Bacteriophage T4 RNase H Removes Both RNA Primers and Adjacent DNA from the 5′ End of Lagging Strand Fragments*

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Bacteriophage T4 RNase H belongs to a family of prokaryotic and eukaryotic nucleases that remove RNA primers from lagging strand fragments during DNA replication. Each enzyme has a flap endonuclease activity, cutting at or near the junction between single- and double-stranded DNA, and a 5′- to 3′-exonuclease, degrading both RNA-DNA and DNA-DNA duplexes. On model substrates for lagging strand synthesis, T4 RNase H functions as an exonuclease removing short oligonucleotides, rather than as an endonuclease removing longer flaps created by the advancing polymerase. The combined length of the DNA oligonucleotides released from each fragment ranges from 3 to 30 nucleotides, which corresponds to one round of processive degradation by T4 RNase H with 32 single-stranded DNA-binding protein present. Approximately 30 nucleotides are removed from each fragment during coupled leading and lagging strand synthesis with the complete T4 replication system. We conclude that the presence of 32 protein on the single-stranded DNA between lagging strand fragments guarantees that the nuclease will degrade processively, removing adjacent DNA as well as the RNA primers, and that the difference in the relative rates of synthesis and hydrolysis ensures that there is usually only a single round of degradation during each lagging strand cycle.

During the replication of duplex DNA, the leading strand is synthesized continuously, but the lagging strand is synthesized as a series of fragments, each initiated by a short RNA primer made by a primase. Ultimately these RNA primers must be removed, the resulting gaps filled in by polymerase, and the adjacent fragments sealed together by DNA ligase. For bacteriophage T4 DNA replication, the primers are removed by a phage-encoded nuclease, T4 RNase H, that degrades both RNA-DNA and DNA-DNA duplexes from their 5′ termini, giving short oligonucleotide products (1). T4 phage with a deletion in the RNase H gene have a reduced burst on a wild type host and cannot replicate in an Escherichia coli host defective in the 5′- to 3′-nuclease of pol I (2).

T4 RNase H is a protein of 305 amino acids with significant sequence similarity to other prokaryotic and eukaryotic enzymes that remove RNA primers during DNA replication (3). These include the T7 gene 6 exonuclease, the N-terminal domain of E. coli pol I, Saccharomyces cerevisiae Rad27, and human FEN-1 proteins. These enzymes can degrade DNA-DNA as well as RNA-DNA duplexes. This raises the possibility that some DNA adjacent to the primers is removed and repaired in all of these replication systems. If there is reduced fidelity in the initial elongation of the primers, removing the adjacent DNA would improve replication accuracy.

T4 RNase H by itself is a nonprocessive nuclease, removing a single short oligonucleotide (1–4 nucleotides) each time it binds to the substrate (4). In multiple turnover reactions, this degradation of the DNA duplex from the 5′ end continues until a limit product of 8–11 nucleotides remains at the 3′ end. T4 gene 32 protein, binding on single-stranded DNA behind T4 RNase H, converts it into a processive exonuclease that removes multiple short oligonucleotides with a combined length of about 10–50 nucleotides each time it binds to the substrate. In reactions where RNase H can bind multiple times, 32 protein increases the rate of degradation to the same 8–11-base limit product. T4 RNase H can remain on the DNA for more than 30 s when 32 protein is on the DNA behind the nuclease (4). Since T4 gene 32 protein would be covering the single-stranded DNA between lagging strand fragments, there must be some mechanism to control the extent of DNA degradation by RNase H at the replication fork.

In addition to the exonuclease activity producing short oligonucleotide products, all of the 5′-nucleases with a role in lagging strand processing have a flap endonuclease activity that moves 5′ to 3′ to cut near the junction of single- and double-stranded DNA on branched substrates (4–7). Branched substrates would be created if the polymerase elongating the most recent lagging strand fragment displaces the 5′ end of the previous fragment, before the primer is removed from the fragment. Thus it is important to determine whether the exonuclease or the flap endonuclease activity is primarily responsible for processing the 5′ end of the lagging strand fragments.

