The 5′-Exonuclease Activity of Bacteriophage T4 RNase H Is Stimulated by the T4 Gene 32 Single-stranded DNA-binding Protein, but Its Flap Endonuclease Is Inhibited*

Medha Bhagwat, Lisa J. Hobbs‡, and Nancy G. Nossal§
From the Laboratory of Molecular and Cellular Biology, NIDDK, National Institutes of Health, Bethesda, Maryland 20892-0830

Bacteriophage T4 RNase H is a 5′- to 3′-nuclease that has exonuclease activity on RNA-DNA and DNA-DNA duplexes and can remove the pentamer RNA primers made by the T4 primase-helicase (Hollingsworth, H. C., and Nossal, N. G. (1991) J. Biol. Chem. 266, 1888–1897; Hobbs, L. J., and Nossal, N. G. (1996) J. Bacteriol. 178, 6772–6777). Here we show that this exonuclease degrades duplex DNA nonprocessively, releasing a single oligonucleotide (nucleotides 1–4) with each interaction with the substrate. Degradation continues nonprocessively until the enzyme stops 8–11 nucleotides from the 3′-end of the substrate. T4 gene 32 single-stranded DNA-binding protein strongly stimulates the exonuclease activity of T4 RNase H, converting it into a processive nuclease that removes multiple short oligonucleotides with a combined length of 10–50 nucleotides each time it binds to the duplex substrate. 32 protein must bind on single-stranded DNA behind T4 RNase H for processive degradation. T4 RNase H also has a flap endonuclease activity that cuts preferentially on either side of the junction between single- and double-stranded DNA in flap and fork DNA structures. In contrast to the exonuclease, the endonuclease is inhibited completely by 32 protein binding to the single strand of the flap substrate. These results suggest an important role for T4 32 protein in controlling T4 RNase H degradation of RNA primers and adjacent DNA during each lagging strand cycle.

During the replication of duplex DNA, the synthesis of the discontinuous lagging strand fragments is initiated by short RNA primers that must subsequently be removed and replaced by DNA. In the bacteriophage T4 system, the pentamer RNA primers made by the T4 primase-helicase are removed by a phage-encoded enzyme with RNase H activity that has been called T4 RNase H (1). Our initial characterization of this nuclease showed that it also had 5′- to 3′-exonuclease activity on double-stranded (ds) DNA, raising the possibility that some DNA adjacent to the RNA primers is removed from the lagging strand fragments. In this paper we show that interaction between T4 gene 32 protein and RNase H controls how much DNA is removed each time T4 RNase H binds to its substrate.

T4 DNA replication is carried out by a multienzyme system in which T4 DNA polymerase (gene 43) is tethered to the template by the gene 45 clamp protein that has been loaded on the DNA by the gene 44/62 protein complex. The gene 32 single-stranded (ss) DNA-binding protein covers unwound DNA at the fork and increases the processivity of the lagging strand polymerase. The primase-helicase composed of the gene 61 primase and gene 41 helicase makes the RNA primers on the lagging strand and unwinds the duplex DNA ahead of the leading strand polymerase. The gene 59 helicase assembly protein facilitates the loading of the primase-helicase, especially on DNA covered with 32 protein. After removal of the primer and perhaps adjacent DNA by RNase H, lagging strand fragments are joined by DNA ligase (gene 30) (for review, see Ref. 2).

The genes for these T4 DNA replication proteins were identified in early screens for T4 mutants with phenotypes of arrested, delayed, or no DNA synthesis (3). In contrast, T4 phage with a deletion within the rnh gene encoding T4 RNase H can replicate in a wild type Escherichia coli host (4). However, the rnh mutant phage cannot replicate in a host with the polA12 mutation, which disrupts coordination between the polymerase and 5′- to 3′-nuclease activities of E. coli DNA polymerase I. Wild type T4 phage production is not decreased by the polA12 mutation and/or by mutations in E. coli RNase H1. Thus T4 RNase H is able to sustain the high rate of T4 DNA replication in the absence of the enzymes that can remove RNA primers in the uninfected host. Although these host enzymes can substitute for T4 RNase H, replication is slower and less accurate in the absence of the phage nuclease. T4 RNase H also plays a role in DNA repair and recombination. The rnh mutant T4 phage is more sensitive than the wild type to ultraviolet radiation and to 4′-(9-acridinylamino)methanesulfonyl-m-anisidide, an aminooaridine inhibitor of T4 topoisomerase I.

