Replication Mechanisms

From our discussion of polymerases and accessory proteins, we should already have a reasonable understanding of how replisomes operate at the replication fork. However, for a more complete understanding of chromosomal replication, a few gaps remain to be filled. Here we will discuss how lagging strands are processed, and how chromosomal replication is initiated.

Completing the lagging strand: Processing Okazaki fragments
Lagging strands are synthesized discontinuously as a series of Okazaki fragments 1-2 kb long. Each fragment is initiated by a primer. In *E. coli*, primers are short RNAs 10-20 nt long, synthesized by DnaG protein (primase). In eukaryotes, Okazaki fragments are somewhat shorter. Primers consist of iRNA/iDNA and are about 40 nt long, made by Pol α:primase.

In *E. coli*, primers are extended by Pol III for ~1-2 kb. Extension continues until the next primer is encountered. In eukaryotes, primer extension is done by Pol δ (or ε).

How are the RNA primers removed and replaced with DNA? It turns out that the mechanisms differ between prokaryotes and eukaryotes.

Lagging strand processing in *E. coli*
The nascent lagging strand is at first a patchwork of RNA and DNA. The 5'-P ends of the RNA primers are adjacent to the 3'-OH ends of the next Okazaki fragment. In essence, they are separated by a nick (Fig. 54).

The nick is a substrate for DNA Pol I. With its 5'→3' exonuclease activity, Pol I performs a nick translation reaction, simultaneously removing the RNA primer and replacing it with DNA. The translated nick is a substrate for DNA ligase. Ligase seals the nick and completes the joining of the Okazaki fragments into an intact lagging strand.

Note that this mechanism requires a switch from Pol III to Pol I. This explains why Pol I is an essential enzyme for DNA replication, and in particular why *E. coli* strains with inactivating mutations in the 5'→3' exonuclease domain of Pol I are conditional lethals. The 5'→3' exonuclease activity is essential for processing Okazaki fragments. Other polymerases are capable of filling gaps in the absence of Pol I polymerase activity, but the exonuclease is unique and essential.
RNase H, an enzyme which specifically degrades the RNA strand of a DNA-RNA hybrid, is also present in *E. coli*. RNase H is another activity that is capable of removing the RNA primer, except for the ribonucleotide at the RNA-DNA junction. Removal of the last ribonucleotide would no doubt require a 5′→3′ exonuclease activity. It is not yet clear how important RNase H is for primer removal in *E. coli*.

**Lagging strand processing in eukaryotes**

Unlike the situation in *E. coli*, eukaryotic DNA polymerases lack an intrinsic 5′→3′ exonuclease activity, and therefore polymerase switching is not used to join Okazaki fragments. Instead, the mechanism employs multiple 5′→3′ exonuclease/endonuclease activities that (via PCNA and RPA) can associate with Pol δ or (Pol ε).

**Dna2**

This protein was initially characterized as a weak helicase, and later demonstrated to have a 5′→3′ endonuclease activity. That is, it sequentially degrades ssDNA from a 5′ end to produce short oligonucleotides. The endonuclease activity is single-strand specific. However, in the presence of ATP the enzyme will degrade duplex DNA, since the helicase activity can unwind the duplex and expose single strands. The protein is often referred to as Dna2 helicase/endonuclease. The endonuclease activity, but not the helicase activity, is essential for yeast viability. Dna2 interacts with several other proteins involved in Okazaki fragment processing, including FEN-1 and the medium-sized (middle) subunit of RPA.

**FEN-1**

FEN-1 (five' exonuclease-1, or flap endonuclease-1) is one another enzyme required for Okazaki fragment processing. FEN-1 is also sometimes called MF-1 (maturation factor-1). FEN-1 plays important roles in both DNA replication and repair. In yeast, FEN-1 null mutants are impaired in replication, but the enzyme is not required for viability. In addition, mutants show a strong mutator phenotype and are sensitive to UV light. FEN-1 (flap endonuclease) interacts with a number of proteins, including PCNA and Dna2. FEN-1 is also an essential component of the *in vitro* SV40 replication system.

