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Assembly of base excision repair complex on abasic DNA and role of adenomatous polyposis coli on its functional activity

Aruna S. Jaiswal and **Satya Narayan**
Department of Anatomy and Cell Biology, University of Florida, Gainesville, Florida 32610

Abstract

Assembly and stability of base excision repair (BER) proteins *in vivo* onto abasic DNA and the role of adenomatous polyposis coli (APC) protein in this process is currently unclear. We have studied the assembly of a multiprotein BER complex onto abasic DNA (F-DNA) and characterized the physical and functional activity of the associated proteins. We found that the BER complex contained all the essential components of the Long-patch BER system, such as APE1, Pol-β, Fen-1 and DNA ligase I. Interestingly, wild-type APC was also present in the BER complex. Kinetics of the assembly of BER proteins onto the F-DNA were rapid and appeared in sequential order depending upon their requirement in the repair process. Presence of wild-type APC in the BER complex caused a decrease in the assembly of BER proteins and negatively affected long-patch BER. These results suggest that major BER proteins in the complex are assembled onto F-DNA, and are competent in performing DNA repair. Wild-type APC in the BER complex reduces the repair activity, probably, due to interaction with multiple components of the system.

Keywords

Adenomatous polyposis coli; DNA damage; DNA repair complex; Long-patch base excision repair

The mammalian genome suffers from continuous insult from free radicals generated by both endogenous (reactive oxygen species) and exogenous sources (tobacco smoke); thereby, undergoing approximately 100,000 modifications per day per cell. These modifications are continuously repaired so that genomic integrity is preserved by a delicate balance between highly accurate DNA replication and a network of DNA repair pathways (1). Defects in DNA repair activity can perturb this balance and result in excessive damage beyond the threshold capacity of the cells. This could result in an increased incidence of cancer (2). DNA damage is sensed by DNA repair enzymes in the cell and the damage is repaired in sequential steps. However, the signaling process that actually operates in response to DNA damage within cells remains largely unknown. The factors that trigger the activation of repair proteins and their assembly at the damage sites have been a center of investigation in recent years. The various DNA repair pathways operating in cells include base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), non-homologous end-joining (NHEJ), homologous end-joining and DNA inter-strand crosslink repair (3–6). Among these, the BER pathway is most prevalent for the removal of damaged bases generated by alkylation, oxidation or reduction (7). In most cases, excision of a damaged base by a DNA glycosylase enzyme leads to the formation of a potentially cytotoxic

To whom correspondence should be addressed: snarayan@ufl.edu, or ajaiswal@ufl.edu, Telephone: 352–273–8163; Fax: 352–293–3305.

apurinic or apyrimidinic site (AP site) intermediate. AP sites are very unstable and degrade spontaneously into DNA-strand breaks by β-elimination (8). In addition to their unstable nature, they are highly mutagenic and result in non-template DNA and RNA synthesis. AP sites are first recognized and removed by APE1 generating a strand break and a flap. If AP sites are not repaired efficiently, they can lead to tumor initiation and progression (9). In the subsequent step, Pol-β acts upon the strand break and inserts the correct nucleotide(s), and after removal of the flap, the gap is sealed by DNA ligase I or III (10– 12). BER can be completed by either single nucleotide (SN)-BER or long-patch (LP)-BER sub-pathways. If the AP site flap is dRP (deoxyribose phosphate) then it is removed by Pol-β, and the damage is repaired by SN-BER (5). If the reduced or oxidized AP site flaps are present then they are removed by Fen1, and the damage is repaired by LP-BER (13). Both SN-BER and LP-BER pathways have been reconstituted *in vitro* using purified human proteins (14).

In the past, the BER mechanism has been extensively studied using cell-free extracts or reconstituted systems with a synthetic DNA substrate containing a single lesion (14–22). During these studies, the interaction of BER proteins with accessory proteins was established by either co-elution of BER proteins or by *in vitro* pull-down experiments (21–26). In one of these studies, multiple activities of BER proteins were observed that were bound to the affinity column of Pol-β during its purification process (22). Recently, the interaction between the AP site analog 3-hydroxy-2-hydroxymethyltetrahydrofuran (tetrahydrofuran or F)-containing AP site DNA and APE1 was established (20, 21, 27, 28). In another study, an APE1-independent BER pathway was described in which the DNA glycosylases NEIL-1 and NEIL-2, following base excision cleave DNA at the AP site by βδ-elimination (29). A number of *in vitro* reconstituted studies have been carried out to precisely determine the physical and functional interaction between BER proteins to define the BER mechanisms. However, an intrinsic limitation of reconstitution assays is that the mechanism of BER pathway may not be an accurate representation of how damaged DNA is processed within the cell.

Loss of function of adenomatous polyposis coli (APC) leads to severe abnormalities in many organs and tissues. Mutations in the central area of the *APC* gene, the mutation cluster region (MCR), are strongly associated with colon cancer (30, 31). Such mutations in the MCR region result in a truncated APC protein of about half the size of full-length APC that lacks the normal binding to β-catenine, end-binding 1 protein, and axin (32, 33). In our previous studies, we have shown that the DNA repair inhibitory (DRI)-domain of APC protein is located towards the N-terminus and interacts with Pol-β and blocks SN-BER by blocking 5′-deoxyribose phosphate (dRP)-lyase activity (26). The DRI-domain of APC also interacts with the enzyme Fen1 and prevents LP-BER by blocking strand-displacement synthesis (24–26, 34). Whether APC affects the stability and assembly of BER proteins or modulates their activity *in vivo* remains an open question.

In the present investigation, we provide evidence indicating that BER proteins rapidly assemble in a biologically active BER complex onto F-DNA from the nuclear extract of colon cancer cells. The results obtained from these studies provide information on the role of APC in BER complex assembly and biological function. Our results show that the BER proteins are recruited through an abasic DNA and perform their individual function as necessary. APC is physically present in the BER complex and modulates its repair activity.

Materials and Methods

Maintenance of mammalian cell lines

LoVo, colon cancer cells expressing truncated APC protein lacking DRI-domain, were grown in Hams F12 medium at 37°C under a humidified atmosphere of 5% CO_2. The

medium was supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/mL of penicillin, and 100 μg/mL of streptomycin.

Transfection and nuclear extract preparation

LoVo cells were transfected with pCMV-APC plasmid using Lipofectamine (Invitrogen, Carlsbad, CA). Cells were harvested 48 h post-transfection and nuclear extract was prepared from control and wild-type APC-overexpressed cells as described earlier (35).

Oligonucleotides and chemicals

All oligonucleotides were purchased from Sigma-Genosys (Woodlands, TX). T4-polynucleotide kinase (PNK) was purchased from New England Biolabs (Ipswich, MA) and [γ-^{32}P]ATP was obtained from Perkin Elmer (Waltham, MA, USA).

Isolation of multiprotein BER complex from nuclear extract

For the isolation of multiprotein BER complex, Dynal-M270 streptavidin-magnetic beads were blocked with 5% BSA and subsequently washed four-times with wash-buffer I (10 mM Tris-HCl, pH 7.6; 1 M NaCl and 1 mM EDTA) using a magnetic separator rack (Dynal, Invitrogen, Carlsbad, CA). Beads were then incubated with double-stranded 60-mer biotinylated/F-DNA-substrate at room temperature with agitation for 30 min in binding buffer (10 mM Tris-HCl, pH 8.0, 1 M NaCl and 1 mM EDTA). Subsequently, these beads were washed four-times with wash-buffer I and then resuspended in resuspension buffer (30 mM Hepes, pH 7.5, 30 mM KCl, 8 mM $MgCl_2$, 5% glycerol, 0.2 mM ATP and 0.5 mM DTT). The DNA beads (30 pmole of DNA per reaction) were then included in a binding-buffer (final reaction volume of 80 μL) containing 30 mM Hepes, pH 7.5, 30 mM KCl, 0.5 mM DTT, 5% glycerol and nuclear extract (30 μg) at 37°C for different periods of time as indicated in the figure legends. Reactions were terminated at indicated times by quickly removing the biotinylated/F-DNA-streptavidin beads from unbound proteins by applying the magnetic field using the separator rack. Beads were washed four-times with 250 μL of wash-buffer II (30 mM Hepes, pH 7.5, 30 mM KCl, 8 mM $MgCl_2$, 5% glycerol, 0.5 mM EDTA and 0.5 mM DTT). The washed beads containing active BER proteins were resuspended in the BER reaction buffer (30 mM Hepes, pH 7.5; 30 mM KCl, 8 mM $MgCl_2$, 1 mM DTT, 0.01 % (v/v) Nonidet P-40) and directly used for measuring the activities of APE1, Fen1 and DNA ligase I proteins and LP-BER. For the analysis of BER proteins, beads were resuspended in SDS-PAGE sample-buffer (25 mM Tris-HCl, pH 6.8, 2.5% mercaptoethanol, 1% SDS, 5% glycerol, 1 mM EDTA, and 0.15 mg/mL bromophenol blue) and analyzed by immunoblotting. Proteins were resolved on a 10% SDS-polyacrylamide gel followed by transfer to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was immunoblotted with the indicated antibodies.