T4 RNase H has sequence similarity to other enzymes with a demonstrated role in removing RNA primers, including phage T7 gene 6 exonuclease, the 5′- to 3′-nuclease domain of E. coli DNA polymerase I, and human Fen-1 (flap endonuclease; also called MF-1 or RAD2 analog). All of these enzymes have 5′- to 3′-exonuclease activity on both RNA-DNA and DNA-DNA duplexes. In addition, most of the enzymes have a flap endonuclease activity that removes the 5′-ssDNA tail of flap or fork structures (6–11). In the recent crystal structure of T4 RNase H (11), a large number of acidic amino acids that are highly conserved in this enzyme family are located in a cleft surrounding two Mg2+ ions in the proposed active site of the nuclease. (The sequence similarity and the structure of T4 RNase H are described in detail in Ref. 11 and in the accompanying paper (12)).

Our long range goals are to determine how primer removal...
by T4 RNase H is coordinated with other steps in lagging and leading strand synthesis and to understand what controls how much DNA is removed along with the RNA primer. In this paper, we show that T4 RNase H also has a flap endonuclease activity. We have characterized the exonuclease and flap nuclease activities of T4 RNase H alone and in conjunction with the T4 gene 32 ssDNA-binding protein. We find that the exonuclease cuts duplex DNA from the 5'-end, releasing short oligonucleotide products until it reaches 8–11 nucleotides from the 3'-end. This exonuclease activity is nonprocessive but becomes processive in the presence of the gene 32 ssDNA-binding protein, removing short products with a combined length of 10–50 nucleotides in a single encounter with the DNA. Binding of 32 protein to the single strand of the flap prevents cleavage by the T4 RNase H endonuclease.

**EXPERIMENTAL PROCEDURES**

### T4 Replication Proteins—Wild type T4 RNase H was purified to apparent homogeneity as described by Nossal et al. (13). The T4 polymerase accessory proteins 44/62 and 45, and the T4 gene 32 ssDNA-binding protein preparations used were those described in Hollingsworth and Nossal (1). The purification of wild type T4 DNA polymerase is described by Spacciapoli and Nossal (14).

**Substrates**—The 34-mer RNA and DNA (LH5), complementary to nucleotides 3493–3526 of M13mp19 viral ssDNA, and the 84-base DNA complementary to nucleotides 6197–6281 were made on an Applied Biosystems 381A DNA synthesizer. The oligonucleotides were 5'-end labeled with γ-32P[ATP using T4 polynucleotide kinase, annealed to the M13 DNA, and separated from ATP and free oligonucleotide on Sephadex CL-2B as described previously (15). Oligonucleotides annealed to M13 ssDNA were 3'-end labeled by the exonuclease-mutant form of T4 DNA polymerase (D219A) (16). The 34-mer was extended to a 36-mer by the addition of 2[α-32P]dCMP and the 84-mer to an 86-mer with 2[α-32P]dTTP.

The following oligonucleotides, used to make the flap, fork, and related substrates (see Fig. 2), were synthesized and reverse phase purified by Cramachem.

- MB25 (30-mer) GGACTCTGCTTACAGGATCAGTGCAGCCG
- MB27 (34-mer) GAGTCTGATGTCATGTCATGTCATGTCATGTCATG
- MB28 (16-mer) CAGGCTTACAGCCG
- MB29 (14-mer) TTTGACGAGCTTG

The oligonucleotides were 5'-end labeled with poly(U)-kinase and separated from ATP by filtration on Sephadex G-50 or G-25. Complementary oligonucleotides in 10 mM Tris, pH 8.0, 1 mM EDTA, and 0.2 mM NaCl were heated to 100 °C for 3 min, 67 °C for 60 min, and then cooled slowly to room temperature.

**Nuclease Activity**—Unless otherwise indicated, reaction mixtures (10 μl) contained 1.8 nM substrate, 25 mM Tris acetate, pH 7.5, 63 mM potassium acetate, 6 mM magnesium acetate, 20 mM dithiothreitol, 1 mM EDTA, and 200 μg/ml bovine serum albumin. Proteins were diluted in a solution containing 50 mM Tris acetate, pH 7.5, 100 mM KCl, 5 mM MgCl2, 0.2 M NaCl were heated to 100 °C for 3 min, 67 °C for 60 min, and then electrophoresed for 40 min at a constant voltage of 100 V at 4 °C. The gels were dried on DE81 paper before autoradiography.

**Processivity of the Exonuclease Activity of T4 RNase H**—The substrate was the 34-mer LH5 annealed to M13 circular ssDNA and 3'-end labeled by adding two μl of [α-32P]dCMP. In the control reaction in the absence of competitor DNA, 1.4 nM T4 RNase H was incubated with 1.5 nM LH5 for 60 min at 20 °C, and the resulting mixture described above, except that magnesium acetate was omitted. The reaction was started by adding Mg2+ to 6.25 mM, and samples were taken at 0.25 and 2 min. In other reactions, competitor DNA (200 nM, φX174 ssDNA) was added as a trap to prevent the nuclease from rebinding to the substrate. The competitor DNA was added either at the same time the labeled substrate or at the same time as the Mg2+ was added to begin the reaction or 0.25 min after the reaction had started.