In this paper we use the T4 DNA replication system to determine the extent of DNA degradation during lagging strand synthesis and the factors controlling this process. T4 DNA polymerase is held on the template by the T4 gene 45 clamp protein that is loaded behind the polymerase by the T4 gene 44/62 clamp loader complex (reviewed in Ref. 8). On gapped DNA model substrates for lagging strand synthesis, we find that RNase H functions as an exonuclease removing short RNA and DNA oligonucleotides, rather than as an endonuclease removing longer flaps created by the advancing polymerase. The combined length of the oligonucleotides released from each fragment ranges from about 3 to 30 and thus corresponds to one round of processive degradation by T4 RNase H with 32 protein behind it. Likewise, we show that an average of about 30 nucleotides is removed from each lagging strand fragment during coupled leading and lagging strand synthesis with the
complete T4 replication system. We conclude that the presence of 32 protein on the ssDNA between lagging strand fragments guarantees that the nuclease will degrade processively, removing adjacent DNA as well as the pentamer RNA primers. However, 32 protein also increases the rate and processivity of the lagging strand polymerase (8). The much higher rate of synthesis than hydrolysis on the 32 protein-covered lagging strand template ensures that there is usually only a single round of degradation during each lagging strand cycle.

**EXPERIMENTAL PROCEDURES**

**T4 Replication Proteins—** T4 RNase H, wild type, and exonuclease-defective D219A (9) T4 DNA polymerase, T4 gene 45 clamp, genes 44/62 clamp head, gene 7.5 single-stranded DNA-binding protein, 84-mer complementary DNA, gene 41 helicase, gene 59 helicase-loading protein, and gene 61 primase were purified to apparent homogeneity as described by Nossal et al. (10). T4 DNA ligase was obtained from Amersham Pharmacia Biotec.

**DNA Substrates—** The 84-mer DNA complementary to nucleotides 6,198–6,281 of M13mp19 viral single-stranded DNA was made on an Applied Biosystems 381A DNA synthesizer and purified by denaturing acrylamide gel electrophoresis. The 41-mer complementary to nucleotides 6282–6322, the 42-mer complementary to 510–551, and the 43-mer complementary to 6309–6351 were made, and reverse phase purified by Genosys Biotechnologies, Inc. Where indicated, the oligonucleotides were 5′-end labeled with [γ-32P]ATP using T4 polynucleotide kinase, annealed to the M13 DNA with or without additional oligonucleotides or free oligonucleotide. For the oligonucleotide on Sepharose CL-2B as described previously (11), the full duplex was washed with the 84-mer and the nicked substrate made with the 84- and 41-mers. The substrates with gaps of 28 or 147 nucleotides were made with the 84-mer and the 43- or 42-mer, respectively. Annealing mixtures contained M13mp19 ssDNA, labeled oligonucleotide, and unlabeled oligonucleotide in a ratio of 1:2.5. The 5′-labeled gapped substrates were made by first annealing the 84-mer (5′-OEIP or P) to the M13 ssDNA, adding two [32P]dTMP using the exonuclease-defective mutant of T4 DNA polymerase (D219A), separating the DNA from dNTP on a Sepharose CL-2B column, and then annealing it to the unlabelled 43- or 42-mer.

**Nuclease and Polymerase Assays—** Unless otherwise indicated, reaction mixtures (10 μl) contained 1.0 mM 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. The concentrations of T4 RNase H are indicated in the figure legends. When present, gene 32 single-stranded DNA-binding protein was 2 μM, T4 wild type or D219A mutant DNA polymerase, gene 45 clamp protein (trimer), and gene 44/62 (4:1 complex) clamp loader were 60, 240, and 160 nM respectively, and T4 DNA ligase was 67 Weiss units/ml. In experiments that included polymerase, clamp and clamp loader, T4 was present at 1 nM and each dNTP at 250 μM. Unless otherwise indicated, reaction mixtures without T4 RNase H or DNA polymerase were incubated for 2 min at 30 °C, and the reaction was begun by the addition of the nuclease, and/or polymerase, as noted in the figures. Aliquots were taken at the times indicated, and the reaction was stopped by addition of 1.5 volumes of a solution of 83% (v/v) formamide, 0.01% xylene cyanol and bromphenol blue, and 33 mM EDTA. Products of the flap endonuclease if T4 RNase H digestion follows flap formation by the polymerase. Our experiments indicate that, in most cases, T4 RNase H degradation before polymerase fills the gap.

