Okazaki Fragment Metabolism

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Cellular DNA replication requires efficient copying of the double-stranded chromosomal DNA. The leading strand is elongated continuously in the direction of fork opening, whereas the lagging strand is made discontinuously in the opposite direction. The lagging strand needs to be processed to form a functional DNA segment. Genetic analyses and reconstitution experiments identified proteins and multiple pathways responsible for maturation of the lagging strand. In both prokaryotes and eukaryotes the lagging-strand fragments are initiated by RNA primers, which are removed by a joining mechanism involving strand displacement of the primer into a flap, flap removal, and then ligation. Although the prokaryotic fragments are ~1200 nucleotides long, the eukaryotic fragments are much shorter, with lengths determined by nucleosome periodicity. The prokaryotic joining mechanism is simple and efficient. The eukaryotic maturation mechanism involves many enzymes, possibly three pathways, and regulation that can shift from high efficiency to high fidelity.

Replication of cellular chromosomal DNA is initiated by the multienzyme replisome machinery, which unwinds the DNA helix to create a replication fork. The antiparallel structure of double-helical DNA and the 3' end extension specificity of all DNA polymerases confine the mechanisms that can be used by the cell for DNA duplication. One copied strand, called leading, can conveniently be extended in a continuous manner in the same direction that the helix must open to allow exposure of templates for polymerization. The other, or lagging strand, must be periodically extended away from the opening helix. This can only be accomplished

if the strand is made discontinuously (Kornberg and Baker 1992). The strand is synthesized in short segments, named Okazaki fragments, after their discoverer (Sakabe and Okazaki 1966; Okazaki et al. 1968) and the segments are then joined. This requirement has two fundamental consequences: (1) The lagging strand must have evolved priming and fragment joining mechanisms involving many additional steps and reactions than needed for leading-strand extension. (2) Mechanisms of lagging-strand replication must have developed means of avoiding mutagenesis while handling the necessary strand manipulations.

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PARALLELS BETWEEN PROKARYOTIC AND EUKARYOTIC REPLICATION

Although replication of eukaryotic DNA on a chromatinized DNA template is a relatively more complex process than replication in bacteria, involving more types of proteins and reactions, the fundamental processes of DNA duplication have striking parallels in all cells. The replisome machineries of both organisms are minimally composed of helicases, which unwind the duplex strands, primase, which initiates synthesis and DNA polymerases, which duplicate the parental strands of the DNA. In bacteria, DNA replication proceeds within a fork, wherein the lagging strand loops into a “trombonelike” structure allowing for the replication enzymes to be continually recycled on the DNA for repeated synthesis and joining (Alberts et al. 1982). Presumably, use of a similar mech-

anism in eukaryotes allows coordination of synthesis between the leading and lagging strands (Fig. 1) (Pandey et al. 2009). Because DNA polymerases cannot incorporate dNTPs without a primer terminated by a 3' hydroxyl, the leading strand and each Okazaki fragment are primed by RNA to initiate synthesis (Hubscher et al. 2002). On the lagging strand the primer is extended by the addition of dNMPs to form short segments of DNA. These segments need to be further processed to form a fully functional strand of DNA. Specifically, the RNA primers have to be excised from the fragments. Removal of RNA primers is performed partly by a ribonuclease H (RNase H). There are two distinct classes of RNase H enzymes in bacterial and eukaryotic systems (type 1: RNase HI [*Escherichia coli*] and RNase H1 [eukaryotes] and type 2: RNase HII [*E. coli*] and RNase H2 [eukaryotes]) (Cerritelli and Crouch 2009). Although

Figure 1. Eukaryotic replisome. A DNA helicase initially unwinds the duplex DNA (red and blue strands) to separate the DNA and form a replication fork. The single-stranded DNA (ssDNA) is coated by the single-strand binding protein, replication protein A (RPA). On the leading strand, replication factor C (RFC) loads proliferating cell nuclear antigen (PCNA) and DNA polymerase ε to continuously synthesize the leading strand. The lagging strand is initially primed by DNA polymerase α (Pol α), which synthesizes a short RNA/DNA initiator primer (orange strand). RFC then displaces Pol α from the lagging strand to initiate the switch from the priming mode to the elongation mode. The initiator primer is extended by PCNA/DNA polymerase δ complex to form short segments of DNA known as Okazaki fragments.

type 1 RNases H require a minimum of four ribonucleotides for hydrolysis, type 2 RNases H can recognize a single ribonucleotide (Cerritelli and Crouch 2009). In eukaryotes, the initiator RNA primers are removed, apparently by two partly redundant processes (Kao and Bambara 2003). The first is the action of cellular RNase H2, which can begin degrading the primer as soon as it is made, but presumably not so soon that it interferes with initiation of DNA synthesis (Turchi et al. 1994; Murante et al. 1998). RNase H2 degrades between ribonucleotides of an RNA strand annealed to DNA. It cannot cleave between the 3'-most ribonucleotide and the initial deoxynucleotide. A second nuclease is needed for this. The second nuclease operates after strand displacement synthesis resulting from extension of the 3' end of the adjacent fragment. This synthesis raises what remains of the RNA primer into a single-stranded flap structure that is removed by endonuclease action. The resulting nick is then ligated to make a continuous strand. The absence of RNase HI in *E. coli* leads to initiation of replication at sites other than the replication initiation site *oriC* (Hong and Kogoma 1993). Genetic deletions of the RNase H enzymes in *Saccharomyces cerevisiae* did not yield a distinct phenotype, leading to the suggestion that RNase H is not the primary pathway for RNA removal in those cells (Frank et al. 1998; Qiu et al. 1999). Fundamental differences among organisms include structural variations in the proteins involved, and length variations in the fragments. There are also as many as three pathways in eukaryotes, which involve different but overlapping sets of proteins (Balakrishnan and Bambara 2011b). Most recently, there is evidence that flow through these pathways is regulated to optimize fidelity and rate of synthesis (Balakrishnan and Bambara 2011b).

KORNBERG SIXTH COMMANDMENT: "DEPEND ON VIRUSES TO OPEN WINDOWS"

Most of our initial understanding of the process of DNA replication was obtained from studies in vitro using cellular extracts to replicate Simian Virus 40 (SV40) DNA, a short double-stranded

circle (Waga et al. 1994; Bambara et al. 1997; Waga and Stillman 1998). Because SV40 encodes only the minimal proteins required for a viral assembly, it utilizes the cellular machinery from the host cell for replication (Tooze 1981). Studies by Stillman and coworkers (Waga et al. 1994; Waga and Stillman 1998) using SV40 as a model system showed that T antigen (encoded by the early viral genes), along with host single-stranded DNA-binding protein, called replication protein A (RPA), and topoisomerases, initiates DNA replication by binding to the origin (*ori*) and unwinding the duplex DNA (Tsurimoto et al. 1990). Reconstitution of SV40 DNA replication using purified proteins helped in the identification of specific enzymatic mechanisms used by the eukaryotic replication fork (Dean et al. 1992; Eki et al. 1992; Ishimi et al. 1992). Individual steps of this process and the enzymes involved are described in the sections below. Although the SV40 replication system in vitro provided valuable insights into eukaryotic replication, the absence of Pol ϵ as the leading-strand polymerase and the use of viral T antigen as the helicase for SV40 replication made it difficult to consider it as an absolutely analogous system to the actual more complex mammalian eukaryotic system.

PRIMING THE LAGGING STRAND—THE INITIAL STEP

Because DNA polymerases cannot initiate synthesis *de novo*, the replicative polymerase has evolved a partnership with primase to enable synthesis on a DNA template. DnaG acts as the primase in bacteria and in eukaryotes the primase is part of DNA polymerase α (Pol α) (Hubscher et al. 2002; Kuchta and Stengel 2010). Pol α is a four-subunit complex, comprised of the polymerase subunit (Pol I, p140), a regulatory subunit (Pol 12, p79), and two primase subunits. The Pri1 subunit (p48) acts as the fundamental initiator of replication by catalyzing the formation of the short RNA primers, which are subsequently elongated by Pol I (Hubscher et al. 2002). The second primase subunit, Pri2 (p58) acts as a scaffold, which holds together the primase and the polymerase subunits.

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Primases frequently associate with helicases, greatly improving their affinities for ssDNA and increasing the number of primer initiation sites (Kuchta and Stengel 2010). Priming does not occur randomly and, with the exception of archaeal and *Aquifex aeolicus* primase, priming is generally initiated at pyrimidine residues because the eukaryotic primase binds more tightly to pyrimidine-rich than to purine-rich DNA (Holmes et al. 1985). The RNA/DNA primer synthesized by the primase/polymerase is known as the “initiator primer.” Typically, during eukaryotic replication, primase synthesizes an RNA segment known as “initiator RNA (iRNA),” which is ~8–10 nt in length. A shorter primer may increase the possibility of slippage in the polymerase active site causing mutagenesis (Zhang and Grosse 1990; Kornberg and Baker 1992). Concentration of nucleotide triphosphates (NTPs) and deoxynucleotide triphosphates (dNTPs) in the cell can also account for varying primer lengths (Hauschka 1973). Priming of the DNA is the rate-limiting step in lagging-strand replication, with the rate of NTP polymerization by primase being at least two orders of magnitude slower than the rate of dNTP polymerization by Pol α (Sheaff and Kuchta 1993).

TRANSFER FROM PRIMASE TO DNA POLYMERASE

The ability of the primase to count the number of NTPs incorporated allows for the switch from the primase subunit to elongation by the DNA polymerizing part of Pol α (Qimron et al. 2006). Although the T7 primase synthesizes only a 4-nt-long primer, eukaryotic primases typically synthesize primers longer than 7 nt to enable efficient hand off of the primer-template from the primase to DNA Pol α (Sheaff et al. 1994). This is performed by the p58 subunit, which acts as the point of contact between the primase and the polymerase (Copeland and Wang 1993). The *E. coli* primase also switches the primer-template to Pol III directly but requires the assembly of the β clamp and the ssDNA-binding protein (SSB) (Yuzhakov et al. 1999). Because it lacks 3′–5′ exonuclease proof-reading activity, Pol α is considered to be an

error-prone polymerase. Pol α elongates the initiator RNA primer by the addition of 20–22 nt of “initiator DNA” (iDNA).

THE LEADING-STRAND REPLICATIVE POLYMERASE

Although studies using SV40 identified DNA polymerase δ (Pol δ) as the polymerase responsible for replicating both the leading and lagging strands, much recent evidence from the Kunkel laboratory has definitively shown DNA polymerase ϵ (Pol ϵ) to be the polymerase involved in leading-strand replication (Pursell et al. 2007). On account of its high processivity and association with PCNA, Pol ϵ can continually synthesize the leading strand (Waga and Stillman 1998). The lagging-strand polymerase, Pol δ , is made up of three subunits in *S. cerevisiae* (Pol 3, Pol 31, and Pol 32), and with the addition of a fourth subunit (Cdm1) in *Schizosaccharomyces pombe* and humans (Garg and Burgers 2005a). This fourth subunit acts to stabilize the polymerase holoenzyme (Podust et al. 2002). In addition to its polymerase function, Pol δ also possesses 3′–5′ exonuclease activity, allowing it to be a higher fidelity polymerase than Pol α (Pavlov et al. 2006).

THE MINIMAL ENZYMES INVOLVED IN BASIC OKAZAKI FRAGMENT PROCESSING

Helicases initially unwind the double-stranded DNA at specific sequences on the genome known as origins. In eukaryotes the replication protein A (RPA) coats the ssDNA to prevent it from reannealing, degradation by nucleases, recombination with other cellular ssDNA, or formation of hairpin structures that would normally obstruct replication fork progression (Wold 1997). RPA is a heterotrimeric protein composed of three subunits (70, 32, and 14 kDa). RPA also coordinates the assembly and disassembly of replication-associated proteins. The ATP-dependent replication factor C (RFC) binds to Pol α and triggers the switch from the priming mode to the elongation mode, the making of iDNA (Tsurimoto and Stillman 1990). RFC loads the proliferating cell nuclear antigen (PCNA)

along with Pol δ to initiate the elongation on the lagging-strand DNA template (Tsurimoto and Stillman 1990). PCNA, a functional homolog of the prokaryotic β clamp, having a similar ring-shaped structure, is opened up with the help of RFC to be loaded onto the double-stranded iDNA (Burgers 2009). PCNA binds to the back of the Pol δ and acts as a sliding clamp, increasing the processivity of the polymerase. Pol δ adds ~ 100 nt of DNA in humans and ~ 250 nt of DNA in *S. cerevisiae* to form short Okazaki fragments, which need to be further matured to form a functional strand of DNA. During a single round of nuclear DNA replication in *S. cerevisiae* $\sim 100,000$ Okazaki fragments are made and matured (Garg and Burgers 2005b).

SIGNIFICANCE OF FRAGMENT SIZE

Despite the much larger DNA content of eukaryotic compared with prokaryotic cells, Okazaki fragments are ~ 1200 nt long in bacteria but only about 200 nt long in eukaryotes (Ogawa and Okazaki 1980). This means that to prepare for every human cell division, > 10 million fragments must be made and joined. The bacterial fragments might be larger because the ligation process is much slower than the time needed to synthesize each fragment. Pol III holoenzyme elongates primers at 1200 nt/sec. This is consistent with the ability of bacteria to double in the range of a half-hour. Possibly the joining of the lagging strand could not keep up this pace if the fragments were shorter. Another possibility is that the nucleosomal structure of DNA influences the frequency of fragment priming. Is it coincidence that the average fragment size is similar to the length of DNA associated with a mononucleosome? Recent evidence using high-resolution analysis has shown that Okazaki fragments are sized according to chromatin repeats (Smith and Whitehouse 2012).

PATHWAYS OF EUKARYOTIC OKAZAKI FRAGMENT PROCESSING

Short Flap Pathway

Because eukaryotic lagging-strand DNA is primed at short intervals, Pol δ frequently en-

counters the downstream primed Okazaki fragment and displaces the RNA/DNA initiator primer into a 5' flap structure. This strand displacing activity is very similar to that reported for bacterial Pol I. However, unlike Pol I, Pol δ does not possess a nuclease activity to cleave the displaced flap. Flap endonuclease 1 (FEN1; or scRad27), a structure-specific 5'-3' endonuclease, recognizes the displaced 5' flap and cleaves at the base creating a nicked substrate for ligation (Bambara et al. 1997; Lieber 1997). This is the predominant method of removing the Pol α -synthesized initiator primer during the maturation process. Initial studies characterizing the mechanism of FEN1 suggested a "tracking" model for FEN1 in which the nuclease moves from the 5' end of the flap to its base where it performs a specific cleavage (Bambara et al. 1997). This mechanism was proposed because studies in vitro showed that endonucleolytic cleavage by FEN1 is inhibited when the 5' end of the flap is blocked either with a complementary primer or a biotin-conjugated streptavidin moiety (Murante et al. 1995). However, recent work has shown that FEN1 initially binds to the base of the flap causing a change in the substrate conformation that orients FEN1 in a manner that allows for precise cleavage (Gloor et al. 2010; Tsutakawa et al. 2011). The free 5' end of the flap is then threaded past or through the helical arch and active site of FEN1 permitting a single cleavage event (Gloor et al. 2010).

DNA ligase I (cdc9 in *S. cerevisiae*) seals the nick generated by FEN1 to create a fully functional continuous double-stranded DNA (Bambara et al. 1997). PCNA interacts with both FEN1 and DNA ligase I and stimulates the enzymatic functions of both these proteins (Rossi et al. 2006). Interactions with PCNA are critical for the creation and proper ligation of the lagging-strand DNA. It has been previously suggested that PCNA serves to recruit the core enzymes to the replication fork and functions to sequentially hand off the proteins to perform their enzymatic tasks during the maturation process (Kao and Bambara 2003). Sequential strand displacement and cleavage by Pol δ and FEN1, respectively, helps to remove the entire

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initiator RNA before nick ligation. The wild-type Pol δ shows reduced strand displacement activity compared with its exonuclease mutants (Pol δ -5DV and Pol δ -01) (Garg and Burgers 2005b). Apparently the exonuclease regulates displacement (Garg and Burgers 2005b). It is still not very clear how the wild-type enzyme senses how many nucleotides of the RNA/DNA primer it has displaced. However, analysis of lagging-strand replication using purified *S. cerevisiae* proteins has shown that the majority of the substrates Pol δ exonuclease activity and FEN1 cleavage functions cooperate to create and cleave flaps shorter than 10 nt long (Ayyagari et al. 2003; Rossi and Bambara 2006). Several displacement and cleavage reactions are required to remove the initiator primer. A flap created and processed via this mechanism has matured by the “short flap pathway” (Fig. 2).

Long Flap Pathway

In some instances FEN1 transiently disengages from the replication complex. This can cause enough of a delay in cleavage that flaps displaced by Pol δ become long. When they reach lengths >22 nt, RPA can bind stably (Rossi and Bambara 2006). RPA-bound flaps are refractory to FEN1 cleavage, requiring the action of another nuclease for proper processing (Bae et al. 2001). Budd and Campbell identified this alternate nuclease, Dna2, in a genetic screen in *S. cerevisiae* (Budd and Campbell 1995). Dna2 nuclease/helicase is a multifunctional protein containing 5′–3′ endonuclease and minor 3′–5′ exonuclease activities, plus 5′–3′ helicase and ATPase functions (Kang et al. 2010). Campbell and colleagues showed that overexpression of Dna2 compensated for defects in FEN1 and overexpression of FEN1 did the same for Dna2 (Budd and Campbell 1997). More recent work showed that the double mutant of 5′–3′ nuclease-defective Dna2 (*dna2-1*) and 3′ nuclease-deficient Pol δ (*pol3-01*), which has augmented strand displacement activity, is lethal (Budd et al. 2005). These results imply that Dna2 works with FEN1 specifically to process long flaps. In addition, Dna2 was recently found to be complexed with FEN1 in human cell extracts,

suggesting it as a physical and functional partner of FEN1 (Balakrishnan et al. 2010).

S. cerevisiae Dna2 can dissociate the RPA from a long flap (Bae et al. 2001; Stewart et al. 2008). Using a mechanism very similar to that of FEN1, Dna2 binds the flap base, and then threads the free 5′ end of the flap (Stewart et al. 2010). However, this nuclease cleaves periodically up to a terminal product flap ~ 5 –6 nt in length. This is too short to bind RPA, so the short flap is readily available for cleavage by FEN1 and subsequent ligation. This method of flap processing is known as the “long flap pathway” (Fig. 2). Reconstitution experiments have shown that although the majority of the displaced flaps are processed by the short flap pathway, a minority require the long flap pathway. Seo and colleagues originally proposed the Dna2 pathway as the primary means of Okazaki fragment processing (Bae et al. 2001). Their proposal was likely influenced by genetic evidence in *S. cerevisiae* that Dna2 inactivation in cells was lethal, whereas FEN1 mutants only showed a slow-growing phenotype. However, these observations can be misleading. Exo1, a 5′–3′ exonuclease interacts with both FEN1 and Dna2 and can specifically act as a backup for FEN1 nuclease activity. Although this is an inefficient process, and probably not biologically relevant, it explains why *rad27 Δ* (FEN1) mutants are viable. Also, recent work from the Campbell laboratory showed that Dna2 interacts with Rad9, the damage checkpoint activator, participating in the double-strand break repair response. The double deletion *dna2 Δ* and *rad9 Δ* rescued *dna2 Δ* lethality suggesting that Rad9-dependent activation of the checkpoint contributed to the lethality in *dna2 Δ* cells (Budd et al. 2011). In the absence of Dna2 and Rad9, the damage response utilizes the Exo1 pathway for repairing the damaged DNA (Balakrishnan and Bambara 2011a).

EVOLVED INTERACTIONS OF FEN1, Dna2, AND Pif1

Why did both FEN1 and Dna2 develop a mechanism in which the nucleases bind to the base of the flap and thread the free 5′ end through their

Figure 2. Okazaki fragment maturation. During Okazaki fragment maturation, (i) Pol δ displaces a short segment of the initiator primer into a 5' flap; (ii) FEN1 recognizes the displaced flap, binds to the base of the flap and cleaves the flap; (iii) DNA ligase seals the nick; (iv) certain flaps are elongated by the action of the 5'–3' helicase, Pif1; (v) the long flaps are stably coated by RPA; and (vi) Dna2 displaces RPA and cleaves the flap at multiple sites leaving a terminal product ~5–6 nt in length.

active site? A reasonable explanation is that the requirement to enter of a free 5' end of a flap prevents these very active endonucleases from cleaving the single-stranded templates between Okazaki fragments, resulting in dangerous double-stranded breaks in the chromosome. Why should this minor long flap pathway have evolved? Reconstitution experiments showed that if flaps were being displaced in the presence of FEN1 and RPA, then FEN1 was able to overcome the inhibition by RPA (Rossi and Bambara 2006). This was likely owing to FEN1 binding the base of the flap and orienting itself in a position allowing for cleavage, before RPA binding could be inhibitory. This observation appeared to remove the need for Dna2 in the processing pathway. Hence, why did this protein evolve to interact with the replication proteins?

The answer to this question was provided by genetic studies in *S. pombe* wherein Seo and colleagues showed that Pfh1 (a homolog of

S. cerevisiae Pif1) enhanced the strand displacement capabilities of Pol δ . Pif1 was capable of binding ahead of Pol δ to enhance flap creation in the downstream Okazaki fragment creating a longer 5' flap substrate that would attract RPA binding. Importantly, in *S. cerevisiae*, deletion of the *PIF1* gene rescued the lethality of the *dna2 Δ* strains (Budd et al. 2006). This result indicates that expression of Pif1 creates a need for Dna2. A reasonable interpretation is that Pif1 makes Dna2 necessary for long flap processing. In fact, inclusion of Pif1 in reconstitution assays augmented the amount of long flaps, and resulted in inhibition of FEN1 by RPA. Because Pif1 complicates fragment processing, why has it evolved to interact with the lagging-strand synthesis machinery? An answer was also suggested by Seo and colleagues (Ryu et al. 2004). Pif1 may fully displace some fragments. Additional reconstitution experiments suggest that fragments with sequences having

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the potential to form 5' end region secondary structure are difficult to process. When the flap is created, it folds in a way that prevents cleavage by either FEN1 or Dna2. However, Pif1 can bind between the structure and the flap base and fully displace the fragment. The upstream fragment can then extend through the structured region. This is effectively a third pathway of fragment processing (Pike et al. 2010). Although the long flap pathway has evolved elegantly to process such flaps, evidence in vitro suggests that the short flap pathway is more commonly used, with each protein from the long flap pathway (Dna2, Pif1, and RPA) stimulating the function of FEN1, and so promoting the use of the short flap pathway (Henry et al. 2010).

