Eukaryotic Lagging Strand DNA Replication Employs a Multi-pathway Mechanism That Protects Genome Integrity

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In eukaryotic nuclear DNA replication, one strand of DNA is synthesized continuously, but the other is made as Okazaki fragments that are later joined. Discontinuous synthesis is inherently more complex, and fragmented intermediates create risks for disruptions of genome integrity. Genetic analyses and biochemical reconstitutions indicate that several parallel pathways evolved to ensure that the fragments are made and joined with integrity. An RNA primer is removed from each fragment before joining by a process involving polymerase-dependent displacement into a single-stranded flap. Evidence in vitro suggests that, with most fragments, short flaps are displaced and efficiently cleaved. Some flaps can become long, but these are also removed to allow joining. Rarely, a flap can form structure, necessitating displacement of the entire fragment. There is now evidence that post-translational protein modification regulates the flow through the pathways to favor protection of genomic information in regions of actively transcribed chromatin.

During the S phase of the cell cycle, the genetic material within the eukaryotic cell is duplicated with high efficiency and accuracy. One might expect nuclear DNA replication, an ancient and fundamental cellular requirement, to be a relatively simple process. In reality, it is very complex, with the evolution of several parallel pathways to ensure the maintenance of genome stability. Based on the 5’→3’-directionality of DNA polymerases, replication proceeds by continuous synthesis on the leading strand, growing in the same direction as the opening of the parental strands, and discontinuous synthesis on the lagging strand, growing in the opposite direction (1). The lagging strand is first made as short fragments of DNA, which are initiated by RNA/DNA primers. These fragments, ~100–150 nucleotides (nt) in length, are known as Okazaki fragments (2, 3). Prokaryotic DNA replication occurs within a fork structure in which the lagging strand loops around in a proposed “trombone model” to help orient the replication enzymes for repeated synthesis and joining (4). A similar structure may be employed by eukaryotes. Synthesis of DNA strands occurs at identical rates, suggesting a coordination between leading and lagging strand synthesis (5).

Prokaryotic DNA Polymerases

Both strands of the parental prokaryotic genome are replicated by DNA polymerase (pol) III holoenzyme (reviewed in Ref. 6). A multisubunit complex of 10 components, the α-, ε-, and δ-subunits make up the catalytic core, acting to polymerize and edit the newly synthesized DNA. Association with the β-subunit, which encircles the DNA as a sliding clamp, completes the holoenzyme, which carries out highly processive synthesis. The triple-DNA polymerase replisome model suggests that three core polymerases assemble at the replication fork, with one working to extend the leading strand and two synthesizing on the lagging strand DNA. Because synthesis on the lagging strand is more complex, it is possible that the presence of two polymerases might help to coordinate the replication rates on the leading and lagging strands to avoid uncoupling of the replication process (7).

On the lagging strand, the holoenzyme quickly releases from the template as it encounters the 5’-end of the preceding downstream Okazaki fragment. This has been termed the “collision model.” New lines of evidence from O’Donnell and co-workers (8) argue against the 5’-end primer recognition to trigger collision release by the polymerase holoenzyme. Instead, it has been proposed that the lack of single-stranded DNA (ssDNA) on encountering the preceding Okazaki fragment triggers polymerase release from the β-clamp and the DNA (8). Dissociation of pol III results in polymerase switching, whereby pol I takes over the maturation of Okazaki fragments. Pol I has intrinsic 5’→3’-exonuclease and polymerase activities, so it can mediate both the removal of RNA primers from the 5’-end and the synthesis of nucleotides onto Okazaki fragments. Pol I performs nick translation for RNA removal, a combination of strand displacement synthesis and cleavage of resulting short flaps suggested by Setlow et al. (9), who showed that products of 5’-exonuclease activity were both mono- and oligonucleotides.