**RESULTS**

**The Size of the Products Released by T4 RNase H during the Processing of the Lagging Strand RNA Primers—** Because T4 RNase H has both exonuclease and flap endonuclease activities, there are two possibilities for the product size during the processing of the lagging strand fragments (Fig. 1). If T4 RNase H removes the primers and adjacent DNA before polymerase fills in the gap, then the exonuclease activity of T4 RNase H would release only small oligonucleotide products. However, if polymerase fills in the gap first and forms a flap by displacing part of the next Okazaki fragment, then the flap endonuclease activity of T4 RNase H would release a longer oligonucleotide product. We tested these possibilities on a model gapped substrate. To avoid degradation of the DNA products by the 5′-to-3′ exonuclease of the wild type T4 DNA polymerase, we used the D219A mutant polymerase that lacks this exonuclease activity (9).

**Strand Displacement Synthesis on Lagging Strand Substrates Is More Extensive with the Exonuclease-defective (D219A) Than with Wild Type T4 DNA Polymerase—** Previous studies have shown that wild type T4 DNA polymerase is released quickly when it reaches a duplex, unless there is a fork formed by a noncomplementary single strand (13, 14). The clamp protein is released along with the polymerase (15, 16). Fig. 2 compares synthesis on a gapped substrate by the wild type and exonuclease-defective polymerases. Elongation of the 43-mer to fill the 28-base gap would yield a 71-base product. Longer products result from strand displacement synthesis as expected, there is little strand displacement synthesis by the

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1 The abbreviations used are: kb, kilobases; ss, single-stranded.
Wild type DNA polymerase (lanes 2–5 and 17–20), in agreement with the earlier studies. However, there is extensive displacement synthesis with the mutant polymerase (lanes 6–9 and 21–24). Strand displacement synthesis by the D219A polymerase is increased by addition of the 45 clamp and 44/62 clamp loader (compare lanes 6 and 8 and lanes 21 and 23) and is most extensive in reactions with the 32, 45, and 44/62 proteins (lanes 9 and 24). In the absence of dCTP, wild type polymerase extended the 43-base downstream primer to the first template G, giving a 54-base product. On some molecules there was additional readthrough to 57 bases where there are 2 Gs in the template (lanes 25–28). As expected there was more extensive readthrough by the polymerase lacking the proofreading exonuclease (lanes 29–32).

Size of the T4 RNase H Products—The products of T4 RNase H digestion on a model lagging strand substrate, coupled to replication by the exonuclease-defective polymerase, are shown in Fig. 3. In this experiment the reaction conditions and template were the same as those in Fig. 2, except that in this case it was the downstream 84-mer that was 5’ end-labeled. The reactions were terminated after 0.25 min. Although by this time the mutant polymerase had displaced a flap of about 10 nucleotides in the reactions with the clamp protein, with or without 32 protein (Fig. 2, lanes 8 and 9), the oligonucleotides removed by T4 RNase H were predominantly the trimer products of exonuclease digestion (Fig. 3, lanes 13–16). There were also some longer products, up to 10 bases, in the reactions containing the 45 clamp and 44/62 clamp loader (lanes 15 and 16). There were fewer long products in the reaction with RNase H, polymerase, and 32 protein (lane 14), consistent with our previous finding (4) that 32 protein inhibits the flap exonuclease activity of T4 RNase H on fork substrates. There were no flap products when dCTP was omitted to prevent complete gap filling by the polymerase (lanes 23–26). We conclude that the exonuclease activity, rather than the flap exonuclease activity of T4 RNase H, is responsible for most of the degradation on the fragment ahead of the polymerase on this model template.

Hydrolysis of RNA Primers on Lagging Strand Fragments Initiated by the T4 Primase—The RNA primers made by the T4 gene 61 primase, acting in conjunction with the gene 41 helicase, are pentamers, with the sequence ppp(A/G)pCpNpNpN (17, 18). In the experiment shown in Fig. 4, the RNA primers were labeled by synthesis with [α-32P]dCTP, in a primer-dependent DNA synthesis reaction on M13 single-stranded circular DNA by the T4 replication system. The reaction products (Fig. 4, top) were extracted with phenol to remove the replication proteins and filtered on a column of Sepharose CL-2B to separate the primer-labeled DNA from free primers that had not been extended by polymerase. Hydrolysis by T4 RNase H was then measured in a second reaction with the indicated proteins (see “Experimental Procedures”). T4 RNase H removed the RNA primers, as RNA oligonucleotides of 2–5 bases (Fig. 4, lane 3). There was no evidence of longer products that would have contained the initial primer and some adjacent DNA. The size distribution of these RNA products was not altered by addition of the 45 clamp, 44/62 clamp-loader, and 32 protein (lane 4) or by addition of the exonuclease-defective T4 DNA polymerase as well as these other proteins (lane 5). Thus RNA primers, like the DNA at the 5’ side of a gap (Fig. 3), are removed by the exonuclease, rather than the flap nuclease activity of T4 RNase H.

