ligated (reaction 4), showing that the original 5′-hydroxyl group was still present. This suggests that the interaction between 32 protein and the wild type RNase H helps to load the nuclease at the junction between the 5′ end of the fragment and the 32 protein-covered ssDNA behind it, in addition to increasing the processivity of the nuclease once it is loaded.

DISCUSSION

T4 RNase H, which removes the RNA primers and some adjacent DNA from lagging strand fragments, is stimulated by both the T4 gene 45 replication clamp (Figs. 2 and 3) and 32 ssDNA-binding protein (8). It interacts with the clamp via a conserved clamp binding motif at the N terminus of the nuclease (Fig. 6). A C-terminal helical bundle at the C terminus is needed for its stimulation by 32 protein (Fig. 7). By using mutant T4 RNase H with deletions in the binding site for either the clamp or 32 protein, we have shown that it is the 32 protein, rather than the clamp, that most affects the maturation of
lagging strand fragments during DNA replication in vitro (Figs. 9 and 10), and phage production in vivo (Table I).

Binding Sites for the Clamp, 32 Protein, and DNA—Although the binding sites for the clamp and 32 protein are on separate termini of T4 RNase H (Fig. 1A, shown in purple), they are close together in the crystal structure of the nuclease (41), away from the active site residues (shown in red), surrounding the magnesium in a large cleft at the top of the protein (27). The N-terminal residues 1–11, which include the clamp binding site, were disordered in the original structure (41) but are ordered in a more recent structure of the metal-free enzyme. The analogous PCNA-binding motif is close to the C terminus of FEN1 (40), but is in a similar location relative to the active site cleft, as the N-terminal clamp binding site on T4 RNase H (compare Fig. 1, A and B).

There is unfortunately no crystal structure of a member of this 5'-nuclease family with DNA present in the active site of the enzyme. The recent structure (40) of the co-crystal of A. fulgidus FEN1 with the DNA that would be behind the clamp binding site is shown in (40), and those in the A. fulgidus FEN1 contact the archaeal clamp (40). References for the clamp interaction sequences are as follows: T4 DNA polymerase (36); T4 gp33 and gp55 transcription factors (37); RB69 DNA polymerase (38); human p21 (39); A. fulgidus FEN1 (40); and human FEN1 (40). See Refs. 34 and 35 for more extensive alignments of clamp interaction motifs.

A

| Protein            | Clamp interaction sequences | Closest terminus |
|--------------------|----------------------------|-----------------|
| T4 RNase H         | 345678 MDLemmMLDEDK         | N               |
| RB69 DNA Polymerase| 345678 KLFSLFMDF           | C               |
| T4 DNA Polymerase  | 345678 EKASLDFLF           | C               |
| T4 GP33 Transcription| 345678 KTNTLDFL           | C               |
| T4 GP55 Transcription| 345678 DSPSLDFLYEAND       | C               |
| Human p21          | 345678 ROTSMDTDFHSSKRR---  | C               |
| human FEN-1        | 345678 TGRPLDDFF-----     | C               |
| A. fulgidus FEN-1  | 345678 TGRPLDDFF-----     | C               |

B

![Figure 6](image-url)

Fig. 6. Mutations in a conserved clamp interaction sequence at the N terminus of T4 RNase H eliminate stimulation of the exonuclease by the clamp. A, alignment of selected clamp interaction sequences in phage, eukaryotic, and archaeal proteins. Consensus residues are shown in boldface. Shaded residues in RB69 DNA polymerase contact the RB69 gene 45 clamp in a crystal structure of the clamp and polymerase peptide (38). Shaded residues in p21(WAF1/CIP1) contact the human PCNA clamp (39), and those in the A. fulgidus FEN1 contact the archaeal clamp (40). References for the clamp interaction sequences are as follows: T4 DNA polymerase (36); T4 gp33 and gp55 transcription factors (37); RB69 DNA polymerase (38); human p21 (39); A. fulgidus FEN1 (40); and human FEN1 (40). See Refs. 34 and 35 for more extensive alignments of clamp interaction motifs. B, exonuclease activity of wild type (WT) and mutant T4 RNase H on the 3' end-labeled nicked substrate, with or without the T4 44/62 clamp loader and 45 clamp (left and center), and on the partial duplex by T4 RNase H alone (right). Reactions were carried out with 10 nM T4 RNase H for the time indicated with the nicked substrate and with 25 nM T4 RNase H for 2 min with the partial duplex. Reaction conditions and the preparation of the substrates and mutant proteins are described under “Experimental Procedures.” * marks the position of 3' end 32P. Products are displayed on 10% polyacrylamide gels.

2 T. C. Mueser, unpublished data.
active site shows that this DNA is bound on the surface of FEN1 close to the PCNA-binding site (primer strand blue and template strand magenta as shown in Fig. 1, B and C). Biochemical analysis of mutant human FEN1 proteins is also consistent with this location for the upstream DNA (45). The FEN1 residues making contact with the template strand (Thr63, Lys317, Asn67, Lys321, Arg64, Phe35, and Arg64 as shown in magenta in Fig. 1B) are located on helices H9231, H9233, and the C-terminal helices H9214 and H9215, and the loops connecting these helical pairs. Structural alignment of T4 RNase H and A. fulgidus FEN1 using the Combinatorial Extension (CE) program (46) shows that these correspond to T4 RNase H helices H1 and H2 and the C-terminal helices H12 and H13. The T4 RNase H residues that align structurally with the A. fulgidus FEN1 template-binding residues (Lys52, Asn300, Lys56, Glu304, Lys53, Thr27, and Thr30, respectively) are shown in magenta on Fig. 1A. Deletion of helix H13 (T4 RNase H 8295–305) was sufficient to prevent 32 protein stimulation of the nuclease (Fig. 7). In the absence of DNA, we observed only a weak interaction between N-terminal His or glutathione S-transferase-tagged T4 RNase H and 32 protein in pull-down experiments (data not shown). However, a tight complex of T4 RNase H and 32 protein on a gapped DNA, like that diagramed in Fig. 1C, top, can be shown in gel mobility shift experiments.3

Assuming that T4 RNase H binds DNA as shown for the A. fulgidus FEN1, the binding sites for both 32 protein and the 45 clamp are located close to the predicted position for the DNA behind the catalytic site surrounding the magnesium (Fig. 1). This is consistent with the expected positions of the clamp and ssDNA-binding protein during lagging strand synthesis (Fig. 1C), as well as our experimental finding that both 32 protein (8) and the clamp (Fig. 2) stimulate only when they can be loaded behind the nuclease.

The PCNA, E. coli β, and T4 45 protein DNA replication clamps have similar circular structures (47, 48) and recognize a conserved clamp-interaction sequence on the DNA polymerases, 5’-nucleases, ligases, and mismatch repair proteins that are affected by the clamp (reviewed in Refs. 34 and 35). There are now several crystal structures of clamp proteins bound to peptides with the clamp-binding sequences from interacting proteins (see Fig. 6A). In all of these structures, including that of the phage RB69 gene 45 clamp with the peptide from RB69 DNA polymerase (38), the peptide is found in the same location in a hydrophobic pocket on the interdomain loop. However, there is evidence that the clamp-interacting peptide from T4 polymerase can also bind at the subunit interface of the 45 clamp in solution (49). When the structure of T4 RNase H was docked on the phage RB69 clamp by putting its N-terminal clamp interacting residues in the position of the RB69 DNA polymerase peptide, it was clear that the nuclease could not bind the clamp, if 32 protein was bound near the C terminus (not shown). On a gapped substrate, 32 protein stimulates the nuclease to a greater extent than the clamp, and there is no further stimulation by adding the clamp, as well as 32 protein (Fig. 2).

The preferred flap substrate for FEN1 is a so-called double flap with a single nucleotide displaced at the 3’ end, in addition to the 5’ flap cut by the nuclease (Fig. 1C, bottom) (50). Although FEN1 cuts at several positions around the junction between single- and double-stranded DNA in a single flap, it

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3 C. E. Jones, unpublished data.
cuts the double flap predominantly in the duplex one nucleotide beyond the junction, thus making a nicked duplex product that can be sealed by ligase. In contrast, T4 RNase H cuts the double flap substrate at a significantly slower rate than a single flap of the same sequence, although like FEN1, T4 RNase H cuts the double flap most often at the position giving a nicked duplex product.3 The primer strand in the DNA in the A. fulgidus FEN1 structure had an unpaired 3'/H11032 nucleotide, corresponding to the 3'/H11032 flap (Fig. 1, B and C, bottom). Three residues in the loop between H9251 and H9251 that bind this unpaired 3'/H11032 nucleotide (His308, Phe310, and Ser311) have no match in T4 RNase H, in the structural alignment between these two proteins, because helices H12 and H13 in T4 RNase H are shorter than helices α14 and α15 in A. fulgidus FEN1.