MECHANISTIC SIMILARITIES OF OKAZAKI FRAGMENT PROCESSING AND LONG PATCH BASE EXCISION REPAIR

Cells are constantly being exposed to endogenous and exogenous stresses that cause oxidative damage to DNA bases. Base excision repair (BER) is the most commonly utilized means of dealing with these damaged bases. It proceeds via two pathways. In the short patch pathway (SP-BER) a damaged base is recognized by a DNA glycosylase and removed. The abasic site is cleaved by apurinic/apyrimidinic endonuclease 1 (APE1). The one-nucleotide gap is filled by DNA polymerase β (Pol β), which also removes the 5' deoxyribose phosphate (dRP) using an intrinsic lyase activity. However, if the dRP is oxidized, reduced, or otherwise altered, the lyase function does not work. The Pol β then displaces the damaged site into a 2–12 nt 5' flap (Balakrishnan et al. 2009). These flaps are directed down the long patch pathway (LP-BER) in which they are cleaved by FEN1 and sealed by DNA ligase I. It is not clear whether the longest flaps would bind RPA with sufficient avidity to require Dna2; however, the recent report of Dna2 involvement in mitochondrial LP-BER suggests that some do (Zheng et al. 2008). The overlap of proteins used for Okazaki fragment processing and LP-BER suggests that the two processes evolved from the same ancestral basic pathway. The overlap also suggests that regula-

tory mechanisms for one process will similarly influence the other.

POSTTRANSLATIONAL REGULATION OF REPLICATION PROTEINS

Most of the proteins involved in eukaryotic DNA replication experience various posttranslational modifications, regulating their enzymatic functions, subcellular localizations, or participation in a specific pathway. Pol α /primase has been reported to be phosphorylated both in humans and *S. cerevisiae*. Phosphorylation of Pol α occurs late in S phase, thereby possibly coordinating the S phase with the mitotic phase. Results obtained in vitro suggest that although the polymerase activity of Pol α is not altered by phosphorylation, the primase function is slightly stimulated without affecting the length of the initiator primer (Waga and Stillman 1998). Recent evidence from the Lee laboratory has shown that phosphorylation of Pol δ on the p68 subunit decreases the binding affinity of the polymerase to PCNA (Rahmeh et al. 2012). This in turn decreases the processivity of polymerization. Pol δ was also found to be acetylated on the catalytic subunit in a mass spectrometric analysis (Choudhary et al. 2009). PCNA is modified by a diverse range of modifications such as acetylation, phosphorylation, and ubiquitination. Although acetylation improves the binding affinity to PCNA to DNA polymerases (Naryzhny and Lee 2004), tyrosine phosphorylation of PCNA controls the protein stability (Wang et al. 2006). Ubiquitination and sumoylation alter the pathways in which PCNA functions (Papouli et al. 2005). FEN1 is posttranslationally modified by phosphorylation, methylation, and acetylation. Phosphorylation of FEN1 decreases its binding affinity to PCNA, whereas methylation prevents phosphorylation of FEN1 (Zheng and Shen 2011). Acetylation greatly diminishes the cleavage function of FEN1 (Hasan et al. 2001). Interestingly, a recent report shows that phosphorylation of FEN1 stimulates its sumoylation. This subsequently helps in the ubiquitination of the protein leading to degradation via the proteasome pathway, thereby regulating the levels of FEN1 in the cell

(Guo et al. 2012). Dna2 is also phosphorylated and acetylated. Phosphorylation of Dna2 improves recruitment to sites of double-strand breaks (DSBs) (Chen et al. 2011), whereas acetylation of Dna2 greatly alters its enzymatic activities (Balakrishnan et al. 2010). RPA is also modified by phosphorylation (on the 70 and 32 kDa subunits) in response to DSBs, which in turn allows the RPA to help in the recruitment of DSB response proteins (Wold 1997). Finally, RPA was found to be acetylated on the 70 kDa subunit (Choudhary et al. 2009).