Eukaryotic DNA Polymerases

Initial studies of eukaryotic DNA replication used SV40 as a model system. Reconstitution of SV40 replication effectively represents cellular processes because the virus makes extensive use of cellular replication proteins. Work with SV40 suggested that the cell initiates replication using pol α/primase (10). Consisting of four subunits (p180, p70, p58, and p48), pol α is the only eukaryotic polymerase that displays primase activity. The primase catalyzes the synthesis of ~8–10 nt of RNA; the DNA polymerase then further extends the initiator segment by adding ~20–20 nt of DNA (11). Lacking a proof-reading 3’→5’-exonuclease activity, polymerization by pol α is error-prone.
The studies with SV40 further indicated that pol δ was the main replicative polymerase on both the leading and lagging viral strands, initiating from the primers made by pol α (12). However, a number of recent studies in yeast by Kunkel et al. (13) have definitively identified pol ε as the polymerase responsible for synthesis of the cellular leading strand. Consisting of four subunits (Pol2, Dpb2, Dpb3, and Dpb4), pol ε is highly processive and is made more processive by proliferating cell nuclear antigen (PCNA), as appropriate for synthesis on the leading strand (14).

After initial priming by pol α, pol δ takes over the synthesis on the lagging strand. In Saccharomyces cerevisiae, pol δ is composed of three subunits (Pol3, Pol31, and Pol32); however, in Schizosaccharomyces pombe and mammalian cells, a fourth subunit functions to stabilize the polymerase holoenzyme. pol δ displays both polymerase and 3′→5′-exonuclease activities. Using its proofreading function, pol δ can correct errors made by pol α, thereby protecting genome stability (15).

**Lagging Strand Replication Machinery Accessory Factors**

Replication protein A (RPA) assembles on the ssDNA immediately after the helicases unwind double-stranded DNA, creating a replication fork. Binding of RPA protects the ssDNA from cellular nucleases and also prevents formation of hairpin structures that might impede the progression of the replication fork (16). Additionally, RPA coordinates the assembly and disassembly of replication-associated proteins on the ssDNA, pol α/primase recognizes RPA-coated ssDNA and initiates primer synthesis on the DNA template (16). Association of the ATP-dependent replication factor C with pol α triggers the switch from replication initiation to replication elongation, wherein pol α is displaced, and PCNA (a functional homolog of the prokaryotic β-clamp) and pol δ are loaded onto the replicating segment of DNA (12). PCNA improves the processivity of pol δ and stimulates strand displacement synthesis (17).

**Eukaryotic Okazaki Fragment Maturation**

**Short Flap Pathway**—As pol δ fills in the gap between Okazaki fragments, it encounters the downstream fragment and displaces it into a short 5′-flap, similar to the action of prokaryotic pol I. Whereas pol I contains both polymerase and exonuclease functions in a single polypeptide, pol δ does not possess 5′→3′-exonuclease function. This role in eukaryotes is filled by FEN1 (flap endonuclease 1; Rad27 in S. cerevisiae) (18, 19). FEN1 binds to the base of the displaced flap and threads the flap through its active site, to cleave back at the base, creating a nick that can be joined by DNA ligase I (LigI) to complete the maturation process.

**Biochemical reconstitutions of Okazaki fragment maturation** performed by Burgers and co-workers (23) and us (24) have shown that the majority of the flaps that are displaced and processed are <10 nt long. Several of these flap cleavages are needed to remove the initiator RNA/DNA primer. Our work suggests that the 3′-proofreading function of pol δ and
the action of FEN1 work coordinately to limit the length of the displaced flap because pol δ mutants lacking a 3’-nuclease show increased strand displacement synthesis, resulting in longer flaps (24). Processing of Okazaki fragments by strand displacement synthesis, followed by cleavage of short flaps, is known as the “short flap pathway” or the “one-nuclease (FEN1-only) pathway” (Fig. 2).