Control of Hydrolysis of RNA Primers and Adjoining DNA on
the Lagging Strand Fragments—T4 RNase H becomes a pro-
cessive exonuclease in the presence of 32 protein, removing
about 10–50 nucleotides in each interaction with the substrate
(4). If the enzyme can bind repeatedly, degradation continues
to a limit product of 8–11 nucleotides. How is this exonuclease
reaction controlled to avoid extensive removal of DNA from the
downstream Okazaki fragment? We have used the experimental
approach diagrammed in Fig. 5 (left) to determine how
much DNA is removed from the downstream fragment in reactions
with T4 polymerase, 45 clamp, 44/62 clamp-loader, 32
protein, and DNA ligase. During DNA synthesis the down-
tream 3'-labeled fragment is extended past the BstNI site,
giving a 143-base restriction product when BstNI is added
later. Simultaneous elongation of the unlabeled upstream frag-
ment creates a nicked DNA that cannot be sealed by DNA
ligase, because there is a hydroxyl group rather than a phos-
phate 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. The number of
nucleotides removed by T4 RNase H, under conditions needed
for ligation, is measured by the decreased size of the 143-base
fragment in reactions where ligase is omitted.

In the experiment shown in Fig. 5, the reactions were carried
out at 30 °C for the indicated time, heated at 60 °C to denature
the replication enzymes, and the products then digested with
BstNI at 60 °C. In the control reactions (lanes 1–9) in which the
downstream 86-mer had a 5'-phosphate, gap filling and liga-
tion were complete by 0.5 min, as shown by the similar quan-
tities of the 191-base restriction fragment at 0.5 and 5 min,
with or without T4 RNase H (lanes 4, 5, 8, and 9). Under these
conditions, wild type T4 DNA polymerase completely filled the
gap on this substrate by 0.25 min, with or without the 32, 45,
and 44/62 proteins (see Fig. 2, lanes 2–5). In the reactions with
the gapped substrate with a 5'-OH 86-mer, there was no join-
ing of the adjacent fragments in the absence of T4 RNase H
(lanes 13 and 14). There was substantial ligation in reactions
with T4 RNase H (lanes 17 and 18), roughly similar to the
fraction of 5' label removed from this gapped substrate by the
same concentration of the nuclease (Fig. 3). In the reactions
with T4 RNase H without ligase (lanes 15 and 16), most of the
downstream fragments that had been digested were 3–15 nu-
cleotides shorter than the 143-base undigested restriction frag-
ment. Some of the products were up to 50 bases shorter than the
143-base fragment. Most of the fragments digested by the
nuclease were joined to the upstream fragment when ligase
was added, as shown by the decrease in labeled products
shorter than 143 bases in the reactions with RNase H and
ligase (lanes 17–18).

We have carried out a similar experiment with the same
3'-32P-labeled 86-mer annealed to M13mp19, in which the
gap between the oligonucleotides is initially 1479 bases (Fig. 6).
This is a more realistic model for the T4 replication system,
since the lagging strand fragments average 1.5 kb both in vivo
and in vitro. Even with this large gap, 36% of the labeled
fragments were ligated to the upstream fragment in 0.5 min
(191-base products, reaction 5). The length of DNA removed
from the downstream fragment before ligation can be deter-
mined by comparing the size distribution of fragments shorter
than 143 bases in the reactions with RNase H without ligase
(reactions 4 and 6) with those in the reactions with both RNase
H and ligase (Fig. 6A, reactions 5 and 7). On the PhosphorIm-
ager scan (Fig. 6B) of the gel in Fig. 6A, the positions of the size
markers are shown above the figure. The sizes of products
present at a higher level in the reaction with RNase H without
ligase (reaction 4, red line) than in the reaction with both
RNase H and ligase (reaction 5, green line) are shown beneath
a bracket. At 0.5 min (top panel) these products ranged from
141 to 114 bases, indicating that 2–29 nucleotides had been
removed before the fragments were ligated. By 2 min (Fig. 6B,
bottom panel), these products ranged from 141 to 96 bases,
indicating the removal of 2–47 bases. Once a nick is formed,
further degradation is slow even without ligase. There is little
degradation between 1 and 2 min on the template with two
oligonucleotides (Fig. 6A, reaction 4), compared with the degrada-
tion of the template with a single oligonucleotide during the
same time (reaction 8).