Western blot analysis

The protein levels of wild-type APC, APE1, Pol-β, Fen1, proliferating cell nuclear antigen (PCNA) and DNA ligase I in the BER complex were determined by Western blot analysis with our previously described procedures (36). Antibodies directed against APE1, Fen1 and DNA ligase I were procured from Novus Biologicals (Littleton, CO), anti-PCNA antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), anti-APC antibody was from Millipore (Billerica, MA) and anti-Pol-β antibody was kindly provided by Dr. Samuel H. Wilson (NIEHS, North Carolina).

Synthesis and labeling of *in vitro* BER substrates

To examine LP-BER activity, 3-hydroxy-2-hydroxymethyltetrahydrofuran (F) was introduced at the 24th position of the 63-mer DNA (5′-

CTAGATGCCTGCAGCTGATGCGC***F***GTACGGATC-CACGTGTACGGTACCGAGGGCGGGTCGACA-3′) called F-DNA (25). This substrate was radiolabeled at the 5′-end with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs, Woburn, MA) and annealed to a 63-mer complementary template. The radiolabeled probe was purified using NICK column (GE Healthcare, Piscataway, NJ).

APE1 activity

APE1 activity was assayed in a total reaction volume of 30 μL, containing 30 mM Hepes buffer, pH 7.5, 30 mM KCl, 8 mM $MgCl_2$, 5% glycerol, 0.5 mM DTT, and ^{32}P-63-mer double-stranded F-DNA. The reaction was initiated by addition of indicated concentrations of multiprotein BER complex isolated at different time intervals, and incubated at 37°C. The cleaved DNA product was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and recovered by ethanol precipitation in the presence of 0.1 μg/mL tRNA. The recovered DNA was resuspended in 10 μL of gel-loading dye (95% formamide, 20 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanol) and denatured at 85°C for 5 min. The DNA products were separated by electrophoresis in a 15% polyacrylamide gel, containing 7 M urea (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA, pH 8.8) and visualized by autoradiography.

Fen1 activity

The Fen1 substrate for 5′-flap endonuclease activity was made by annealing a 23-mer (5′-CTAGATGCCTGCAGCTGATGCGC-3′) and a 5 1-mer (5′-***F***AACATTTTTTTGTACGGATCCACGTGTACGGTACCGAGG-GCGGGTCGACA-3′) oligonucleotides to a 63-mer complementary template as described previously (37). The 51-mer oligonucleotide has a flap of 11-nt, which was cleaved by Fen1. The 51-mer oligonucleotide was radiolabeled at the 5′-end with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs, Woburn, MA). All three oligonucleotides were annealed at a molar ratio of 1:1:1.

Assay for 5′-flap endonuclease activity of Fen1 was performed in a final volume of 30 μL as described earlier (26, 37). Briefly, the reaction mixture contained 30 mM Hepes, pH 7.5, 30 mM KCl, 8 mM $MgCl_2$, 1 mM DTT, and the indicated amounts of multiprotein BER complex from different time intervals. After addition of the BER complex, the reaction mixture was incubated at room temperature for 5 min and then 2.5 nM of ^{32}P-labeled flapped-DNA substrate was added to the mixture followed by incubation at 37°C for 15 min. Reaction was terminated with a stop solution containing 0.4% (w/v) SDS and 5 mM EDTA. DNA was recovered by phenol/chloroform/isoamyl alcohol (25:24:1, v/v) extraction followed by ethanol precipitation. The 11-nt DNA product generated from the Fen1 activity was separated on a 15% acrylamide-7 M urea gel and visualized by autoradiography.

