Acetylation of Dna2 Endonuclease/Helicase and Flap Endonuclease 1 by p300 Promotes DNA Stability by Creating Long Flap Intermediates

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Lata Balakrishnan,1 Jason Stewart1,2, Piotr Polaczek3, Judith L. Campbell1, and Robert A. Bambara1,3

From the 1Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642 and 3Braun Laboratories, California Institute of Technology, Pasadena, California 91125

Flap endonuclease 1 (FEN1) and Dna2 endonuclease/helicase (Dna2) sequentially coordinate their nuclelease activities for efficient resolution of flap structures that are created during the maturation of Okazaki fragments and repair of DNA damage. Acetylation of FEN1 by p300 inhibits its endonuclease activity, impairing flap cleavage, a seemingly undesirable effect. We now show that p300 also acetylates Dna2, stimulating its 5′→3′ endonuclease, the 5′→3′ helicase, and DNA-dependent ATPase activities. Furthermore, acetylated Dna2 binds its DNA substrates with higher affinity. Differential regulation of the activities of the two endonucleases by p300 indicates a mechanism in which the acetylase promotes formation of longer flaps in the cell at the same time as ensuring correct processing. Intentional formation of longer flaps mediated by p300 in an active chromatin environment would increase the resynthesis patch size, providing increased opportunity for incorrect nucleotide removal during DNA replication and damaged nucleotide removal during DNA repair. For example, altering the ratio between short and long flap Okazaki fragment processing would be a mechanism for better correction of the error-prone synthesis catalyzed by DNA polymerase α.

Eukaryotic DNA is tightly compacted into the nucleus with the help of histone proteins that form chromatin, consequentially making the DNA less accessible to the various biological machineries that process its genetic information. DNA replication and repair are complex processes involving the assembly and coordinated function of a multitude of proteins. The limited accessibility of the chromatin environment to these proteins provides a challenge to their efficient operation. Chromatin-remodeling enzymes such as histone acetyltransferases play a significant role in modifying the structure of the chromatin during various biological activities. SV40 replication in vitro has been shown to occur with higher frequency in minichromosomes that contain hyperacetylated histones, indicating easier access and movement of the replisome on the nucleosomal DNA (1).

In addition to modifying histones, the acetyltransferase p300 also interacts with and acetylates many nonhistone proteins. Proliferating cell nuclear antigen (PCNA), a vital component of DNA replication and repair machinery, recruits p300 to the DNA, where it likely acetylates histone proteins in preparation for replication or repair (2). Significantly, PCNA is acetylated by p300, and the modification increases PCNA binding efficiency to the main polymerase involved in Okazaki fragment maturation, DNA polymerase δ (3). In addition to modifying PCNA, p300 also acetylates many proteins involved in base excision repair such as DNA polymerase δ, apurinic/apyrimidinic endonuclease 1, and DNA glycosylases, modifying their enzymatic functions (4–6).

Current work suggests that Okazaki fragment processing and long patch base excision repair involve strand displacement synthesis that creates flaps for the removal of priming RNA/DNA in replication and damage in repair (7, 8). In most cases, flap endonuclease 1 (FEN1) cleaves the flaps while short; however, there is a potential for some flaps, which escape FEN1 cleavage, to exceed 20 nt in length (9, 10). These longer flaps are coated by replication protein A (RPA), inhibiting FEN1 and creating the requirement for the Dna2 nuclease/helicase (Dna2). RPA stimulates Dna2, which displaces RPA and cleaves the flap to a length of 5–6 nt, allowing FEN1 access (11–13). This is known as the “two-nuclease” Okazaki processing pathway. Dna2, the second endonuclease, is a multifunctional protein possessing 5′→3′, 3′→5′ endonuclease activity, ATPase activity, and limited helicase activity (14, 15). Many biochemical and genetic studies have shown that Dna2 and FEN1 interact and have sequential functions in processing flap substrates (16).

FEN1 plays a central role in essential cellular functions including Okazaki fragment maturation (8), long patch base excision repair (17), and nonhomologous end joining (18). FEN1 is also postranslationally modified after forming a complex with p300 in vitro and in vivo (19). Acetylation inhibits FEN1 exo- and endonuclease activities and also reduces its DNA binding efficiency (19, 20). Inactivation or haploinsuffi-

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1 The abbreviations used are: PCNA, proliferating cell nuclear antigen; FEN1, flap endonuclease 1; nt, nucleotide(s); h, human; RPA, replication protein A; Dna2, Dna2 nuclease/helicase; ssDNA, single-stranded DNA; SCBT, Santa Cruz Biotechnology; Ac-, acetylated; BER, base excision repair.

2 Both authors contributed equally to this work.

3 To whom correspondence should be addressed: Dept. of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, 601 Elmwood Ave., Box 712, Rochester, NY 14642. Tel.: 585-275-3269; Fax: 585-275-6007; E-mail: robert_bambara@urmc.rochester.edu.
iciency of FEN1 has been previously linked to increased tumor formation and rapid progression to cancer (21, 22). Therefore, the finding that acetylation of FEN1 reduces its activity has been difficult to rationalize physiologically because it is a vital endonuclease in various DNA metabolic processes.

Previously, it was proposed that FEN1 acetylation inhibits its enzymatic function to avoid the premature processing of Okazaki fragments (19). Inhibition of FEN1 would promote the formation of longer flaps requiring the two-nuclease pathway. Because acetylation of FEN1 should promote utilization of Dna2, we considered the possibility that Dna2 is also acetylated, allowing it to accommodate a greater flow of substrates through the two-nuclease pathway. In this study, we examined whether Dna2 could be acetylated, the biochemical effects of such a modification, and the likely regulatory effects in the cell.

EXPERIMENTAL PROCEDURES

Oligonucleotides—Synthetic oligonucleotides were synthesized by Integrated DNA Technologies and subjected to 3’ end labeling as described previously (23). The 53-nt flap substrate, 32P-labeled on the 3’ end of the downstream primer, was used to study 5’–3’ nuclease activity and binding efficiency of Dna2. The substrate sequence and annealing conditions were the same as those described previously (11). The helicase assay was measured using a substrate that contained a 32P-labeled 5’, 42-base oligonucleotide annealed to M13mp18 ssDNA (New England Biolabs), resulting in a partial duplex substrate with an 18-nt 5’ noncomplementary strand as described previously (15).

Purified Proteins—hDna2 (15) and hFEN1 (24) were purified as described previously. Full-length recombinant p300 (31124) and recombinant catalytic domain mapping amino acids 965–1810 (31205) were purchased from Active Motif. Recombinant catalytic domain mapping 1284–1673 (SE451-0100) was purchased from Biomol International.

In Vitro Acetylation—Two micrograms of purified hDna2 was incubated with 0.1 µCi of [14C]acetyl coenzyme A (PerkinElmer Life Sciences) in 20 µl of histone acetyltransferase buffer (50 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol, 150 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium butyrate) for 30 min at 30 °C along with 100 ng of either the recombinant full-length or the catalytic domain of p300 (Active Motif). The catalytic domain was used for acetylating Dna2 used in vitro. The acetylated and unacetylated forms were separated on a 4 – 15% SDS-polyacrylamide gel (Bio-Rad). The gels were subjected to Coomassie stain, dried in a vacuum, and then subjected to autoradiography.

Binding Assay—Purified Dna2 (1 ng) and 1 ng of either the catalytic domains (965–1810 or 1284–1673) or 1 ng of full-length p300 were allowed to bind together in a coupling buffer consisting of 25 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol for 2h at 4 °C (in a 1:1 ratio). Antibody to p300 (Santa Cruz Biotechnology (SCBT) sc-585 or sc-32244) and control IgG (SCBT sc-2027) were pre-bound to protein A-agarose for 1 h at room temperature and washed twice with phosphate-buffered saline. The bound proteins were then added to the washed protein A-agarose-antibody complex and incubated overnight at 4 °C with end-over mixing. The following day, the proteins were released from the protein A-agarose using elution buffer. The immunoprecipitates were separated on precast 10% gels (Bio-Rad). Western blot analysis was performed with anti-Dna2 polyclonal antibody (Abcam 42439), anti-p300 antibody (Abcam 17993), and anti-acetylated lysine antibody (Cell Signaling 9441).

Nuclease Assay—Five fmol of substrate (53-nt flap substrate) labeled at the 3’ end of the downstream primer was incubated with various amounts of proteins, as indicated in a reaction volume of 20 µl, at 37 °C for 10 min. The reaction buffer consisted of 50 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 30 mM NaCl, 0.1 mg/ml bovine serum albumin, 2 mM MgCl2, and 1 mM ATP. The reactions were terminated using 2 × termination dye (90% formamide (v/v), 10 mM EDTA, 0.01% bromphenol blue, and 0.01% xylene cyanol). After termination, samples were heated at 95 °C for 5 min and loaded onto a denaturing gel (7 M urea) 15% polyacrylamide gel and electrophoresed for 1 h 30 min at 3000 V.

Helicase Assay—Helicase assay was performed using the nuclease-deficient mutant of hDna2 (D294A). The helicase substrate was incubated with various amounts of proteins, as indicated in a reaction volume of 20 µl, at 37 °C for 60 min. The reaction buffer consisted of 50 mM Tris-HCl (pH 7.5), 25 mM NaCl, 2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, 4 mM MgCl2, 4 mM ATP. The reactions were terminated using 5 × stop solution (60 mM EDTA, 40% sucrose, 0.6% SDS, 0.25% bromphenol blue, and 0.25% xylene cyanol). After termination, samples were loaded on an 8% native polyacrylamide gel and electrophoresed for 1 h at 150 V.

ATPase Assay—ATPase reactions were performed as described previously (15). The reactions were incubated with or without 1 µg of oligonucleotide (ssDNA, 30 bases) for 30 min and stopped by 10 mM EDTA/10% acid-washed charcoal (Norit Protocol). The reactions were allowed to sit on ice overnight and spun at 13,000 rpm for 30 min the following day. Duplicate 100-µl supernatant samples were counted by the Cerenkov method. ATP hydrolysis was determined by dividing the adjusted protein-dependent counts by the specific activity of ATP in each reaction.

Electrophoretic Mobility Gel Shift Assays—Binding efficiency of unacetylated and acetylated Dna2 to a 53 nt flap substrate was assessed using electrophoretic mobility gel shift assays. Five
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**[A]** In Vitro Acetylation

![Coomassie stain and autoradiograph with lanes labeled with Dna2 or AcDna2](image)

**[B]** In Vitro Binding

![Western blot with lanes labeled with Input, IgG, IP:p300, IP:Dna2](image)

**[C]** In Vivo Binding

*HeLa Whole Cell Extracts*

![Western blot with lanes labeled with IP:p300, IP:Dna2, and IP:FEN1](image)

**[D]** UV Treated HeLa Cell Extracts

![Western blot with lanes labeled with Unimmunoprecipitated, UV Treated, IP:Dna2, and IP:Ac-K](image)

**FIGURE 1.** *p300 binds and acetylates Dna2 both in vitro and in vivo.* A, 2 μg of unmodified hDna2 (wtDna2) and 14C-acetylated in vitro hDna2 (AcDna2) was separated on a 4–15% SDS-polyacrylamide gel. The upper panel shows the Coomassie stain of the separated proteins and acts as a loading control for the unmodified and modified Dna2. The same gel was subsequently exposed to x-ray film for 1 week, and the acetylation status of Dna2 was analyzed (lower panel). B, 1 ng of purified hDna2 was incubated with 1 ng of recombinant catalytic domain (amino acids 1284–1673), 1 ng of recombinant catalytic domain (amino acids 965–1810), or 1 ng of recombinant full-length p300 in a binding assay. The bound proteins were immunoprecipitated (IP) with antibody against full-length p300, separated by gel electrophoresis, and Dna2 was detected by Western blotting (IB) with antibody against Dna2. C, HeLa whole cell extracts were used either to immunoprecipitate p300 and immunoblot for p300 and Dna2, and FEN1 or immunoprecipitate Dna2 and immunoblot for p300, Dna2, and FEN1. The co-immunoprecipitated proteins and their molecular weights are indicated in the gel. ML, molecular weight ladder. D, HeLa whole cell extracts from either untreated or UV-treated cells were used to immunoprecipitate Dna2 and immunoblot for Dna2 and acetylated lysines (Ac-K). The proteins associated with the acetylated lysines detected by Western blotting were confirmed by stripping the blot and reprobing it with either anti-Dna2 or anti-FEN1 to confirm the detected protein.

RESULTS

hDna2 Is Acetylated in Vitro and in Vivo—To understand the cellular rationale for inhibition of FEN1, we initially investigated whether its functional interacting partner, Dna2, was also affected by acetylation. Either the full-length p300 or the catalytic (acetyltransferase) domain of p300 was incubated with purified Dna2 and [14C]acetyl coenzyme A. Similar to FEN1, the purified Dna2 protein was robustly acetylated in vitro by both full-length p300 and the catalytic domain of p300 (Fig. 1A, lane 2). To confirm the association of p300 and Dna2 in vitro, binding assays using the purified human recombinant proteins were performed. Dna2 and either full-length p300 or truncation mutants, 1284–1673 or 965–1810, of p300 containing the catalytic domains were incubated together. Using an antibody that recognizes the full-length p300 and the different length catalytic domains, an immunoprecipitation was performed to assess Dna2 binding to the different forms of p300. The immunoprecipitates were separated on a SDS-polyacrylamide gel, and Western blotting was performed using antibody against Dna2. The nonspecific IgG showed no contamination with Dna2 (Fig. 1B, lane 2). Both the 1284–1673 (Fig. 1B, lane 3) and 965–1810 (Fig. 1B, lane 4) p300 catalytic domains were as efficient at binding Dna2 as full-length p300 (Fig. 1B, lane 5). The association of the purified proteins was not mediated by DNA, and a molar excess of purified FEN1 in the assay did not abrogate the binding between Dna2 and p300 (data not shown).

Because p300 can acetylate Dna2 in vitro, we next examined whether these two proteins interact in HeLa whole cell extracts. Endogenous p300 was co-immunoprecipitated with antibody against Dna2 and FEN1, and *vice versa*, Dna2 was co-immunoprecipitated with antibody against p300 and FEN1 (Fig. 1C). FEN1 and p300 have previously been shown to co-immunoprecipitate together. Using an antibody that separates the different forms of p300, Dna2 was co-immunoprecipitated with antibody against Dna2 and p300 (Fig. 1C). FEN1 and p300 have previously been shown to co-immunoprecipitate together (19). Our results suggest that p300 can form a complex with both Dna2 and FEN1, although the exact organization of binding of these proteins with respect to one another could not be determined.

Because FEN1 acetylation is increased during UV damage (19), and yeast dna2 mutants are mildly sensitive to UV, we analyzed the levels of Ac-Dna2 in HeLa cells exposed to UV (Fig. 1D). Western blot analysis of immunoprecipitated Dna2 using an antibody against Dna2 showed that a similar amount of protein was expressed and immunoprecipitated in both untreated and UV-treated cells. When immunoprecipitated Dna2 was immunoblotted with an antibody raised against acetylated lysine residues, we observed an significant increase in Ac-Dna2 after UV treatment. Increased acetylation of the immunoprecipitated FEN1 on UV treatment was also observed, as reported previously (19).

Effect of Acetylation on hDna2 Nuclease Function—We next defined the effects of acetylation on the activities of Dna2 in vitro. Dna2 is a tracking enzyme, whereby it enters the flap at its 5' end and cleaves endonucleolytically while tracking toward the base, leaving a terminal 5–6-nt flap (23). Although Dna2 can bind directly onto the flap without tracking, the cleavage activity of Dna2 is dependent on the tracking mode (15). The
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In addition to the 5′–3′ endonuclease activity, hDna2 also exhibits a 3′–5′ directional nuclease activity, similar to but more efficient than that of yeast or Caenorhabditis elegans Dna2 (14). Compared with yeast or C. elegans, the 3′–5′ nuclease activity of hDna2 on ssDNA is significantly higher than the 5′–3′ activity. We compared the 3′–5′ endonuclease activity of unmodified and acetylated hDna2 using a 30-nt ssDNA blocked at the 5′ end using biotin-conjugated streptavidin to prevent 5′–3′ cleavage. We observed only a minor (1.5-fold) stimulation in the 3′–5′ endonuclease activity in Ac-Dna2 (supplemental Fig. S1). Notably, the increase in the 3′–5′ endonuclease activity resulting from acetylation was significantly lower than the stimulation of the 5′–3′ endonuclease activity though the same active site is proposed to be utilized for both activities (15).