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![Diagram](Image)
major products are the dephosphorylated dimers and trimers expected from hydrolysis by the exonuclease activity of T4 RNase H. The hydrolysis products are decreased when ligase is added to seal the nick formed when the elongating polymerase fills in the gap. Note that the RNA primers were not labeled, and the monomer DNA products could not be detected by this assay, because all of their 32P would be removed by alkaline phosphatase. The bands of the isolated products were excised and counted (Fig. 8D). The total DNA removed from each fragment was calculated, assuming that 50% of the trichloroacetic acid precipitable product was in lagging strand fragments with an average length of 1.5 kb (Fig. 8C). In the reaction with both T4 RNase H and DNA ligase, about 30 nucleotides were removed from each fragment.

**DISCUSSION**

At T4 replication forks the leading and lagging strands are synthesized coordinately at the rapid rate of about 400 nucleotides per s (reviewed in Ref. 8). Thus the challenge on the lagging strand is to accurately remove and replace the primers initiating each lagging strand fragment, using a mechanism that is compatible with the rapid cycling of the lagging strand polymerase from the end of one fragment to the primer initiating the next fragment. T4 RNase H belongs to a family of prokaryotic and eukaryotic nucleases, with similar structures, that cuts at, or near, the junction between single- and double-stranded DNA, as well as a 5’-to-3’exonuclease that degrades both RNA-DNA and DNA-DNA duplexes. These dual activities are consistent with two general mechanisms for primer removal. The 5’-exonuclease could act first, removing the RNA primer and some adjacent DNA in time for the resulting gap to be filled by the polymerase completing the upstream fragment. Alternatively, strand displacement synthesis by this polymerase could first create a flap, including the primer, that is subsequently removed by the flap endonuclease (Fig. 1). Since all of these nucleases can degrade DNA-DNA duplexes, it is important to know how much adjacent DNA is removed along with the RNA primers and what limits the extent of this degradation.

Within the multienzyme T4 replication system, wild type T4 DNA polymerase catalyzes strand displacement synthesis on forked leading strand templates but stops and is rapidly released when it reaches an annealed duplex on model lagging strand DNA templates (13–16). It thus seemed unlikely that T4 DNA polymerase would create flaps for the nuclease unless there was more strand displacement synthesis when it encountered a duplex terminated with a 5’-triphosphorylated RNA primer. To distinguish between the exonuclease and flap endonuclease models for lagging strand processing, we have used an exonuclease-defective (D219A) mutant of T4 DNA polymerase that can carry out strand displacement to form flaps at a duplex (Fig. 2 and Ref. 19). In reactions with this mutant polymerase, our experiments show clearly that it is the exonuclease rather than the flap endonuclease activity of RNase H that is predominantly responsible for removing the 5’ end of either DNA fragments or fragments terminated with 5’-triphosphorylated RNA pentamers made by the T4 primase. Since the mutant polymerase can displace the 5’ end of the fragment ahead, these experiments indicate that when T4 RNase H and polymerase are added simultaneously to the template, in most cases the nuclease begins degradation before the upstream fragment is completed. This mechanism has the
The presence of some longer products, characteristic of the flap endonuclease, in reactions with both T4 RNase H and the mutant polymerase, indicates that if polymerase arrives first, the flap can be removed by the endonuclease. The flap endonuclease may improve fidelity when the 5' end of the duplex fragment is destabilized by mispairing or DNA damage. In addition, the flap endonuclease may be important in removing 5'-terminated single strands from strand invasion structures, because most T4 DNA replication takes place at forks created by recombination (20, 21). Although 32 protein inhibits the flap endonuclease, presumably because it covers the single-stranded flap (4), there is more flap product in reactions in which the T4 45 clamp and 44/62 clamp loader are present in addition to 32 protein, polymerase, and RNase H (Fig. 3).

### Control of Degradation of DNA Adjacent to the RNA Primers

If the accuracy of adding the first nucleotides to the RNA primer is lower than subsequent additions, it is desirable to remove the DNA adjacent to the primers before the gap between fragments is filled in by polymerase. However, it is important to limit the extent of degradation by the RNase H exonuclease to avoid removing more newly synthesized DNA than is required to maintain fidelity. There must be some mechanism to control degradation, because, under multiple turnover conditions, the exonuclease will continue degradation of a duplex to a limit product of 8–11 nucleotides (4).

At the T4 replication fork (Fig. 9), 32 protein covers the single-stranded lagging strand template between Okazaki fragments, increasing the processivity of both hydrolysis of the downstream fragment by RNase H and synthesis of the upstream fragment by polymerase. Under single turnover conditions, the nonprocessive nuclease makes a single cut, releasing a short oligonucleotide, usually a dimer or trimer. When 32 protein is present behind RNase H, the exonuclease becomes moderately processive. It then removes a series of short oligonucleotides (mainly dimers and trimers) with a combined length of 2–50 nucleotides during a single interaction with the substrate (4).

In reactions with T4 RNase H, 32 protein, polymerase, clamp, and clamp loader, the number of nucleotides removed before ligation in model DNA templates with gaps between

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### Table: Length of DNA removed from lagging strand fragments by T4 RNase H during gap filling and ligation of adjacent fragments on a model template with a 1479 nucleotide gap

| Reaction | Polymerase | RNaseH (nM) | Ligase | Minutes |
|----------|------------|-------------|--------|---------|
| 1        | +          | 13          | +      | 2       |
| 2        | +          | 13          | +      | 2       |
| 3        | +          | 26          | +      | 2       |
| 4        | +          | 13          | +      | 2       |
| 5        | +          | 26          | +      | 2       |
| 6        | +          | 13          | +      | 2       |
| 7        | +          | 26          | +      | 2       |
| 8        | +          | 13          |        | 2       |
| 9        | +          | 26          |        | 2       |
| 10       | +          | 13          |        | 2       |
| 11       | +          | 26          |        | 2       |

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### Figure 6

**A**. Length of DNA removed from lagging strand fragments by T4 RNase H during gap filling and ligation of adjacent fragments on a model template with a 1479 nucleotide gap. A, 10% polyacrylamide, 7 M urea gel. The template in reactions 1–7 had a 32P-labeled 86-mer 1479 nucleotides downstream from an unlabeled 42-mer on M13mp19 ssDNA (see "Experimental Procedures"). There was no 42-mer on the template in reactions 8–11. The DNA was incubated with the T4 32 ssDNA-binding protein, 45 clamp, 44/62 clamp loader, and when indicated T4 DNA ligase, for 2 min at 30 °C, before the addition of T4 RNase H and wild type DNA polymerase for the times shown. The reactions were stopped by heating to 60 °C, and the products were digested with BstN I nuclease and then heated for 3 min at 95 °C before electrophoresis. The 32P-labeled 86-mer was elongated to the BstN I site, giving a 143-base product in the absence of T4 RNase H (reactions 2 and 3). The products shorter than 143 bases are a measure of hydrolysis by the exonuclease activity of T4 RNase H (reactions 4–11). The 191-base products are molecules ligated following T4 RNase H digestion to expose a 5'-P, and gap filling by polymerase (reactions 5 and 7). ● marks the position of 32P on the 86-mer. B, PhosphorImager scan of the gel in A, showing the products of reactions 3 (ligase without RNase H, black), 4 (RNase H without ligase, red), and 5 (RNase H and ligase, green). The positions of the size markers are shown above the figure. The brackets in each panel show the sizes of products present at a higher level in reactions with RNase H without ligase (red) than in the reactions with both RNase H and ligase (green). At 0.5 min (top panel) these products ranged from 141 to 114 bases, indicating that 2–29 nucleotides had been removed before the fragments were ligated. At 2 min (bottom panel) these products ranged from 141 to 96 bases, indicating the removal of 2–47 nucleotides. PSL is the detected radiation in arbitrary units.
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fragments of either 28 or 1479 nucleotides (Fig. 5 and 6 respectively) ranged from 2 to 50, with most strands shortened by 3–29 nucleotides. Similarly, an average of about 30 DNA nucleotides, in addition to the pentamer RNA primers, were removed from each lagging strand fragment during coupled leading and lagging strand synthesis on a rolling circle template by the complete T4 replication system (Fig. 7 and 8). These products are consistent with the hydrolysis expected during a single round of processive degradation by RNase H, with 32 protein behind it. Further cycles of processive degradation are prevented because the rate of synthesis by polymerase extending the upstream fragment is so much faster that the rate of degradation by the nuclease (1–2 nucleotides/s (4)). RNase H can remain bound to a model lagging strand substrate for more than 30 s when 32 protein is on the ssDNA behind the nuclease (4). In the time needed for the first nuclease cycle, polymerase fills in the gap, displaces 32 protein, and forms a nick that can be sealed by DNA ligase. T4 RNase H by itself gives very little degradation by the nuclease (1–2 nucleotides/s (4)). RNase H would increase as more single strand is unwound by the helicase. Although T4 DNA polymerase is a monomer in solution, there is evidence that polymerase molecules associate with each other (23). If the leading and lagging strand polymerases at the replication fork are in contact with each other, and the leading strand polymerase is in contact with the helicase, there may be considerable torsional stress on the structure compacting the ssDNA closest to the fork, as more ssDNA is unwound by the helicase. It is possible that this compact structure shields the primer from the nuclease or alters the conformation of 32 protein on the ssDNA enough to prevent its stimulation of the nuclease. Alternatively, the primer closest to the fork may be accessible to the nuclease during the elongation and termination stages of the lagging strand cycle, in which case hydrolysis would be controlled simply by the large difference (about a hundredfold) in the rates of polymerization and degradation. Hydrolysis would be limited to that catalyzed by T4 RNase H in the short time necessary to prime and extend the next fragment. In prokaryotic systems, with lagging strand fragments of 1–2 kb, this limited hydrolysis of the most recent fragment would not be a serious problem. However, tighter control would be expected in eukaryotic systems where polymerization is slower, and the fragments are about 10-fold shorter (reviewed in Ref. 24).