In vitro LP-BER assays

The LP-BER reaction mixture contained 30 mM Hepes buffer, pH 7.5; 30 mM KCl, 8 mM $MgCl_2$, 1 mM DTT, 100 μg/mL BSA, 0.01 % (v/v) Nonidet P-40, 0.5 mM ATP, and 20 μM each dATP, dCTP, dGTP, dTTP in a final reaction volume of 30 μL. Ten μL of multiprotein BER complex from different time points was taken and incubated with 2.5 nM of ^{32}P-labeled 63-mer F-DNA (pre-incubated with 1 nM of APE1 to create an incision at the repair site) and 0.8 nM DNA ligase I was supplemented as indicated in the figures. Each reaction was incubated at 37°C for 60 min and then terminated by the addition of 30 μL of a stop solution containing 5.0 mM EDTA, 0.4% (w/v) SDS with 1 μg proteinase K and 5 μg carrier tRNA. After incubation for an additional 20 min at 37°C, the DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) followed by

ethanol precipitation. The reaction products were resolved on a 15% polyacrylamide-7 M urea gel and visualized by autoradiography.

Results

APC and basic BER proteins are present in a multiprotein complex

In previous studies, using an *in vitro* reconstituted system we have shown that 20-amino acids fragment of APC (1250-KVSSINQETIQTYCVEDTPI-1269) containing the DNA repair inhibitory domain (DRI-domain) blocked BER (24–26, 34). Whether full-length wild-type APC can block BER is currently unclear. Thus, to determine whether full-length APC is present in the BER complex and to explore how it modulates BER activity, we purified the multiprotein BER complex from nuclear extract of control and wild-type APC-overexpressed LoVo cells. We chose LoVo cells for the isolation of multiprotein BER complex because these cells express truncated APC protein (120-kDa) lacking the DRI-domain which does not interact with Pol-β or Fen1 and will not affect the BER pathway (24–26). First, to ascertain that the BER proteins do not interact non-specifically with the control biotinylated/F-DNA-streptavidin beads, we used unmodified DNA substrate immobilized to these beads and incubated with nuclear extracts. We could not detect the presence of APC and other BER proteins associated with control biotinylated/F-DNA-streptavidin beads (data not shown). After optimizing the amount of nuclear extract and immobilized biotinylated/F-DNA-beads required for the assembly of the BER complex, we examined the time dependence of assembly by incubating biotinylated/F-DNA-streptavidin beads with nuclear extracts for varying interval of time (Fig. 1A). We expressed full-length wild-type APC in LoVo cells to examine whether it can assemble with BER complex onto the F-DNA through interaction with Pol-β and Fen1. Results showed the association of wild-type APC protein in the BER complex onto the F-DNA, which was maximum at 2.5 min of the complex assembly and then gradually decreased with time (Fig. 1A and B).

Next, we determined the presence of APE1 in the complex, as the interaction of APE1 with F-DNA is the first step in the LP-BER pathway, in which APE1 acts by one-step associative phosphoryl transfer mechanism on a F-containing DNA substrate (21). It is known that APE1 can stably bind to AP sites in DNA which can be detected by DNA-protein complexes. However, it is not known whether the presence of wild-type APC in the BER complex influences the binding of APE1 to AP site lesion. We found that the association of APE1 to the F-DNA was very rapid reaching maximal levels at 0.5 min, the earliest time interval we examined, and gradually diminishing thereafter to its lowest level at 10 min (Fig. 1A and C). The presence of full-length APC prevented association of the APE1 protein level in the complex, but did not prevent its loss in a time-dependent manner (Fig. 1A and C). These results suggest that the level of APE1 onto the F-DNA decreases with time. The presence of full-length APC affects the assembly of APE1 onto F-DNA.

Subsequently, we analyzed Pol-β protein levels in the multiprotein BER complex. Pol-β protein level increased in a time-dependent manner up to 2.5 min and then decreased to its lowest level at 10 min of incubation (Fig. 1A and D, compare lanes 2–6). Pol-β level was decreased in the wild-type APC containing complex as compared to the control (Fig. 1A, compare lanes 2–6 with 8–12, respectively), which could be due to either the presence of APC or to the loss of APE1. Since Fen1 plays an important role in LP-BER, we also analyzed its level in the multiprotein BER complex. The results showed that Fen1 assembled very rapidly and stably onto F-DNA (Fig. 1A and E). Since the level of Fen1 did not change in the BER complex isolated from the wild-type APC containing nuclear extract (Fig 1A and E), it appears that APC does not interfere with the *binding* of Fen1. We then determined the level of DNA ligase I in the complex and found that a very low level of this protein was

present. The presence of wild-type APC slightly decreased the assembly of DNA ligase I onto the F-DNA (Fig. 1A and F).