Long Flap Pathway—Although the majority of the flaps are processed through the short flap pathway, our results also showed that a small percentage of flaps reach lengths >20–30 nt before cleavage (24). It is currently unknown why some flaps escape FEN1 cleavage. Possibly, FEN1 transiently disengages from the replication protein complex. As the flaps reach lengths >22 nt, RPA can stably bind and coat them (25). Bound RPA prevents cleavage by FEN1, necessitating an additional nuclease component (26). Budd and Campbell (52) identified this second nuclease, Dna2, in *S. cerevisiae*. Dna2 is a multifunctional enzyme, displaying ssDNA endonuclease, ATPase, and 5’–3’-helicase activities. Genetic studies showed that *dna2-1* (5’–3’-nuclease-deficient mutant) and *pol3-01* (3’-exonuclease-deficient mutant of pol δ), which increases strand displacement activity, are a lethal combination. This result suggests that Dna2 is needed to process long Okazaki fragment flaps.

RPA binding acts as the critical switch between the short and long flap-processing pathways in *S. cerevisiae* (26). Dna2 functionally and physically interacts with RPA (27). Although RPA inhibits FEN1 activity, the endonucleolytic cleavage function of Dna2 is significantly stimulated on RPA-coated flaps (26, 28). Independent of its helicase and endonuclease activities, Dna2 displaces RPA from the flaps and, using its endonuclease activity, cleaves multiple times until the flap is shortened to ~5–6 nt (26, 28). RPA cannot rebind on this short flap, thereby allowing for FEN1 activity. After displacement of RPA, Dna2 can remain nonproductively bound to the short flap substrate. FEN1 is capable of disengaging Dna2 from these flaps and then cleaving to create a nick for proper ligation.

Our reconstitutions of Okazaki fragment processing produced the surprising result that if the flap is created by pol δ in the presence of FEN1 and RPA, FEN1 could acquire the flap for cleavage before inhibition by RPA (24). Why then was it necessary to evolve Dna2? A possible answer lies in the properties of the 5’–3’-DNA helicase Pif1. Genetic evidence...
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from *S. pombe* suggested that Pfh1 (homolog of *S. cerevisiae* Pif1) plays a role in Okazaki fragment maturation by promoting strand displacement synthesis by pol δ. If so, Pif1 might promote rapid flap formation, creating a need for Dna2. In support of this hypothesis, genetic studies in *S. cerevisiae* showed that deletion of *PIF1* rescued the lethal phenotype of *dna2Δ* strains (29). However, *pif1 dna2Δ* strains grew only at lower temperatures (30°C), suggesting that Dna2 was still required for most efficient DNA replication. Notably, the *pif1 dna2Δ* strain, combined with a deletion in the *POL32* gene, which encodes a pol δ subunit needed for efficient strand displacement, was able to grow at normal temperatures (37°C) (29). Moreover, when Okazaki fragment processing was reconstituted with Pif1, a subset of flaps grew so rapidly that RPA bound them and blocked FEN1 (30). Apparently, although nick translation by pol δ and FEN1 supports the short flap pathway, the cooperative action of Pif1 and pol δ makes long flaps needing Dna2/FEN1 for processing. This is the “long flap pathway” or the “two-nuclease (Dna2/FEN1) pathway” (Fig. 2). Despite the mechanistic elegance of the long flap pathway, evidence suggests that the short flap pathway has evolved to act as efficiently as possible. Recent results show that all three protein components of the long flap pathway (Dna2, RPA, and Pif1) strongly stimulate cleavage by FEN1, promoting short flap processing (31).