Comparison of Okazaki Fragment Maturation in T4 and Other Systems—Like T4 DNA polymerase, the E. coli replicative polymerase pol III does not form flaps because it dissociates rapidly when it reaches an annealed fragment (25). The 5'-to 3'-nuclease of E. coli pol I, which has both flap and exonuclease activities, plays the major role in removing the primers, with an auxiliary role for RNase HI in vivo (26–28), and during the replication of a plasmid with the oriC origin in vitro (29). Because the polymerase activity of pol I does catalyze strand displacement synthesis, it has been proposed that synthesis by pol I creates the flaps that are removed by the associated 5'-nuclease (30). Recent studies on model gapped templates with flaps showed that the polymerase and 5'-nuclease activities operate independently, although the nuclease was more likely to cut molecules that had already been extended by polymerase upstream of the most recent fragment. Hydrolysis of the most recent fragment must be limited, since the total hydrolysis during coupled leading and lagging strand synthesis in vitro was equivalent to a single round of degradation on each fragment by RNase H with 32 protein behind it (Figs. 7 and 8).

One possibility is that the most recent primer is shielded from the nuclease by other replication proteins on the lagging strand template. Electron microscopic studies of phage T7 replication forks suggested that the ssDNA on the lagging strand is in a compact protein-covered form. Single-strands were visible, as expected, on deproteinized replication forks, but micrographs of the fork with the replication proteins showed large protein complexes and a double-stranded DNA loop but no extended ssDNA (22). Because recent electron microscopic studies have shown similar structures at replication forks with T4 proteins,3 the 32 protein-covered DNA in Fig. 9 is shown in a compacted, rather than extended, form. During the elongation stage of the lagging strand cycle there will be compacted ssDNA adjacent to each primer. The protein composition and path of the ssDNA in each of these compacted structures remain to be determined. The size of the structure between the two fragments will decrease as the upstream fragment is elongated, whereas the size of the structure closest to the fork, which may be associated with the helicase, primase, and/or helicase loading protein, will increase as more single strand is unwound by the helicase. Although T4 DNA polymerase is a monomer in solution, there is evidence that polymerase molecules associate with each other (23). If the leading and lagging strand polymerases at the replication fork are in contact with each other, and the leading strand polymerase is in contact with the helicase, there may be considerable torsional stress on the structure compacting the ssDNA closest to the fork, as more ssDNA is unwound by the helicase. It is possible that this compact structure shields the primer from the nuclease or alters the conformation of 32 protein on the ssDNA enough to prevent its stimulation of the nuclease. Alternatively, the primer closest to the fork may be accessible to the nuclease during the elongation and termination stages of the lagging strand cycle, in which case hydrolysis would be controlled simply by the large difference (about a hundredfold) in the rates of polymerization and degradation. Hydrolysis would be limited to that catalyzed by T4 RNase H in the short time necessary to prime and extend the next fragment. In prokaryotic systems, with lagging strand fragments of 1–2 kb, this limited hydrolysis of the most recent fragment would not be a serious problem.