PCNA plays an important accessory role in Pol-δ/ε dependent LP-BER. PCNA physically interacts with APE1 (16), and also interacts with Pol-β and Fen1 (23, 38, 39). We were unable to demonstrate the assembly of PCNA in the BER complex assembled onto F-DNA either in the presence or absence of wild-type APC (Fig. 1A).

APC reduces APE1 activity of multiprotein BER complex

APE1 can stably bind to abasic sites in DNA (40, 41) and can be detected in the DNA/protein complexes of BER (42). However, it is not known whether the presence of wild-type APC in the BER complex can affect the binding of APE1 to AP site DNA and thereby changes the dynamics of the components of BER complex assembly. We wished to examine this phenomenon by *in vitro* assays with the isolated BER complex. We standardized the assay conditions for APE1 activity using ^{32}P-labeled F-DNA as a substrate and purified BER complex as a source of APE1 (Fig. 2A). Appearance of a ^{32}P-labeled 23-mer product is an indicator of APE1 activity. We found that the purified complex was highly active and completed the cleavage of ^{32}P-labeled 63-mer DNA by 10 min. Next, we compared the APE1 activity of the purified BER complex from the control and wild-type APC-overexpressed nuclear extracts of LoVo cells at various time-intervals. APE1 activity of BER complex isolated from LoVo control and APC overexpressed nuclear extract matches with the protein level present in the complex, which was maximal at early time point and then decreased thereafter (Fig. 2A and 2B; Lanes 1–6 , respectively). We further observed that the presence of wild-type APC significantly caused a decrease in the APE1 activity of the complex (Fig. 2A and B; compare lanes 1–6 with 7–12, respectively). These results indicate that wild-type APC modulates the activity of APE1.

APC blocks Fen1 activity of the BER complex

In the above experiment, the major loss of APC protein level, but the minor loss of APE 1 activity and the Fen1 reaction was assembled using ^{32}P-labeled flap-DNA substrate. In previous studies, we have shown that Fen1 activity was blocked in an *in vitro* assay by APC fragment (1250-KVSSINQETIQTYCVEDTPI-1269), which subsequently blocked LP-BER (24). In the present study, we examined whether the presence of full-length wild-type APC protein in the BER complex can modulate Fen1 activity. Fen1 activity was determined by cleavage of 11-mer flap from ^{32}P-labeled-51-mer oligonucleotide of the sense strand (see the structure of DNA substrate on the top of Fig. 3A). We found that Fen1 protein level was not significantly affected by wild-type APC in the purified BER complex. However, the BER complex isolated in the presence of wild-type APC showed significantly reduced Fen1 activity at all the time points of complex assembly as compared to the complex assembled with the control nuclear extract (Fig. 3A and B; compare lanes 2–6 with 8–12, respectively). These results suggest that Fen1 activity was decreased by the presence of wild-type APC protein in the BER complex, which supports our previous findings (24).

APC blocks LP-BER activity of the BER complex

The LP-BER reaction was assembled using APE1-precut ^{32}P-63-mer F-DNA and purified BER complex. The incision product of ^{32}P-63-mer F-DNA by APE1 is shown as a 23-mer product (Fig. 4A, lane 2). Pol-β-mediated strand-displacement synthesis of 1 to 7-nt products was partially blocked by the complex from wild-type APC-overexpressed nuclear extract (Fig. 4A, compare lane 3–7 with 9–13, respectively). The BER complex from wild-type APC-overexpressed nuclear extract showed increased level of 1-nt incorporation (24-mer product) by Pol-β and reduced levels of strand-displacement synthesis products (Fig. 4A, compare lanes 3–7 with 9–13, respectively). The overall repair of 63-mer product by the