REGULATION OF PATHWAYS BY ACETYLATION

Although most posttranslational modifications alter the function of individual proteins, recent evidence of the regulation of proteins by acetylation suggests a coherent hypothesis in which acetylation of replication proteins regulates the choice of a specific pathway for Okazaki fragment maturation. It has been known for some time that FEN1 can be acetylated by the histone acetyltransferase p300 (Hasan et al. 2001). Complete acetylation reduces the cleavage activity by about 90%. The reason why the cell would want to down-regulate FEN1 activity to that degree was initially unclear, because when expression of FEN1 was knocked out on one of the two chromosomes in diploid cells, the 50% reduction in cleavage activity resulted in genomic damage (Kucherlapati et al. 2002). We now know that this phenomenon makes more sense when viewed in the context of regulation of most lagging-strand replication proteins by acetylation. Suggestions of a more global regulation mechanism came from analyses of the effects of acetylation on the properties of other lagging-strand replication proteins.

The p300 acetylase also reacts with Dna2, with multifold stimulation of nuclease, helicase, and ATPase activities (Balakrishnan et al. 2010). Notably, alteration of the helicase activity allows Dna2 to drive the nuclease active site to the base of the original flap, and on some flaps even farther. This results in a shifted cleavage distribution farther downstream. Moreover, Pol δ is acetylated on the catalytic subunit. Preliminary

results show that acetylation of the polymerase greatly augments its ability to perform strand displacement synthesis. The combined effect is that flaps are created faster by the modified Pol δ . This would normally be a genome stability problem because long flaps can form secondary structures that inhibit processing, and can recombine at ectopic sites. However, the increased efficiency of Dna2 must prevent the flaps from actually achieving great length. Instead, displacement will occur for a greater distance, and ultimate ligation will be delayed because the lowered activity of FEN1 will not be able to rapidly create nicks. The overall consequence of the modification is that, without actually making long flap intermediates, a longer patch of the downstream fragment would be removed and replaced.

Why would this be desirable? In higher eukaryotes, millions of 150- to 200-nt fragments are needed to make the lagging strand. If an average of 40 nucleotides are removed and replaced from each, then $\sim 23\%$ of the lagging strand has to be made twice. This would only be desirable if it protects DNA that provides the organism with a selective advantage. A reasonable interpretation is that lagging-strand replication is selectively regulated for fidelity. Pol α makes the primer for each fragment, but then adds an additional ~ 20 nt of DNA. Because Pol α lacks a proofreading exonuclease, those nucleotides are added at lower fidelity than the later nucleotides added by the much more accurate Pol δ . As a consequence, each fragment can be viewed as having a gradient of potential errors decreasing from the 5' to the 3' end. Because of this gradient, replacement of a long patch of the Pol α -synthesized nucleotides with extension from an upstream primer by Pol δ would correct a very high proportion of replication errors in the lagging strand.

Why isn't this corrective effect constitutive? The p300 acetylase activates selected areas of chromatin for gene expression. Its distribution to these areas suggests that it preferentially acetylates replication/repair proteins for synthesis of active genes. Possibly Pol α synthesis patch full replacement has evolved into a regulated process because the cell tries to protect actively

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transcribed DNA but replicates most other DNA with unacetylated proteins, in an efficient but less accurate manner.

SUMMARY

Although Okazaki fragment processing is one of the fundamental processes of life, it can be optimized in any particular organism for speed, fidelity, energy consumption, or some combination. Speed and energy consumption would appear to be most important in bacteria because they are competing with other rapidly growing cells. Moreover, occasional lethal mutations should not affect the success of the population. The result is the evolution of a long fragment mechanism. Higher eukaryotes appear to have developed processing that is optimized for fidelity in active genes. This would appear best for survival through development, delay of cancers, and a long average life span.

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