An exclusive role for Pif1 in fragment maturation in creating a need for Dna2 does not seem a likely reason for evolution of the properties of these two proteins. Instead, our recent results suggest that Pif1 allows the cell to properly process Okazaki fragments in regions where the template DNA forms hairpin structures (53). Tandem repeat sequences such as trinucleotide repeats and telomeres tend to form hairpin structures that hinder primer elongation. As the polymerase synthesizes through a fold-back, the displaced flap will have the complementary sequence, with a similar propensity to fold back. Fold-back flaps cannot be processed through either the short or long flap pathway because both FEN1 and Dna2 require a free 5′-end for endonuclease activity. Seo and co-workers (54) had originally proposed that Pfh1 (Pif1 in *S. cerevisiae*) is capable of removing an entire Okazaki segment in the presence of a fold-back flap. Our recent results using templates simulating Okazaki fragments with flaps containing hairpin structures showed that, contrary to our initial hypothesis, Pif1 did not open up the flap in the 5′–3′-direction to allow for RPA binding and flap processing through the long flap pathway. Instead, Pif1 bypassed the hairpin structure, completely displacing the partially synthesized Okazaki fragment. pol δ then resynthesized the complete stretch of DNA template to create a functional DNA strand (Fig. 2).

We did not distinguish whether Pif1 unwound the downstream flap substrate by binding to the base of the flap and unwinding in the 5′–3′-direction or whether it bound the template strand and moved in the 5′–3′-direction, removing the Okazaki segment from the 3′-end. The RecQ helicase family, specifically the BLM (Bloom) protein and the WRN (Werner) protein, has been implicated in lagging strand DNA maturation. These helicas unwind in the 3′–5′-direction driven by ATP. It is possible that on creation of hairpin structures that are refractory to cleavage by FEN1 and Dna2, BLM or WRN binds to the gap between the hairpin and flap base and moves to destabilize the hairpin or binds the gap in the template and displaces the fragment. Potentially, other helicases also act to resolve such secondary structures.

**Involvement of Okazaki Maturation Proteins in Long Patch Base Excision Repair**

A large majority of DNA repair within the cell is processed by the base excision repair (BER) pathway. Depending on the patch length of repair, BER can be divided into short patch BER (correction of a single nucleotide) or long patch BER (LP-BER; correction of a patch 2–12 nt in length) (32–34). Many of the mechanisms and protein components of Okazaki fragment maturation are also involved in LP-BER. pol β is the main BER polymerase, containing both synthesis and lyase functions. Excision of a damaged base by APE1 (apurinic/apyrimidinic endonuclease 1) creates a 5′-deoxyribose phosphate (dRP) residue, which is cleaved by the lyase activity of pol β, creating a nicked substrate. DNA ligase III then seals the nick, completing short patch BER. When the dRP residue is either oxidized or reduced, the lyase activity of pol β is inhibited. In this event, pol β performs strand displacement synthesis to shift the dRP residue into a 5′-flap, which is subsequently recognized and cleaved by FEN1. LigI seals the nick in LP-BER (Fig. 2) (34–37). Although Dna2 has been recently implicated in mitochondrial LP-BER, the function of Dna2 in nuclear LP-BER has not been ascertained (38). Studies using plasmid DNA containing a single base lesion have shown that the repair patch size for LP-BER varies from 6 to 12 nt. This patch length is not long enough to stably bind RPA and push the repair process to require the long flap-processing pathway. However, under specific conditions, as described below, the displaced repair patch length might be sufficient to require proteins for long flap processing.

**Regulation of the Pathways for Okazaki Fragment Maturation and LP-BER by Acetylation**

FEN1, the central component of both the short and long flap pathways, has been reported to be acetylated by p300 acetyltransferase (39, 40). Acetylation decreases its ability to cleave flap substrates by ~90% (39, 40). Haploinsufficiency of FEN1, in which the nuclease is present at 50% of the normal level, has deleterious effects in the cell (41, 42). Consequently, an intentional down-regulation of nuclease function by acetylation seems undesirable in the context of genome stability. We recently discovered that Dna2, the functional and structural interacting partner of FEN1, is also post-translationally modified by p300 (43). However, unlike FEN1, the nuclease, helicase, ATPase, and binding activities of Dna2 are all greatly stimulated by acetylation (43). Notably, we also found that in a small subset of substrates, Dna2 was able to cleave past the base of the flap, potentially eliminating the need for cleavage by FEN1. Apparently, regulation of FEN1 and Dna2 promotes displacement of more nucleotides into each flap before ligation of adjacent Okazaki fragments.