Effect of Acetylation on hDna2 ATPase and Helicase Functions—In addition to the nuclease activity, hDna2 also has a DNA-dependent ATPase function. ATP hydrolysis in the presence of ssDNA was measured using either unmodified Dna2 or Ac-Dna2. The ATPase activity of hDna2 is significantly weaker than that of Saccharomyces cerevisiae Dna2 (ScDna2) (15). However, on modification by p300, Ac-Dna2 showed an ~3-fold stimulation of ATPase activity (Fig. 3A). This increase was independent of the presence of p300 in the absence of acetyl coenzyme A, demonstrating that the modification on Dna2 stimulated its ATPase function.

Helicase activity of hDna2, similar to Xenopus Dna2, has been described as either “moderate” (15) or “extremely minimal” (14) compared with that of ScDna2. Using a substrate consisting of a 42-nt oligomer with 24 bp complementary to M13mp18 and a 5′ noncomplementary flap of 18 nt, we assessed the 5′–3′ helicase activity of modified Dna2. The robust nuclease of Dna2 limits the detection of the weak helicase. To facilitate accurate helicase measurement, the nuclease-deficient hDna2-D294A mutant protein, described previously, was used for the helicase assays (15). Helicase function was measured by the amount of labeled oligonucleotide that was displaced from the annealed substrate. Helicase activity of Ac-Dna2-D294A was stimulated by ~2-fold (Fig. 3B). The moderate increase in helicase and ATPase activity likely accounts for the 5′–3′ cleavage past the base of the flap on a small percentage of 53-nt flap substrates by Ac-Dna2 (Fig. 2).

ScDna2 activity is stimulated by RPA, and in turn it displaces RPA that is bound on ssDNA flaps (11). Although RPA can significantly stimulate hDna2 endonuclease function (15), high levels of RPA were found to inhibit the endonuclease (14). We assessed the ability of Ac-Dna2 to displace RPA from flap substrates using a 53-nt flap substrate containing pre-bound RPA. Either unmodified Dna2 or Ac-Dna2 was added to the reaction and the cleavage pattern of Dna2 was observed. Ac-Dna2 exhibited no differences compared with unmodified Dna2 in its ability to displace RPA (data not shown).

Acetylation of hDna2 Influences Its Binding to DNA Substrates—Because all of the activities of Dna2 were enhanced by acetylation, we investigated whether there was a correlation between the binding affinity of Dna2 and acetylation. FEN1 showed significantly reduced DNA binding activity upon acetylation, which likely correlated with its decreased enzymatic
functions (19). DNA binding efficiency of unmodified and Ac-Dna2 was measured using a 53-nt double flap substrate similar to the substrate employed to study the 5'-3' nuclease activity in Fig. 2. The unmodified and Ac-Dna2 were allowed to bind onto the substrate, and the binding affinity was assessed by electrophoretic mobility gel shift assay. Ac-Dna2 (Fig. 4, lanes 10–13) showed a significant increase in binding (~17-fold) to a 53-nt flap substrate compared with the unmodified Dna2 (Fig. 4, lanes 2–5). This binding was apparently not due to the presence of p300 because p300 in the absence of acetyl-CoA did not increase the ability of Dna2 binding (Fig. 4, lanes 6–9). These results suggest that the enhanced catalytic activities of Ac-Dna2 derive in part from increased DNA substrate affinity.

FIGURE 3. Ac-Dna2 shows increased ATPase and helicase activities. A, DNA-dependent ATPase activity of 500 fmol of unmodified and Ac-Dna2 was analyzed as described under "Experimental Procedures." The ATPase activity of the unmodified and Ac-Dna2 is plotted in the graph. A control without DNA is included. B, helicase activity was assayed as described under "Experimental Procedures." Reactions containing 1 fmol of M13-HPR partial duplex substrate and 1000 fmol of nuclease-defective hDna2-D294A alone (lanes 3 and 4), hDna2-D294A and p300 (lanes 5 and 6), and Ac-Dna2-D294A (lanes 7 and 8) were incubated for 15 min at 37 °C. Lane 1 represents the boiled substrate, and lane 2 represents the annealed M13-HPR partial duplex substrate. The labeled substrate and helicase products are indicated at the side of the gel. Positions of the substrate, helicase products, and nuclease products are indicated on the left of the figure.
DISCUSSION