Different Mechanisms for Coordinating Maturation of Lagging Strand Fragments—Phage T4, E. coli, and eukaryotic cells each have efficient, but different, mechanisms for coordinating the reactions that remove the RNA primers, and those that elongate the discontinuous fragments, on the lagging strand. This coordination is essential to prevent the accumulation of recombinogenic nicks and gaps as a result of the discontinuous nature of lagging strand synthesis. The T4 replication system is the simplest (11, 12). A single clamped polymerase is used to extend the RNA primer made by the phage-encoded primase and to complete the synthesis of the lagging strand fragment.

While polymerase is extending the fragment, a separate nuclease, T4 RNase H, acting as a 5’-exonuclease rather than a flap endonuclease, removes the RNA pentamers and about 30 nucleotides of adjacent DNA. 32 protein, which coats the ssDNA between the nuclease and polymerase, coordinates their activities by increasing the rate and processivity of each of these enzymes (13). The large difference in their rates ensures that in most cases polymerase will complete the upstream fragment before the nuclease can bind a second time (13). T4 DNA polymerase dissociates rapidly when it reaches an annealed duplex (51, 52). Thus the nuclease must act before polymerase finishes, so that a ligatable nick is formed when polymerase leaves. In contrast to the eukaryotic system discussed below, the polymerase and nuclease act separately, although each is affected by 32 protein. The rate of hydrolysis by the nuclease is not changed when polymerase is present (13), and the rate of lagging strand synthesis is the same, with or without the nuclease (Fig. 9).

Our experiments suggest that the interaction between 32 protein and RNase H promotes the binding of the nuclease on 32 protein-coated DNA, in addition to increasing its processivity. We used a model gapped lagging strand substrate, where removing a single nucleotide was sufficient to expose the 5’-phosphate needed for ligation. A C-terminal deletion mutant of RNase H, defective in the 32 protein interaction, was unable to
Sealing of lagging strand fragments in a model lagging strand system is less efficient with the T4 RNase H C-terminal Δ278–305 deletion. The template is M13mp19 ssDNA with a 1479-base (b) gap between the annealed 3′-end-labeled 86-mer and the unlabeled 42-mer. The DNA was incubated with the T4 32 protein, 45 clamp, and 44/62 clamp loader for 2 min at 30 °C, before the addition of wild type (WT) or mutant T4 RNase H (10 nM), T4 DNA ligase, and T4 DNA polymerase for the times shown, as indicated. The reactions were stopped by heating to 60 °C, the products digested with BstNI nuclease, and then heated for 3 min at 95 °C before electrophoresis. The [32P] 86-mer was elongated past the BstNI site, giving a 143-base restriction product in the absence of T4 RNase H (reactions 7 and 8). The products shorter than 143 bases are a measure of hydrolysis by the exonuclease activity of T4 RNase H (reactions 1, 3, and 5). The 191-base products are molecules ligated following T4 RNase H digestion to expose a 5′-phosphate, and gap filling by polymerase (reactions 2, 4, and 6). * marks the position of 32P on the 86-mer. Conditions for the replication and BstNI restriction reactions are described under "Experimental Procedures." Products are displayed on a 12% polyacrylamide gel.

Table I

Complementation of bacteriophage T4 Δrnh by plasmids encoding wild type and mutant T4 RNase H

The E. coli MIC2003 host contains the rnhA339::cat interruption in the gene encoding the host RNase HI and the temperature-sensitive PolA12 mutation affecting the 5′-3′-exonuclease of DNA polymerase I. All host cells also contained a T7 promoter expression plasmid encoding wild type (WT) or mutant T4 RNase H, or the pVex11 vector, as well as a compatible pGP1.2 plasmid, which contains the gene for T7 RNA polymerase under the temperature-controlled P1 promoter. The cells were grown to 1 × 10^8/ml at 30 °C, shifted to 43 °C for 15 min to induce production of T4 RNA polymerase and disrupt the polA12 DNA polymerase-associated 5′-nuclease, and then infected with T4D or T4 Δrnh (Δ118–305) at a ratio of 0.5 phage/cell. Infective centers, the number of cells containing at least one phage, were determined by plating on E. coli CR63, 5 min after infection. Total phage were measured at 60 min, after lysis with chloroform (see "Experimental Procedures").

| Plasmid | Phage | Infective centers ×10^3/ml | Phage/infective center |
|---------|-------|--------------------------|-----------------------|
| I       |       |                          |                       |
| pVex 11 (vector) | T4D | 2.4 | 271 |
| pNQ1004 (WT) | T4D | 3.2 | 310 |
| pNQ1101 (ΔN-(2–10)) | T4D | 3.4 | 121 |
| pMB5002 (ΔC-(278–305)) | T4D | 1.7 | 128 |
| II      |       |                          |                       |
| pVex 11 (vector) | T4D | 3.0 | 215 |
| pNQ1004 (WT) | T4D | 2.0 | 217 |
| pGO1701 (Δ286–305) | T4D | 2.8 | 74 |
| pGO1801 (Δ295–305) | T4D | 2.4 | 82 |

*a Phage/infective center* is the total phage yield per original infective center, rather than the burst size of phage from a single cell, because the infected bacteria were not diluted to avoid additional rounds of infection.
make a single cut on 43% of the molecules in the time needed for polymerase to fill in the 1.5-kb gap (Fig. 10). Under the same conditions, the wild type nuclease and an N-terminal deletion, which could interact with 32 protein but not the clamp, made the cut needed for a ligatable nick on all of the molecules. Control experiments showed that the wild type and both mutant nucleases hydrolyzed the DNA at the same rate in the absence of 32 protein. Fragment sealing during coupled leading and lagging strand synthesis by the complete T4 replication system was also impaired when the C-terminal deletion mutant of RNase H replaced the wild type (Fig. 9). Finally, although plasmids encoding wild type T4 RNase H can have upstream fragment, before the downstream primers are removed by the nuclease. T4 RNase H by itself degrades the 5′ end of a nick very slowly; its activity at a nick is stimulated by the clamp but not by 32 protein (Figs. 2A and 4). The clamp increases loading of the nuclease at a nick, but it does not increase the processivity of the nuclease after it is loaded (Fig. 5). Our finding that a mutant of RNase H, not stimulated by the clamp, can function like the wild type in promoting fragment sealing in vitro, and phage production in vivo, shows that the primers are normally removed before the nick is formed by the upstream polymerase. A role for T4 RNase H in DNA repair is suggested by the increased sensitivity of the T4 rnh mutant to DNA-damaging agents (see Introduction). It seems likely that the interaction of the clamp with RNase H may prove to be important for this function.

In E. coli DNA replication, DNA pol III holoenzyme extends the primer made by primase and remains tightly associated with the primer rather than polymerase (55). The preferred substrate for the clamp is composed of three nonidentical subunits that contact each other in the 5′-nuclease and 3′-nuclease activity of pol I in the former and by the combined activities of FEN1 nuclease and pol δ in the latter. T4 DNA polymerase and RNase H do not carry out nick translation together. Instead, the stimulation of the nuclease by the 32 protein on the lagging strand ensures that the primers will be removed in time for a nick to be formed by the polymerase completing the upstream fragment. In each of these systems, the clamp present on the lagging strand can interact with the polymerase extending the fragment, the 5′-nuclease removing the primer, and the ligase joining adjacent fragments. Because of the subunit composition and symmetry of the clamp structures, there are three of the interdomain binding sites for these proteins on each clamp. An important unanswered question is whether the lagging strand clamp binds more than one of these proteins simultaneously. In this regard, the recent finding that the Sulfolobus solfataricus clamp is composed of three nonidentical subunits that contact either polymerase, ligase, or FEN1 (62) is intriguing.

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