To study the processivity of the exonuclease activity in the presence of the T4 gene 32 protein, similar experiments were performed except that samples were taken at 0.5, 2, or 5 min, as indicated in the figure legends. In this case the substrate was the 54-mer annealed to M13mp19, 3'-end labeled by adding two μl of [α-32P]dTMP. The competitor DNA was the fork described above, at a final concentration of 22.9 μM. T4 RNase H and 32 protein were present at 6.8 nM and 1 μM, respectively. Where indicated, the labeled substrate was cut within the M13 multicycling site by Smal endonuclease (New England Biolabs), and the protein was removed by phenol extraction.

**RESULTS**

### Substrate Specificity of RNase H

T4 RNase H Degradates Both RNA-DNA and DNA-DNA Duplexes to Short Oligonucleotide Products—Although T4 RNase H was originally purified on the basis of its RNase H activity, the enzyme also acts as a 5'- to 3'-nuclease on DNA-DNA duplexes (1). We have compared these two activities directly by using RNA and DNA 34-base oligonucleotides with the same sequence (Fig. 1A). When annealed to the complementary region of M13 viral ssDNA, the 5'-end labeled RNA and DNA oligomers were degraded to short oligonucleotides, mainly monomers to tetramers (panels C and D). There was no detectable degradation of the free RNA and very little degradation of the free DNA 34-mer (panels A and B).

T4 RNase H made the initial cut on the RNA and DNA 34-base oligonucleotides in a blunt end duplex at the same rate as on the duplexes formed by annealing the same oligonucleotides to the circular M13 DNA (Fig. 1B). The products depended on both the sequence and whether the substrate was RNA or DNA. The products from the 34-mer DNA with the 5'-end sequence ACCAATCA (top strand in Fig. 1B), annealed to M13 DNA (panel D) or its 34-mer complement (panel D) were mainly dimers and trimers. In contrast, the complementary DNA 34-mer (bottom strand) with the 5'-end sequence AATGATAA gave predominantly pentamers (panel C). Although there are clearly preferential cutting sites (see Fig. 4A below), it is not yet clear how these are determined. As shown in Fig. 1B, there was also a difference in the size distribution of the products from the 5'-end of RNA and DNA with the same sequence (compare panels A with E, and B with D). Finally, the product size distribution changed with the reaction temperature. The ratio of tri- to dinucleotide products from the substrate shown in panel E (Fig. 1B) increased from 1.0 at room temperature to 5.8 at 45 °C (see Fig. 6 in the accompanying paper (12)).

T4 RNase H Cuts Preferentially At or Near the Branch Point Flap or Fork DNA Substrates—T4 RNase H cut a 20-base 5'-tail on flap DNA (Fig. 2, panel D) or fork DNA (panel E) at positions near the branch point, giving mainly products of 19 and 23 nucleotides. In these experiments the subsequence showing the same flap sequence used previously to characterize murine FEN-1 (6, 17) to compare directly these enzymes which, as indicated in the Introduction, are related in both function and amino acid sequence. In contrast to the T4 enzyme, murine FEN-1 cut this flap 100 times faster than the corresponding fork structure (6, 17). However, calf thymus FEN-1 has been reported to cut fork
structures with other sequences (18).

Under reaction conditions where the T4 nuclease cut a 34-base blunt end duplex (Fig. 1B), it did not cut the shorter blunt end duplex made by a 16-base oligonucleotide in a partial hybrid (Fig. 2, panel A), nicked (panel B), or flap structure (panel C). It did cut short duplexes (14 bases) in which there was either a ss (panel G) or ds (panel F) extension behind the 5'-end, which presumably acted as an attachment site for the enzyme. However, at the very high enzyme and DNA concentrations used to prepare crystals, there was detectable degradation of a 16-base duplex (data not shown).

**T4 RNase H Binds Preferentially to Fork and Flap DNA Structures**—A gel mobility shift assay was used to compare the binding of different types of substrates to T4 RNase H (Fig. 3). We studied this binding in the absence of Mg$_2^+$ ions to prevent the degradation of DNA substrates. Under these conditions, there was significant binding to the fork and flap DNA at a T4 RNase H concentration of 5.6 nM (Fig. 3A). In contrast, at a 100-fold higher enzyme concentration (560 nM) there was little binding to the ds (Fig. 3A) or nicked (Fig. 3B) DNA and no detectable binding to the ss 34-mer (Fig. 3A) or 14-mer (Fig. 3B).

**T4 RNase H Stops Degradation When It Reaches 8–11 Nucleotides from the 3'-End**—After removing a short oligonucleotide product from the 5'-end, T4 RNase H continued to remove other short products until it reached 8–11 nucleotides from the 3'-end (Fig. 4A). The enzyme stopped because the substrate was too short, not because it reached a refractory sequence. When 9 unlabeled nucleotides were added to the substrate by concurrent incubation with T4 DNA polymerase and 3 dNTPs, the label at the 3'-end of the original substrate became internal and was subsequently degraded to very short (1–4 bases) products (Fig. 4B, compare lanes 3 and 4). There was some mononucleotide formed by the 3'- to 5'-exonuclease of wild type T4 RNase H.