FEN-1 is a 40 kDa protein that has 5′→3′ exonuclease/endonuclease activity. As an endonuclease, it specifically recognizes a 5′ single stranded flap on a branched substrate and tracks down the flap strand to the cleavage site, which is located at the junction where two strands of duplex DNA adjoin the flap (Fig. 55). The endonuclease activity is independent of flap length, and the flap can be either DNA or RNA.
FEN-1 can also act as a 5'→3' exonuclease, and will hydrolyze DNA substrates containing a gap or a nick. The exonuclease activity will also hydrolyze RNA substrates. However, a substrate that contains a 5'-triphosphorylated ribonucleotide and is completely annealed to DNA (such as an intact, non-displaced, Okazaki fragment) cannot be degraded by FEN-1. In this case, a ribonuclease or some other nuclease is needed.

Primer removal
There may be more than one mechanism used to process Okazaki fragments in eukaryotes. Two are presented here.

Mechanism 1. This mechanism employs two different nucleases, FEN-1 and RNase H1. These nucleases are required for complete SV40 DNA replication in vitro, and for reconstitution of lagging strand synthesis on certain artificial substrates.

Removal of the RNA portion of an RNA/DNA primer appears to occur via the combined action of RNase H1 (~60 kDa) and the 5'→3' exonuclease activity of FEN-1. (Note: eukaryotic cells contain at least two enzymes with RNase H activity; RNase H1 and RNase H2.) (Fig. 56)

RNase H1, acting as an endonuclease, nicks the iRNA/iDNA primer on the 5' side of the 3'-terminal primer ribonucleotide. (The intact, annealed iRNA is not a substrate for FEN-1 because it is not branched and is 5'-triphosphorylated.) The oligoribonucleotide (iRNA less the 3' terminal ribonucleotide) is now flanked by a nick on either side, and is displaced intact. In vivo, this is probably done by a helicase.

The ribonucleotide that remains is a substrate for FEN-1. FEN-1 (functioning as an exonuclease) then removes the 3' ribonucleotide of the primer. The gap is filled by DNA polymerase (probably Pol δ or Pol ε). Finally, the nick between the two DNA termini is sealed by DNA ligase I after the polymerase disengages from the template.

Mechanism 2. This mechanism employs FEN-1 and Dna2 helicase/endonuclease, which is known from biochemical and genetic experiments to interact with FEN-1. It also relies on strand displacement synthesis by Pol δ complex to peel back the RNA/DNA primer. DNA ligase I is also required.
In this model, continued extension of the upstream Okazaki fragment by Pol δ displaces the rRNA/iDNA primer, creating an ssDNA flap substrate (Fig. 57). Displacement synthesis is very slow, compared to synthesis on a single stranded template (about 1 nt/sec vs. ~50 nt/sec.)

The displaced ssDNA interacts with RPA, which blocks access by FEN-1. However, the ssDNA is a substrate for Dna2, which cleaves and shortens it so that RPA can no longer bind. (RPA requires about 30 nt for binding to ssDNA.) Recall that Dna2 interacts with RPA. This interaction could recruit Dna2 to the substrate.

Displacement synthesis eventually stops (about 12 nt past the RNA-DNA junction) and Pol δ abandons its PCNA clamp, leaving a shorter flap that is a FEN-1 substrate. FEN-1 is targeted to the flap through its interaction with PCNA.

Thus one would predict that only FEN-1 is required to produce a ligatable nick in the absence of RPA. FEN-1 would also be sufficient in the presence of RPA if the displaced flap were less than 30 nt long. However, both FEN-1 and Dna2 are required when RPA is present and flaps are 30 nt long or greater (Fig. 58).

Once FEN-1 cleaves the flap at the ssDNA-duplex DNA junction, DNA ligase I can seal the remaining nick. DNA ligase I can also interact with PCNA and at high concentrations can shorten the nick translation patch size, perhaps by displacing Pol δ from the clamp.

Thus in eukaryotes, Okazaki fragment maturation involves a process that is equivalent to nick translation because there is no net DNA synthesis. But there is no polymerase switch. Instead there is an endonuclease switch: Pol δ associates directly or indirectly with two different 5′→ 3′ nucleases to carry out this reaction. These nucleases, and DNA ligase, are probably components of the replisome as they interact with each other and/or with key pol accessory proteins (PCNA and RPA).

References:

