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## Extra Views

# How the Cell Deals with DNA Nicks

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## KEY WORDS

DNA polymerase, Okazaki fragment, idling, nuclease, nick translation, FEN1, PCNA

## ABBREVIATIONS

Pol $\delta$ , $\epsilon$	DNA polymerase $\delta$ , $\epsilon$
PCNA	proliferating cell nuclear antigen
FEN1	flap endonuclease 1
RAD27	gene for <i>S. cerevisiae</i> FEN1
MMS	methylmethane sulfonate

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## ABSTRACT

During lagging strand DNA replication, the Okazaki fragment maturation machinery is required to degrade the initiator RNA with high speed and efficiency, and to generate with great accuracy a proper DNA nick for closure by DNA ligase. Several operational parameters are important in generating and maintaining a ligatable nick. These are the strand opening capacity of the lagging strand DNA polymerase  $\delta$  (Pol  $\delta$ ), and its ability to limit strand opening to that of a few nucleotides. In the presence of the flap endonuclease FEN1, Pol  $\delta$  rapidly hands off the strand-opened product for cutting by FEN1, while in its absence, the ability of DNA polymerase  $\delta$  to switch to its 3'→5'-exonuclease domain in order to degrade back to the nick position is important in maintaining a ligatable nick. This regulatory system has a built-in redundancy so that dysfunction of one of these activities can be tolerated in the cell. However, further dysfunction leads to uncontrolled strand displacement synthesis with deleterious consequences, as is revealed by genetic studies of exonuclease-defective mutants of *S. cerevisiae* Pol  $\delta$ . These same parameters are also important for other DNA metabolic processes, such as base excision repair, that depend on Pol  $\delta$  for synthesis.

## INTRODUCTION

The simple DNA nick is often overlooked as a potential source of genome instability, yet it occurs vastly more often during a cell cycle than all other DNA changes combined. During most DNA repair processes and DNA recombination, nicks are generated as one of the initial steps in these processes, and they also represent the final step in the pathway before the integrity of double-stranded DNA is restored by ligation. During DNA replication of the lagging strand, the replicating machinery has to generate a chemically correct DNA-DNA nick at each Okazaki fragment and maintain the integrity of this nick for recognition by DNA ligase. This is a process that has to occur with extraordinary high fidelity. In human cells, tens of millions of nicks are generated each cell cycle due to the nature of lagging strand DNA replication. Any nicks left behind, or small gaps or displaced DNA flaps resulting from the failure to maintain the nick, will result into double-strand breaks during the next cell cycle. As the cell has only a limited capacity to repair double-strand breaks, this imposes a high demand upon the machinery for accurate nick maintenance. In the yeast *S. cerevisiae* with its very small genome, an estimated 100,000 Okazaki fragments are matured per cell cycle. Considering that cell lethality ensues when the number of double-strand breaks exceeds the repair capacity of the cell, errors in nick maintenance higher than ~0.02% result in lethality in a wild-type haploid yeast cell.<sup>1</sup> In this review, we discuss the synthetic and degradative forces that are active in the cell in producing a ligatable nick, and those that are active in maintaining this nick in preparation for ligation. In one pathway, a process known as idling allows the polymerase to actively preserve a ligatable nick (Fig. 1). In an alternative pathway, nick translation by the combined action of the DNA polymerase and a flap endonuclease, most commonly FEN1, allows the system to progress along the DNA until a ligatable nick has been reached. The latter process is essential in degrading the initiator RNA during lagging strand DNA replication.

## STRAND OPENING AND DISPLACEMENT SYNTHESIS

Strand opening and strand displacement synthesis by the DNA polymerase are the synthetic forces that drive both idling and nick translation. Our discussion focuses on DNA polymerase  $\delta$  (Pol  $\delta$ ), because as the lagging strand polymerase, it has been uniquely

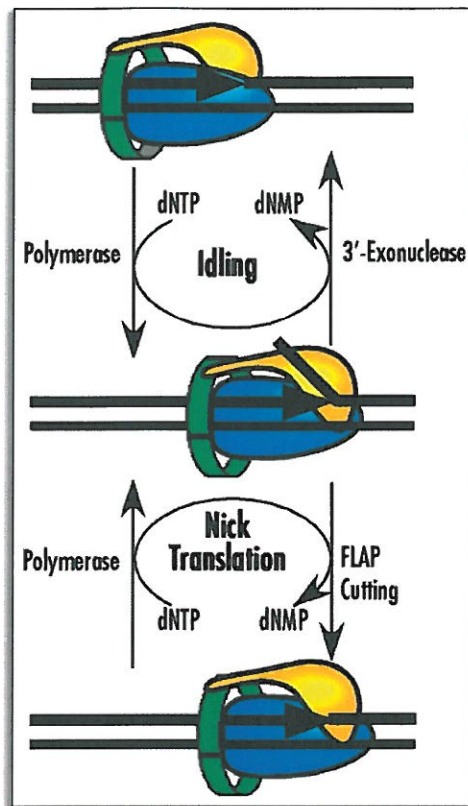


Figure 1. Degradation of flaps by idling and by nick translation. During coupled nick translation with FEN1, the flap is predominantly one nucleotide in size and the product released by FEN1 action is a mononucleotide. During idling, the flap is 2–3 nucleotides in size and products released by the 3'→5'-exonuclease are several mononucleotides.

tailored to deal with the recurring problem of initiator RNA degradation, and nick formation and maintenance. The structural parameters in the polymerase that determine its strand displacement potential remain unknown. From a structural comparison between bacteriophage T7 RNA polymerase and the Klenow fragment of *E. coli* DNA polymerase I, a helix in the fingers domain has been implicated in strand separation of downstream DS DNA.<sup>2</sup> However, the fingers domains of class B DNA polymerases to which Pol δ belongs, are completely different from those in the class A enzymes represented by the Klenow fragment, ruling out the benefit of a structural comparison.<sup>2</sup> Interestingly, in the structure of the B class RB69 replication complex, the downstream template strand exits the active site through a positively charged groove made by the N-terminal domain and the exonuclease domain.<sup>3</sup> Therefore, the exonuclease domain of the structurally analogous Pol δ may participate in regulating strand displacement synthesis.

At this point it is helpful to define two terms relating to strand displacement synthesis: strand opening and strand displacement. The characteristic displacement of the nick-proximal few nucleotides by a DNA polymerase is designated as strand opening (Fig. 2). For wild-type Pol δ with an active 3'→5'-exonuclease, strand opening is a reversible step, because the enzyme can arrive back at the nick position by activating its 3'→5'-exonuclease function. On the other hand, strand displacement is an irreversible reaction whereby ever longer DNA flaps are generated as the enzyme proceeds.

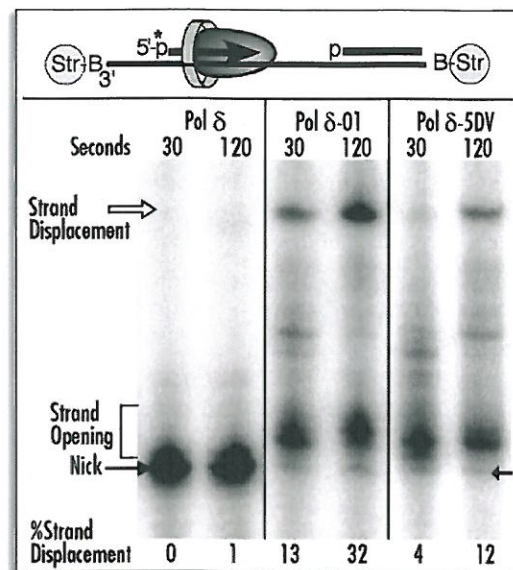


Figure 2. Strand displacement synthesis by two exonuclease-deficient forms of Pol δ. Top, schematic of the substrate. The 113-mer template contains an upstream 5'-<sup>32</sup>P-labeled 30-mer primer and a downstream 27-mer blocking oligo. The biotin-streptavidin bumpers prevent sliding of PCNA off the DNA. Replication by the PCNA-Pol δ complex was carried out as described in reference 6. The complex incorporates 25 nucleotides prior to reaching the downstream oligo position (the nick position), indicated by closed arrows. Strand opening by the polymerases, as indicated by the accumulation of paused molecules, ranges from +1 for wild-type to +4 for Pol δ-01. Full length strand displacement products are indicated by open arrows.

We have studied the catalytic properties of two exonuclease-deficient mutants of Pol δ.<sup>4,6</sup> Based on our extensive knowledge of 3'→5'-exonuclease domains, mutants Pol3-01 (DE321,323AA) and Pol3-5DV (D520V) are both likely deficient for binding a divalent metal ion essential for catalysis.<sup>7</sup> Normal DNA synthesis, i.e., DNA replication of a single-stranded DNA template, by these enzymes is the same as wild-type. However, the two mutant enzymes show very different properties when they encounter downstream double-stranded DNA.

An examination of a DNA replication reaction shows that all three polymerases paused at or near the nick site (Fig. 2). Pausing by the wild-type enzyme was at the precise nick position and 1 nt into the double-stranded DNA. However, Pol δ-5DV paused at positions 2–3 nt into the double-stranded DNA, and Pol δ-01 at positions 3–4 nt into the double-stranded region. Our studies show that in actuality, strand opening by the wild-type enzyme also proceeds to the extent of about 2–3 nucleotides, however, the back reaction catalyzed by the exonuclease predominates, giving the appearance of the enzyme actually pausing at the nick position.

In contrast to the wild-type enzyme, strand opening by the exonuclease-deficient enzymes is irreversible. Significantly, the strand-opened complexes are relatively long lived, indicating that the enzyme actively inhibits further DNA synthesis when just a few nucleotides have been displaced.<sup>6</sup> However, even at the earliest time-point, evidence of complete strand displacement synthesis can be found (Fig. 2). Pol δ-01 is much more prone to strand displacement synthesis than Pol δ-5DV, indicating a participating of structural determinants in the exonuclease domain in limiting excess strand opening. Eventually, upon prolonged incubation, even wild-type Pol δ



will proceed into a strand displacement mode. Interestingly, once the enzyme has been committed to extensive strand displacement synthesis, the rate of strand displacement by wild-type Pol  $\delta$  is comparable to that by an exonuclease-deficient enzyme, suggesting that backup by the exonuclease no longer occurs.<sup>5</sup>

It is not clear how the enzyme senses that a limited number of nucleotides of the downstream DNA, or RNA, have been displaced. One easy explanation would be that the enzyme somehow measures the length of the displaced flap. However, this easily testable hypothesis turns out to be incorrect. When a noncomplimentary 5'-flap of 10 nt was added to the downstream DNA, the exonuclease-defective Pol  $\delta$ -5DV still showed a strong pause site with about 3 nt of double-stranded DNA opened, thereby generating a 13 nt flap.<sup>5</sup> Therefore, it is the number of base-pairs opened rather than the length of the flap that signals to the polymerase to halt further strand opening.

## IDLING AND NICK TRANSLATION

The fate of the strand-opened products depends on the presence of the flap endonuclease FEN1. FEN1 functions primarily during Okazaki fragment maturation where it degrades the initiator RNA.<sup>8</sup> However, FEN1 also functions in long patch base-excision repair. *RAD27* (*FEN1*) deletion mutants are viable, but double mutants which also delete a related nuclease *EXO1* are not, suggesting that Exo1 can substitute, albeit less efficiently, for FEN1 during nick translation.<sup>9,10</sup> However, this assumption still requires direct experimental verification.

With FEN1 present in the replication complex, nick translation predominates, whereas in its absence, the systems idles at the nick (Fig. 1). Idling involves recurring strand opening by the polymerase domain followed by degradation by the exonuclease domain of Pol  $\delta$ , resulting in a net turnover of dNTPs into dNMPs without net DNA synthesis.<sup>6</sup> Turnover of dNTPs into dNMP by Pol  $\delta$  is specifically activated by the presence of a nick. The enzyme rapidly goes through a cycle of synthesis followed by degradation once every 1–2 seconds. All of this is accomplished while the enzyme stays bound to the DNA as a PCNA-Pol  $\delta$  complex. After limited strand opening by the polymerase domain, the nascent DNA primer switches to the exonuclease domain followed by limited degradation until the nick position has been reached again. Further degradation is inhibited and, therefore, gaps are not produced while the enzyme is idling.

Idling is specifically carried out by Pol  $\delta$ . Despite the presence of a strong 3'→5'-exonuclease activity in Pol  $\epsilon$ , this enzyme does not idle at the nick.<sup>6,11</sup> It is the putative leading strand DNA polymerase, and as such usually does not encounter downstream double-stranded DNA. Therefore, it appears that the potent idling capacity of Pol  $\delta$  has been evolved in order for the enzyme to deal with the recurring problem of nicks on the lagging strand.

In the presence of FEN1, idling by Pol  $\delta$  is inhibited and the PCNA-FEN1-Pol  $\delta$  complex sets off into an efficient nick-translation reaction. The coupling between the strand opening reaction by Pol  $\delta$  and the flap cutting reaction by FEN1 is so efficient, that over 90% of the products released by FEN1 are mononucleotides. In other words, when FEN1 is present in the replication complex, the flap generated by only one nucleotide of strand opening is with high efficiency handed off to FEN1 to be cut. In agreement with this tight coupling are studies which show that both FEN1 and Pol  $\delta$  participate in a stable processive complex with PCNA.<sup>12</sup>

From these considerations, it is apparent that there are several operational parameters that are important in generating and maintaining

a ligatable nick. These are the strand opening capacity of Pol  $\delta$  and its ability to limit strand opening to that of a few nucleotides. In the presence of the flap endonuclease FEN1, the ability of Pol  $\delta$  to rapidly hand off the strand-opened product for cutting by FEN1 is crucial for controlled nick translation. While in its absence, the ability of Pol  $\delta$  to switch to its 3'→5'-exonuclease domain in order to degrade back to the nick position is important in maintaining a ligatable nick. This regulatory system has a built-in redundancy so that dysfunction of one of these activities can be tolerated in the cell. However, further dysfunction leads to uncontrolled strand displacement synthesis with deleterious consequences in vivo, as is exemplified by studies with the exonuclease-defective mutants of Pol  $\delta$ .

The coupling between the polymerase and FEN1 is disturbed in these exonuclease-defective mutants. Although the majority of excision products are still mononucleotides, longer excision products are formed with these Pol  $\delta$  mutants, particularly with Pol  $\delta$ -01 which has a much stronger strand displacement capacity than Pol  $\delta$ -5DV (Fig. 2). The formation of longer flaps increases the likelihood that they no longer form substrates for FEN1 in vivo, or for Exo1 in a *RAD27* (*FEN1*) mutant strain.<sup>13</sup> Indeed, this is observed in the cell, where we found that *pol3-01* is not only synthetic lethal with *rad27-Δ*, but also with a mild mutation in FEN1 (*rad27-p*) which decreases its affinity for PCNA.<sup>4</sup> On the other hand *pol3-5DV* is only synthetic lethal with *rad27-Δ*, although the viable *pol3-5DV rad27-p* double mutant accumulates duplication mutations indicative of defects in Okazaki fragment maturation.

Finally, the balance between synthesis and degradation in order to maintain a ligatable nick is not just important for lagging strand DNA replication, but also for other pathways that use Pol  $\delta$  and FEN1, such as in long patch base excision repair.<sup>14</sup> Whereas each of the single mutants of *pol3-5DV* or *rad27-p* is not sensitive to methylmethane sulfonate (MMS), the *pol3-5DV rad27-p* double mutant is exquisitely sensitive to MMS treatment. Simple gap filling as a final step in long patch base excision repair requires a proper functional interaction between FEN1 and Pol  $\delta$ , and in a *pol3-exo- rad27-p* double mutant both degradative mechanisms that provide a ligatable nick would be crippled.

## DNA LIGATION

As during lagging strand DNA replication it appears that the nick translation machinery is keeping a tight grip on this discontinuity to allow DNA ligase to seal the nick as soon as the initiator RNA is degraded, one would expect DNA ligase to function as an integral member of the nick translation complex. In mammalian cells there is evidence for the interaction of DNA ligase I with other replication proteins and the importance of these interactions for proper DNA metabolism. The interaction between PCNA and DNA ligase I is not only critical for the subnuclear targeting of the ligase, but also for proper ligation during lagging strand DNA replication.<sup>15,16</sup>

On the other hand, in both yeasts, mutations of the PCNA-binding domain in DNA ligase I result in only small defects in DNA damage tolerance.<sup>17,18</sup> Even more surprisingly, a *S. cerevisiae* ligase deletion mutant is complemented by the Chlorella virus ligase, the smallest eukaryotic ATP-dependent ligase that has been characterized.<sup>17</sup> This 30 kDa catalytic core, which lacks the large N- and C-terminal domains typical for cellular ligases, not only fully sustains wild-type mitotic growth of a *S. cerevisiae* *CDC9* (ligase I) deletion, it also partially complements the nonhomologous end joining defect of a *LIG4* deletion.<sup>19</sup> Furthermore, the sensitivity to DNA damaging

agents is only slightly increased in strains expressing *Chlorella* ligase as the only source of ligase.<sup>17</sup> Consistent with these genetic studies, biochemical studies have failed to show that DNA ligase I functions as an integral component of the Okazaki fragment maturation complex.<sup>12</sup>

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