**Fig. 1.** T4 RNase H degrades both RNA-DNA and DNA-DNA duplexes to short oligonucleotide products. A, the substrates (approximately 2 nM) were 5'-end-labeled RNA or DNA 34-mers complementary to nucleotides 3493–3526 of M13 viral ssDNA, alone (panels A and B) or annealed to M13mp7 (panels C and D). In each case the top strand shown is complementary to M13, and the 32P-labeled strand is marked with a ●. T4 RNase H concentrations were 0, 33.2, 16.2, 8.3, and 4.2 nM in lanes 1–5, respectively. Reactions were stopped after 2 min at 30 °C, and the products were analyzed by denaturing gel electrophoresis, as described under “Experimental Procedures.” B, the substrates (7 nM) were the 5'-end-labeled RNA or DNA 34-mers annealed to M13mp7 or to a complementary 34-mer DNA. The T4 RNase H concentration was 1.7 or 3.4 nM as indicated, and samples were taken after 0.5 and 2 min at 30 °C.

**Fig. 2.** T4 RNase H cuts preferentially at or near the branch point in flap or fork DNA substrates. The substrates (2 nM) were made by annealing the required 14-, 16-, 30-, and/or 34-mers, as described under “Experimental Procedures.” The 5'-labeled (●) oligomer was the 16-mer in panels A–C, 34-mer in panels D and E, and 14-mer in panels F–H. T4 RNase H was present at 0.34 and 3.4 nM in panels A and B and 0.34, 3.4, and 34 nM in panels C–H. Products formed in 2 min at 30 °C are displayed on a 15% polyacrylamide, 7 M urea gel.

**Interaction of T4 RNase H and Gene 32 ssDNA-binding Protein**
difference in the DNA substrates are shown under "Experimental Procedures." The samples were analyzed on 6% polyacrylamide DNA retardation gels (Novex). Sequences of the DNA substrates are shown under "Experimental Procedures." A, ss 34-mer, fork, flap, and ds 34-mer. B, nicked 30-mer and ss 14-mer.

polymerase (lane 2), but the products of 2–4 bases were found only when both T4 RNase H and polymerase were present (lane 3). Similar T4 RNase H products were found when wild type T4 DNA polymerase was replaced by the exonuclease-defective (D218A) polymerase (lane 5).

T4 Gene 32 ssDNA-binding Protein Converts T4 RNase H from a Nonprocessive to a Processive Exonuclease

Stimulation of the Exonuclease Activity by 32 Protein—The rate of degradation of a 3'-end-labeled 86-mer by T4 RNase H was greatly increased by T4 gene 32 ssDNA-binding protein (Fig. 5). With 3’-labeled substrates, the stimulation by 32 protein was observed over a wide range of T4 RNase H concentrations, with either a hydroxyl or a phosphate group at the 5’-end (Fig. 5, left and center panels). Only the first product released can be observed when the substrate is 5’-end-labeled (right panel). Because T4 RNase H becomes processive in the presence of 32 protein (see below), there was an apparent inhibition by 32 protein when the 5’-labeled substrate (1 nM) was present at a higher concentration than T4 RNase H (Fig. 5, lanes 20 and 21). The nuclease, which remained bound for a longer time on its initial substrate in the presence of 32 protein, was not available to cut other DNA molecules.

Processivity of the Exonuclease Activity of RNase H—To determine whether T4 RNase H is a processive exonuclease, we used the 3’-end-labeled 36-mer annealed to M13 as the substrate and a 133-fold molar excess of φX174 circular ssDNA as a competitor to prevent the enzyme from rebinding to the substrate (Fig. 6A). The enzyme was incubated with the labeled substrate in the absence of Mg2+, and the reaction was started by adding Mg2+. In the absence of competitor, enzyme can bind repeatedly to the substrate, and there was extensive degradation within 2 min (lanes 7 and 8). In the control reaction, where T4 RNase H was allowed to bind to the substrate in the presence of the cold competitor DNA and the reaction was started with Mg2+, there was almost no reaction (lanes 3 and 4). In the reaction in which competitor and Mg2+ were added simultaneously, products 1–4 bases shorter than the initial substrate were formed at the first time point of 0.25 min (lane 5), and there was no further degradation at 2 min (lane 6). Because T4 RNase H removed oligonucleotides of 1–4 bases with a single cut from the 5’-end of this substrate (Fig. 1), the limited degradation in the reaction shown in lanes 5 and 6 indicates that the enzyme made only one cut, fell off the DNA, and was then trapped by the cold competitor. When the reaction was allowed to proceed for 0.25 min before competitor was added, the products at 2 min (lane 2) were 1–4 bases shorter than those present at 0.25 min (lane 7). We conclude that T4 RNase H makes only a single cut each time it binds to the substrate.