Why would the cell want to intentionally displace a greater length of flap? Negative effects include more likely formation...
of secondary hairpin structures or recombination with other complementary sequences. However, a reasonable hypothesis is that regulation by acetylation has evolved because intentional lengthening of the flap would increase the replication and repair patch lengths that are replaced. Since pol α can, on account of its lack of proofreading activity, occasionally synthesize an error-prone initiator primer, if long flaps are displaced, cleaved, and subsequently ligated, it is more likely that mismatches synthesized by pol α will be removed. As a high fidelity polymerase, pol δ would incorporate correct bases into the longer patch. Increased activity of Dna2 would ensure that as the flaps get long, they are efficiently processed to create FEN1 substrates to allow for proper maturation of the Okazaki fragment.

Significantly, a recent report on the mass spectrometric analysis of three different cell types for protein acetylation identified many replication and repair proteins, including pol δ (on the p12 subunit) and RPA (on the 70-kDa subunit) (44). Initial characterization of acetylated replication/repair proteins suggests that the modification regulates the length of the flaps, consistent with our hypothesis. PCNA was previously known to be modified by p300, improving its ability to bind to the polymerases, especially pol δ (45). Because PCNA is the processivity factor for pol δ, on acetylation, PCNA is likely to improve the ability of pol δ to displace strands. Strikingly, our preliminary analyses showed that acetylation of pol δ greatly improved its strand displacement functions on Okazaki fragment substrates. Mechanistic effects of acetylation of these proteins are all consistent with intentional replacement of longer patches in DNA replication and repair. Additionally, both BLM and WRN were shown to be acetylated using mass spectrometric analysis. Acetylation of WRN stimulates its 3′→5′-helicase activity (46). The effect of modification on BLM is currently unknown. A summary of intermediates and products expected in the presence of unmodified and acetylated proteins is depicted in Fig. 3.

With respect to repair, if LP-BER makes a longer patch, it is more likely to remove damage on several adjacent nucleotides. Significantly, BER proteins such as DNA glycosylase OGG1 (47), APE1 (48), and pol β(49) have also been found to be acetylated by p300. Acetylation of pol β selectively abrogates its dRP lyase activity (49). Inhibition of lyase activity would drive the repair into the LP-BER pathway, with the formation of longer repair patch lengths. With a shift toward more LP-BER, it is possible that RPA and Dna2 would be involved in processing these flaps, similar to the situation in Okazaki fragment maturation.

The long patch replacement hypothesis also suggests that the histone acetyltransferase p300 is enriched in regions of active chromatin not only to improve accessibility of the DNA to transcription machinery but also to promote higher fidelity DNA replication and repair to compensate for the destabilizing effects of transcription. Many of the proteins undergoing acetylation are also post-translationally modified by phosphorylation, methylation, and ubiquitination. Distinctively, the modification by acetylation seems to have the uniform effect of increasing the patch length of DNA replication/repair to improve genome stability. Additionally, other forms of modification are likely to regulate protein functions that interact with the effects of acetylation. For example, phosphorylation of FEN1 disrupts binding to PCNA (50), resulting in defects in Okazaki fragment ligation. Conversely, arginine methylation of FEN1 suppresses phosphorylation of FEN1, thereby increasing binding affinity for PCNA (51). As we begin to understand the cross-effects among different forms of modifications on replication and repair proteins, we may be able to map out a combinatorial modification code that regulates the proteins and the pathways for highest fidelity to slow the progression of cancer, neurodegenerative disorders, and aging.

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FIGURE 3. The long flap pathway promotes genome stability by increasing the fidelity of the replication process. The dark blue lines on the DNA substrate represent the primer nucleotides added by pol α, and the red stars represent mismatches on the downstream Okazaki DNA sequence.