6 M. Bhagwat, O. Gangisetty, and N. G. Nossal, unpublished experiments.

3 P. Chastain, S. Markov, N. G. Nossal, and J. Griffith, unpublished experiments.
Primer removal in eukaryotic systems is more complicated. The FEN-1, RNase HI, and Dna2 nucleases all have been implicated in this process. FEN-1 has exonuclease and flap endonuclease activities on both RNA-DNA and DNA-DNA duplexes (5). RNase HI has exonuclease activity only on RNA-DNA hybrids and has little or no activity on the RNA nucleotide adjacent to the DNA (32, 33). Dna2 is an interesting enzyme that is both a 5′-to-3′ helicase and a DNA endonuclease that cuts ssDNA but not RNA (34, 35). Based on these properties of the purified enzymes, pathways for processing lagging strand fragments have been proposed involving either FEN-1 and RNase HI (24, 32, 33), or alternatively, Dna2 and FEN-1 (35). RNase HI and FEN-1 were sufficient to allow complete SV40 replication in vitro (36–39). Genetic studies in S. cerevisiae showed that mutants in the nuclease activity of Dna2 are not viable (40), mutants in FEN-1 (rad27) are temperature-sensitive (33, 41, 42), but mutations in RNase H, the yeast homologue of mammalian RNase HI, have only a small effect on viability (33). On a model gapped template with fragments separated by the distance between eukaryotic Okazaki fragments, Dna2 cut 2 or 3 nucleotides after the 5′-RNA only if pol, the proliferating cell nuclear antigen clamp, and RFC clamp loader were present to give strand displacement synthesis (35).

The salient features of the maturation of T4 discontinuous fragments are that T4 RNase H acts predominantly as an exonuclease rather than a flap endonuclease, removing the primer and about 30 nucleotides of adjacent DNA. We thank Debbie Hinton, Charles Jones, and Erin Green for helpful comments on the manuscript.

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FIG. 8. Isolation and quantitation of RNase H products during coupled leading and lagging strand replication. A, 20% polyacrylamide, 7 M urea gel of the hydrolysis products. Products were labeled by including [α-32P]dCTP in the replication reactions shown in Fig. 7. To improve separation of the short oligonucleotides from residual dCTP, the reaction mixtures were treated with alkaline phosphatase as described under “Experimental Procedures,” before electrophoresis. There were no short products in reactions without primase, indicating that all the RNase H hydrolysis products came from the lagging strand. Markers are the alkaline phosphatase-treated di- and trinucleotide primers made by T4 primase and helicase. The dephosphorylated trimer runs ahead of the dimer. B, quantitation of oligonucleotide products. The dCMP in oligonucleotides was determined by scintillation counting of bands excised from the gel shown in A. C, nucleotides removed per lagging strand fragment. The estimate of nucleotides removed per lagging strand fragment assumes that lagging strand synthesis was 50% of the total incorporation and that the average length of the fragments was 1500, as shown in the alkaline agarose gel in Fig. 7. Mononucleotide products from hydrolysis by RNase H had to be neglected because they would not have remained labeled after treatment with phosphatase. ■, RNase H; ▴, RNase H and ligase; ○, omit both RNase H and ligase.

FIG. 9. Speculative model of T4 DNA replication fork in which the primer at the end of the most recent fragment is associated with a compact protein-ssDNA complex that shields it from hydrolysis by T4 RNase H. The compact structure of the single-stranded regions of the lagging strand is suggested by electron microscopic studies of the T7 and T4 replication systems (22). This model proposes that the compact structure nearest the fork extends far enough to protect the 5′ end of the newly synthesized chain from digestion until the structure is altered when the next primer made upstream fragment is completed by polymerase, and that the extent of this degradation is controlled by the great difference in the 32 protein-stimulated rates of polymerization and hydrolysis. Whether hydrolysis of DNA adjacent to the primers is a general feature of lagging strand synthesis in other replication systems remains to be determined.
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