T4 32 Protein Converts T4 RNase H to a Processive Exonuclease—Similar experiments showed that T4 RNase H becomes much more processive in the presence of the gene 32 ssDNA-binding protein (Fig. 6B). We used fork DNA as the cold competitor in these experiments because it bound tightly to T4 RNase H (see Fig. 3A). The control reactions in Figs. 6B and 7 indicated that the fork DNA effectively trapped unbound nuclease for a longer time than the circular ssDNA used as a competitor in Fig. 6A. When T4 RNase H and gene 32 protein were allowed to bind to the 3’-end-labeled 86-mer-M13 substrate, and the reaction was started by the addition of Mg2+ and competitor fork DNA, nuclease that was already bound to the substrate continued to degrade it to shorter products (lanes 13 and 14, Fig. 6B). This is in striking contrast to the reactions without 32 protein, in which the digestion in the presence of trap was terminated after a single 1–4-base oligonucleotide was removed (Fig. 6A, lanes 5 and 6). Although there was no apparent degradation in reactions with T4 RNase H alone in Fig. 6B (lanes 6 and 7), this gel would not have separated the 86-mer substrate from products that were only 1–4 bases shorter. When both T4 RNase H and 32 protein were present, most of the remaining 3’-labeled substrate was between 34 and 74 bases at 2 min (Fig. 6B, lane 14), indicating that T4 RNase H removed 10–50 bases from the 5’-end during a single binding to the substrate. There was no further degradation between 2 and 5 min (Fig. 7, lanes 12 and 13). The control reaction B (Fig. 6B, lanes 11 and 12) confirmed that the fork DNA acted as an effective trap, blocking the exonuclease reaction when it was added before the Mg2+.

The time course in the absence of competitor showed some products of intermediate size as well as the initial substrate and short limit products. It is likely that the nuclease remained bound to these intermediate sized products because those present at 0.5 min in the absence of competitor (lane 9) were degradated further to shorter products after competitor was added at 0.5 min, and the reaction then stopped at 2 min (lane 15). Thus 32 protein greatly increased the processivity of T4 RNase H but did not eliminate pausing by the nuclease at discrete positions on the substrate.

32 Protein Must Bind behind T4 RNase H for Processive
Degradation—32 protein can bind both behind the 5'-end and ahead of the 3'-end of the duplex region of the substrate (Figs. 6 and 7). To determine whether it was necessary for 32 protein to bind behind the 5'-end of the duplex to stimulate T4 RNase H, we used a linear blunt end substrate made by cutting the 3'-labeled 86-mer annealed to M13 DNA with SmaI endonuclease (Fig. 7). When T4 RNase H and the fork DNA trap were added together, there was no processive degradation on this blunt end, 76-base partial duplex, (Fig. 7, compare the reaction containing T4 RNase H and 32 protein with the circular substrate (lanes 12 and 13) with that on the linear DNA substrates (lanes 26 and 27)). Note that 32 protein did stimulate degra-

Fig. 4. T4 RNase H stops degradation when it reaches 8–11 nucleotides from the 3'-end. The substrate was made by adding two [α-32P]dCMP (●) to the 34-mer annealed to M13mp7. A, T4 RNase H at the concentration shown was incubated with 22 nM substrate. Samples were removed at the indicated time and analyzed as described under “Experimental Procedures.” B, degradation continues when the substrate is elongated by T4 DNA polymerase. Where indicated, T4 RNase H was present at 34 nM and T4 DNA polymerase (wild type (WT) or exonuclease-defective (D219A) (exo•)) at 13 nM. Enzymes and substrate (6.8 nM) were incubated for 5 min. The reactions contained 250 μM dATP, dGTP, and dCTP, which allowed 9 nucleotides to be added to the 36-mer. Lane 1, no enzyme; lane 2, wild type polymerase; lane 3, wild type polymerase and T4 RNase H; lane 4, T4 RNase H; lane 5, exo• polymerase and T4 RNase H; lane 6, exo• polymerase.

Fig. 5. T4 RNase H exonuclease activity is strongly stimulated by the T4 gene 32 ssDNA-binding protein. The 3'-labeled substrates were made by adding two [α-32P]dTMP to the 84-mer (5'-OH or phosphorylated) annealed to nucleotides 6197 to 6281 of M13mp19 (see “Experimental Procedures”). The 3'- or 5'-labeled substrates (1 nM) were incubated for 2 min at 30 °C with T4 RNase H at the concentration shown, with or without 32 protein as indicated. See “Results” for an explanation of the apparent inhibition of degradation of the 5'-labeled substrate by 32 protein at the lowest RNase H concentration (lanes 20 and 21).