BER complex was also blocked in the wild-type APC-overexpressing cells, which correlated with the block in the strand-displacement synthesis (Fig. 4A, compare the 63-mer product in lanes 3–7 with 9–13, respectively, and accumulation of 24-mer product in lanes 9–13). There was a time-dependent increase in the LP-BER activity (63-mer repaired product) when the complex was purified from control cells (lacking wild-type APC) (Fig. 4A and 4B, compare lanes 3–7 , respectively), while the presence of wild-type APC significantly reduced total LP-BER activity (Fig. 4A and 4B, compare lanes 3–7 with 9–13, respectively). The protein levels of APC, APE1, Pol-β, Fen1 and DNA ligase I of LoVo-NE and LoVo(APC)-NE used in these experiments were comparable to each other (Fig. 4C). These results suggest that wild-type APC present in the purified BER complex impaired the LP-BER activity.

Addition of DNA ligase I improved LP-BER of the purified BER complex

The poor appearance of the 63-mer repaired product as compared to the accumulated 1-nt (24-mer product) and strand-displacement synthesis products observed in Figure 4A could be due to limiting amounts of DNA ligase I in the assembled BER complex (Fig. 1). Thus, we examined whether supplementation of exogenous DNA ligase I to the BER complex could improve LP-BER activity of the complex. We first optimized the amount of DNA ligase I needed for the efficient repair of ^{32}P-63-mer F-DNA (data not shown), and then used the optimized amount of DNA ligase I (0.8 nM) in subsequent LP-BER assays (Fig. 5A). Results showed that addition of DNA ligase I to the BER complex increased LP-BER activity and decreased the accumulation of strand-displacement products as compared to the results of Figure 4A. Furthermore, the presence of wild-type APC in the BER complex continued to block LP-BER (Fig. 5A, compare lanes 3–7 with 9–13, respectively). The BER complex assembled from wild-type APC-overexpressed nuclear extract showed greater accumulation of 1-nt (24-mer) incorporation product than the BER complex assembled from the control nuclear extract (Fig. 5A, compare lanes 3–7 with lanes 9–13, respectively). Thus, from these results it was evident that the BER complex with supplemented DNA ligase I showed maximal LP-BER up to 2.5 min time interval then decreased thereafter. The block by wild-type APC was consistent even after the addition of exogenous DNA ligase I to the BER complex assembled onto the F-DNA (Fig. 5A and 5B).

Next, we examined LP-BER to determine whether the incubation period of the LP-BER reaction could be limiting. We incubated APE1-precut ^{32}P-63-mer F-DNA with the BER complex assembled in the absence or presence of wild-type APC and 0.8 nM of DNA ligase I for intervals from 1–3 h at 37°C. The results showed that the supplementation with DNA ligase I to the BER complex improved repair of ^{32}P-63-mer DNA for the complex assembled from the control nuclear extract as compared to wild-type APC-overexpressed nuclear extract (Fig. 6A and 6B, compare lanes 3–6 with lanes 8–11, respectively). It was evident that as the time of reaction increased, accumulation of 24-mer product decreased in both the control and wild-type APC-overexpressed conditions. However the decrease was greater with control nuclear extract than with wild-type overexpressed nuclear extract (Fig. 6A, compare lanes 3–6 with lanes 8–11, respectively). The supplementation of DNA ligase I in the reaction mixture could not overcome the APC-mediated block of LP-BER.

Discussion

The *in vitro* BER systems assembled with purified proteins do not take into account the contribution of various co-factors/accessory proteins which might affect the dynamics of BER complex assembly and its efficiency in the processing of damaged DNA. In the present study, we therefore have attempted to analyze the biologically active BER complex from nuclear extract of colon cancer cells assembled onto AP site DNA. Most of the essential components of the BER machinery were physically associated with the AP site DNA which