Our results indicate that p300 regulates the activities of endonucleases involved in DNA replication and repair to favor long flap formation. The simultaneous inhibition of FEN1 but stimulation of Dna2 imply a switching of pathways from one in which flaps are cleaved and ligated while short, to one in which flaps grow longer. Inhibition of FEN1 uncouples it from strand displacement synthesis by the DNA polymerase, allowing flap displacement without immediate cleavage. Augmentation of Dna2 activities ensures that the resultant longer flaps are processed efficiently and faithfully for proper ligation and may even increase flap displacement through enhanced helicase activity. The overall implication is that the cell is intentionally manipulating flap processing to allow controlled removal of a greater number of nucleotides, resulting in a longer patch of resynthesis.

Why would a longer resynthesis patch be advantageous? DNA polymerase α is a low fidelity polymerase, lacking proof-reading ability, that lays down the initial RNA/DNA primer in Okazaki fragments (25). The 3′–5′ exonuclease activity of DNA polymerase δ, acting at the time of polymerase switching, plays a significant role in removing the errors caused by polymerase α to maintain genome stability (26). We propose that strand displacement synthesis activity of polymerase δ, acting during 5′ RNA/DNA primer removal, also plays a role in removing mismatched base pairs that are laid down by polymerase α. Moreover, the displacement would be more effective in this process if it were augmented by the properties of Ac-Dna2 and Ac-FEN1. We also propose that acetylation of Dna2 and FEN1 would increase the resynthesis patch during long patch BER, increasing the likelihood of complete damage removal. Consistent with this idea, the 5′-2-deoxyribose 5-phosphate activity of DNA polymerase β is inhibited on acetylation, suggesting an influence of acetylation the BER pathway, in which long patch BER would be favored over short patch BER (6). The overall effect of acetylation would then have damage repaired more frequently by a long patch process in which the patch length is intentionally increased. In support of this proposal, p300 is known to modify a number of proteins involved in BER (5, 6, 19, 27, 28), indicating that acetylation is used comprehensively to regulate this pathway.

Why does p300 exert this regulation? The local chromatin environment influences the efficiency of DNA replication and repair. DNA is susceptible to more damage in euchromatin compared with heterochromatin (29), yet the integrity of active chromatin is most important to preserve. We suggest that the cell utilizes the chromatin-associated histone acetyltransferase p300 in active chromatin locally to improve the fidelity of DNA base excision repair and nucleotide excision repair by lengthening resynthesis. DNA replication through active chromatin would also be more accurate because the longer resynthesis patch would ensure complete removal of the RNA/DNA primer. Alternatively, or in addition, the transient long flap could serve as a signal to recruit DNA replication or repair proteins so that they would be present at high concentration in the region. The likelihood that the long flap pathway, involving Dna2, is intentionally induced also provides an answer to the longstanding puzzle of why the Dna2 pathway has evolved. The current belief is that it catches the small fraction of flaps missed by FEN1 while they are short (12). However, its main purpose may be as an extreme accuracy alternative to normal flap processing.

Consistent with the idea that p300 directs location-dependent regulation, acetylation of BER proteins alters their activity and subcellular localization differentially (5, 6, 19, 27, 28). DNA lesions are less accessible to DNA repair complexes in chromatin than in free DNA. In fact, chromatin-remodeling enzymes such as p300 play a significant role in “opening up” the chromatin around DNA lesions. UV irradiation causes an increase in the activity of histone acetyltransferases and a decrease in the activity of histone deacetylases (30). As discussed earlier, PCNA has been implicated in recruiting p300 to DNA damage sites, and p300 in turn acts as a recruiting factor for additional repair complexes (2, 31). Recruitment of p300 to damage sites by PCNA might promote the observed acetylation of relevant proteins. All of these observations are consistent with the existence of a general mechanism for regulation of DNA replication and repair by acetylation. We believe that the long flap induction mechanism described here is part of this overall effort by the cell to protect genetic information in the busy environment of active chromatin.

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