Degradation—32 protein can bind both behind the 5'-end and ahead of the 3'-end of the duplex region of the substrate (Figs. 6 and 7). To determine whether it was necessary for 32 protein to bind behind the 5'-end of the duplex to stimulate T4 RNase H, we used a linear blunt end substrate made by cutting the 3'-labeled 86-mer annealed to M13 DNA with SmaI endonuclease (Fig. 7). When T4 RNase H and the fork DNA trap were added together, there was no processive degradation on this blunt end, 76-base partial duplex. (Fig. 7, compare the reaction containing T4 RNase H and 32 protein with the circular substrate (lanes 12 and 13) with that on the linear DNA substrates (lanes 26 and 27)). Note that 32 protein did stimulate degra-
FIG. 6. T4 gene 32 ssDNA-binding protein converts T4 RNase H from a nonprocessive to a processive nuclease. A, T4 RNase H exonuclease is nonprocessive. The enzyme (1.4 nM) was incubated with substrate (1.5 nM 3'-end-labeled 36-mer annealed to M13 DNA) for 2 min at 30 °C in the absence of Mg²⁺, and the reaction was started by adding magnesium acetate to 6.25 mM. Unlabeled competitor DNA (200 nM circular 8X174 ssDNA) was added either with the labeled substrate (lanes 3 and 4), with Mg²⁺ (lanes 5 and 6), or 0.25 min after the Mg²⁺ (lane 2). A control reaction without competitor is shown in lanes 7 and 8. T4 RNase H made only one cut, releasing a short oligonucleotide product before being trapped by the competitor DNA (compare lanes 5 and 6 with lane 1, and lane 2 with lane 7). Products are displayed on a 10% polyacrylamide, 7 M urea gel. B, T4 RNase H is processive in the presence of 32 protein. Processivity was determined by the protocol described in A, except that the substrate was the 3'-labeled 86-mer annealed to M13 DNA, described in Fig. 5. T4 RNase H (6.8 nM) was added either before Mg²⁺ (lanes 6–9), or with Mg²⁺ (lanes 9–15) added to start the reaction (lanes 6, 7, 13, and 14), or 0.5 min after the Mg²⁺ (lanes 8 and 15). Control reactions without competitor DNA are in lanes 2, 3, 8, and 9 (circular) and lanes 16, 17, 22, and 23 (linear).

FIG. 7. 32 Protein must bind behind T4 RNase H for processive degradation. The products of T4 RNase H digestion of a 3'-end-labeled oligonucleotide annealed to circular or linear DNA were compared, with and without 32 protein. (The circular substrate (see Fig. 6B) was linearized by cutting with SmaI endonuclease.) Reactions were carried out as described in Fig. 6B except that samples were removed at 2 and 5 min. Unlabeled competitor fork DNA (see Fig. 2) was added as a trap at 22.9 μM either before Mg²⁺ (lanes 4, 5, 10, and 11 (circular) and lanes 18, 19, 24, and 25 (linear)) or with the Mg²⁺ added to start the reaction (lanes 6, 7, 12, and 13 (circular) and lanes 20, 21, 26, and 27 (linear)). The control reactions without competitor DNA are in lanes 2, 3, 8, and 9 (circular) and lanes 16, 17, 22, and 23 (linear).

**DISCUSSION**

T4 RNase H belongs to a family of prokaryotic and eukaryotic 5'-nucleases that are required to remove the RNA primers present alone (lanes 2–8) or with 1 μM 32 protein (lanes 9–15). Unlabeled competitor fork DNA (see Fig. 2) was added at 22.9 μM either before Mg²⁺ (lanes 4, 5, 11, and 12), with the Mg²⁺ added to start the reaction (lanes 6, 7, 13, and 14), or 0.5 min after the Mg²⁺ (lanes 8 and 15). Control reactions without competitor DNA are in lanes 2, 3, 9, and 10. The products are displayed on a 10% polyacrylamide, 7 M urea gel. The positions of size markers are shown on the right. Comparison of lanes 13 and 14 indicates that the degradation of the 86-mer continued for more than 0.5 min after the addition of competitor in the reactions with 32 protein. The size of the residual 3'-labeled substrate shows that between 10 and 50 nucleotides had been removed from the 5'-end at the end of 2 min (lane 14). There was no further degradation between 2 and 5 min under these conditions (see Fig. 7).
Interaction of T4 RNase H and Gene 32 ssDNA-binding Protein

This paper shows that T4 RNase H makes the first cut from the 5'-end of RNA-DNA and DNA-DNA duplexes at similar rates and that degradation continues until there is a limit oligonucleotide product of 8–11 bases, which is too short to form a stable duplex (Figs. 1 and 4). Our finding that the size of the oligonucleotides released from the 5'-end of the duplex depends on the sequence (Fig. 1) and increases with increasing reaction temperature (Fig. 6 in the accompanying paper (12)) is consistent with the possibility that destabilizing the duplex allows more of the 5'-end of the strand to move into the cleft, past the catalytic site. In flap or fork structures with 5'-ended single strands of 20 bases, T4 RNase H cuts the phosphodiester bonds near the branch point to give 19- and 21-mer products (Fig. 2). Other 5'- to 3'-nucleases in this family, including E. coli DNA polymerase I, T5 D15 nuclease, and the eukaryotic FEN-1 proteins have been shown to cut at or near the branch point (7–10, 17, 22, 23). The flap endonuclease activity of T4 RNase H is inhibited by 32 protein (Fig. 8). Thus 32 protein would be expected to protect flap DNA structures that may be generated during recombination (for review, see Ref. 24).

It is likely that some DNA adjacent to the RNA primers is removed during lagging strand processing because all of these enzymes can degrade both RNA-DNA and DNA-DNA duplexes. This would clearly improve the fidelity of eukaryotic replication, in which the inaccurate DNA pol I polymerase-primase makes the RNA primer and extends it with a short stretch of DNA (for review, see Ref. 25). In T4 DNA replication, the pentamer RNA primer made by the primase-helicase is extended by T4 DNA polymerase. Although this polymerase is extremely accurate in copying DNA templates (for review, see Ref. 26), it may make more errors as it begins elongating the RNA primer.

Our finding that the T4 gene 32 ssDNA-binding protein stimulates the 5'-nuclease of T4 RNase H by converting it to a processive enzyme (Fig. 6) suggests a mechanism for controlling how much DNA is removed along with the RNA primers. 32 protein must be behind the RNase H on the 5'-end of the chain to stimulate the nuclease activity because there was no increased processivity on blunt end duplexes (Fig. 7). The ssDNA that is behind each lagging strand primer at the replication fork would be covered by 32 protein, whose concentration in the T4-infected cell is regulated to match the concentration of ssDNA (27) (Fig. 9). Thus, each time T4 RNase H binds to the end of the lagging strand fragment, it would cut processively, removing 10–50 nucleotides.

In the presence of 32 protein, T4 RNase H bound repeatedly to a simple partial duplex, degrading it rapidly to a short chain of 8–11 bases (Fig. 5). However at the replication fork, a consideration of the different rates of polymerization and nuclease digestion suggests that the nuclease would be limited to single round of processive degradation on each lagging strand fragment. When T4 45 protein clamps T4 DNA polymerase on a ssDNA template covered with 32 protein, the polymerase copies the template at 200–300 nucleotides/s at 30 °C (for review, see Ref. 2). Thus, synthesis of a 2,000-base lagging strand

![Fig. 8. T4 gene 32 ssDNA-binding protein inhibits the flap endonuclease activity of T4 RNase H. The flap substrate with a 20-base tail (1 nM) (see Fig. 2) was treated with T4 RNase H (6.8 or 1.4 nM) in the absence (lanes 2–5) or presence (lanes 6–9) of gene 32 protein (1 μM). The reactions were performed at 30 °C for 2 min (even numbered lanes) or for 5 min (odd numbered lanes). The products were separated on a 10% polyacrylamide, 7 M urea gel.](image)

![Fig. 9. Model of T4 RNase H and Gene 32 ssDNA-binding protein on the lagging strand.](image)

A. No 32 protein

B. With 32 protein

- RNaseH
- pol 45

32 protein must be behind the RNase H on the 5'-end of the chain to stimulate the nuclease activity because there was no increased processivity on blunt end duplexes (Fig. 7). The ssDNA that is behind each lagging strand primer at the replication fork would be covered by 32 protein, whose concentration in the T4-infected cell is regulated to match the concentration of ssDNA (27) (Fig. 9). Thus, each time T4 RNase H binds to the end of the lagging strand fragment, it would cut processively, removing 10–50 nucleotides.

In the presence of 32 protein, T4 RNase H bound repeatedly to a simple partial duplex, degrading it rapidly to a short chain of 8–11 bases (Fig. 5). However at the replication fork, a consideration of the different rates of polymerization and nuclease digestion suggests that the nuclease would be limited to single round of processive degradation on each lagging strand fragment. When T4 45 protein clamps T4 DNA polymerase on a ssDNA template covered with 32 protein, the polymerase copies the template at 200–300 nucleotides/s at 30 °C (for review, see Ref. 2). Thus, synthesis of a 2,000-base lagging strand
fragment would take about 10 s. In contrast, on a partial hybrid covered with 32 protein, T4 RNase H took more than 30 s to remove 10–50 nucleotides before being released from the substrate, giving a rate of 1–2 nucleotides/s (Fig. 6). During this time T4 polymerase could completely copy the ssDNA between adjacent fragments, creating a nick that would be sealed by DNA ligase.

Our recent studies of the interaction of T4 RNase H with other proteins in the T4 replication system are in agreement with this prediction of a single round of T4 RNase H digestion during each lagging strand cycle. We find that 10–50 nucleotides are removed from the downstream fragment before ligation either in model reactions on gapped DNA substrates with T4 RNase H, DNA polymerase, polymerase accessory proteins (gene 45 polymerase clamp and gene 44/62 clamp loader), 32 protein and DNA ligase, or during coupled leading and lagging strand synthesis by the complete T4 DNA replication system. Inhibition of the flap endonuclease activity of T4 RNase H by 32 protein does not create problems for the T4 DNA replication system. Our recent studies show that there is little strand displacement synthesis on the lagging strand by wild type T4 DNA polymerase with the polymerase accessory proteins and 32 protein. Moreover, in similar reactions with the exonuclease-defective T4 DNA polymerase, in which there is more strand displacement synthesis, T4 RNase H is able to cut short flaps.

Acknowledgments—We thank Deborah Hinton, Edith W. Miles, and Timothy Mueser for suggestions that improved the manuscript.

REFERENCES
1. Hollingsworth, H. C., and Nossal, N. G. (1991) J. Biol. Chem. 266, 1888–1897
2. Nossal, N. G. (1994) in Molecular Biology of Bacteriophage T4 (Karem, J., ed) pp. 43–53, American Society for Microbiology, Washington, D. C.
3. Epstein, R. H., Bolle, A., Steinberg, C. M., Kellenberger, E., Boy de la Tour, E., Chevalley, R., Edgar, R. S., Susman, M., Denhardt, G. H., and Lielauasis, A. (1963) Cold Spring Harbor Symp. Quant. Biol. 28, 375–392
4. Hobbs, L. J., and Nossal, N. G. (1996) J. Bacteriol. 178, 6772–6777
5. Woodworth, B. L., and Kreuzer, K. N. (1996) Genetics 143, 1081–1090
6. Harrington, J. J., and Lieber, M. R. (1994) EMBO J. 13, 1235–1246
7. Harrington, J. J., and Lieber, M. R. (1995) J. Biol. Chem. 270, 4509–4508
8. Lyamichev, V., Brow, M. A. D., and Dahlberg, J. E. (1993) Science 260, 778–783
9. Murante, R. S., Huang, L., Turchi, J. J., and Bambara, R. A. (1994) J. Biol. Chem. 269, 1191–1196
10. Ceska, T. A., Sayers, J. R., Stier, G., and Suck, D. (1996) Nature 382, 90–93
11. Mueser, T. C., Nossal, N. G., and Hyde, C. C. (1996) Cell 85, 1101–1112
12. Bhagwat, M., Meara, D., and Nossal, N. G. (1997) J. Biol. Chem. 272, 26531–26538
13. Nossal, N. G., Hinton, D. M., Hobbs, L. J., and Spacciapoli, P. (1995) Methods Enzymol. 262, 560–584
14. Spacciapoli, P., and Nossal, N. G. (1994) J. Biol. Chem. 269, 438–446
15. Venkatasesan, M., and Nossal, N. G. (1992) J. Biol. Chem. 267, 12435–12443
16. Frey, M. W., Nossal, N. G., Capson, T. L., and Benkovic, S. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2579–2583
17. Harrington, J. J., and Lieber, M. R. (1994) Genes Dev. 8, 1344–1355
18. Murante, R. S., Rumbaugh, J. A., Barnes, C. J., Norton, J. R., and Bambara, R. A. (1996) J. Biol. Chem. 271, 25888–25897
19. Johnson, R. E., Kovvali, G. K., Prakash, L., and Prakash, S. (1995) Science 269, 238–240
20. Sommers, C. H., Miller, E. J., Dajin, B., Prakash, S., and Prakash, L. (1995) J. Biol. Chem. 270, 4193–4196
21. Tishkoff, D. X., Filosi, N., Gaida, G. M., and Kolodner, R. D. (1997) Cell 88, 253–263
22. Robbins, P., Pappin, D. J., Wood, R. D., and Lindahl, T. (1994) J. Biol. Chem. 269, 28535–28538
23. Xu, Y., Derbyshire, V., Ng, K., Sun, X. C., Grindley, N. D. F., and Joyce, C. M. (1997) J. Mol. Biol. 268, 284–302
24. Kowalczykowski, S. C., and Egglerston, A. K. (1994) Annu. Rev. Biochem. 63, 991–1043
25. Stillman, B. (1994) Cell 78, 725–728
26. Goodman, M. F., Creighton, S., Bloom, L. B., and Petruska, J. (1993) Crit. Rev. Biochem. Mol. Biol. 28, 83–126
27. Russel, M., Gold, L., Morrissett, H., and O’Farrell, P. Z. (1976) J. Biol. Chem. 251, 7283–7270
28. Spacciapoli, P., and Nossal, N. G. (1994) J. Biol. Chem. 269, 447–455

2 M. Bhagwat and N. G. Nossal, in preparation.