might partially reflect an *in vivo* situation. The results of assembled BER complex analysis clearly demonstrated that the BER complex contained the basic essential proteins of BER such as APE1, Pol-β, Fen1 and DNA ligase I. Interestingly, we found that wild-type APC was assembled with other BER proteins onto the F-DNA, which blocked Pol-β-directed LP-BER. These results support our previous findings that APC blocks Pol-β-directed LP-BER (24, 26). However, earlier studies were done in a reconstituted LP-BER system using a 20-amino acid fragment of APC (containing the DRI-domain) in the *in vitro* competition assays. For these studies the question remained whether wild-type APC (full-length protein, 310 kDa) would perform in a similar fashion. Our current results clearly indicate that the wild-type APC is present in the BER complex assembled onto F-DNA and blocks LP-BER. Thus, our previous findings with the 20-amino acid fragment of APC to block LP-BER in reconstituted *in vitro* assays are similar to the results of wild-type APC protein assembled to the BER complex. Many other proteins including, Werner's syndrome protein (43, 44), poly(ADP-ribose) polymerase-1 (45, 46), replication protein A (47) and X-ray cross-complementing group 1 (XRCC1) (48) interact with Pol-β and modulate its activity. Other proteins that interact with Fen1 also modulate BER, for example, Blooms syndrome (49), arginine methyltransferase 6 (50) and high-mobility group box 1 protein (HMGB1) (51). We now add APC which interact with both Pol-β and Fen1 to this list. Whether APC directly interacts with the AP site DNA is currently unknown. However, in previous studies it has been shown that APC interact with genomic DNA, preferentially with A/T rich sequences (52). The interaction of APC with DNA has functional consequences, such as it inhibits DNA replication that negatively regulates cell cycle progression (53). Recently, it has also been suggested that induction of DNA double-strand break results in the accumulation of APC at damaged chromatin structures through interaction with DNA double-strand break repair factor, DNA-dependent protein kinase catalytic subunits (54). Thus, based upon these and our findings it can be suggested that APC participates in DNA repair and replication through protein/DNA or protein/protein interactions.

Another objective of this study was to examine whether BER occurs by sequential assembly of different proteins at the site of DNA damage or through a precursor BER complex. Earlier studies showed that APE1 stably binds to DNA containing an AP site and serves as an assembly and coordination factor for LP-BER proteins (27). Our findings support the notion of sequential and rapid assembly of proteins onto F-DNA. The results indicate that the assembly of APE1 and Fen1 come first, followed by Pol-β, APC and finally DNA ligase I. As the APE1 executes function, its level starts decreasing and the level of Pol-β starts increasing. After 2.5 min, the level of Pol-β also starts decreasing. Interestingly, the level of APC onto the F-DNA follows the pattern of the assembly of Pol-β. Whether the assembly of APC and Pol-β onto F-DNA takes place as a complex, as suggested earlier for APE1 and Pol-β (42), or as individual proteins is not clear from these studies. However, since APC interacts with Pol-β (25, 26, 34) one may suppose that APC and Pol-β might enter onto F-DNA as a complex. The rapid recruitment of APE1 and Pol-β on early time points and then decrease at the later time points both in the presence or absence of APC suggests that APE1 and Pol-β after executing their function are dissociated from the AP site DNA.

APC also interacts with Fen-1 (24); however, the recruitment of APC onto the F-DNA does not follow the pattern of Fen1. The recruitment of Fen1 onto the F-DNA is as rapid as APE1 and does not change with the time-course of the assembly of the complex. Nonetheless, the presence of APC on F-DNA decreases the activity of both Pol-β and Fen1. The role of the recruitment of Fen1 protein as early as APE1 onto F-DNA is not clear, but could provide a stabilization function.

PCNA plays multiple roles in the BER pathway, and one of the roles being as a scaffold protein (55). However, our results show that PCNA is not detectable in the BER complex

under these conditions. This raises the possibility that PCNA has a limited role in Pol-β-dependent LP-BER and it may be more involved with the function of Pol-δ/ε (56–58).

We show here that even the small amount of APC results in diminished APE1 binding and diminished Fen1 activity. The block of LP-BER caused by APC remains the same even after the exogenous supplementation with DNA ligase I. In summary, these interaction studies confirm that BER proteins are rapidly recruited in an orderly fashion at AP site DNA as suggested by earlier studies (28), and demonstrate that APC is an important regulator of this process. Our studies can be instrumental for uncovering the kinetics of recruitment of BER proteins *in vivo* to DNA damage. These results also suggest an unexpected role of APC in DNA repair and carcinogenesis.

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Abbreviations

APE1	apurinic/apyrimidinic endonuclease 1
Pol-β	DNA polymerase β
DRI- domain	DNA repair inhibitory domain
Fen1	5′-flap endonuclease 1
BER	base excision repair
LP-BER	long-patch base excision repair
SN-BER	single nucleotide base excision repair

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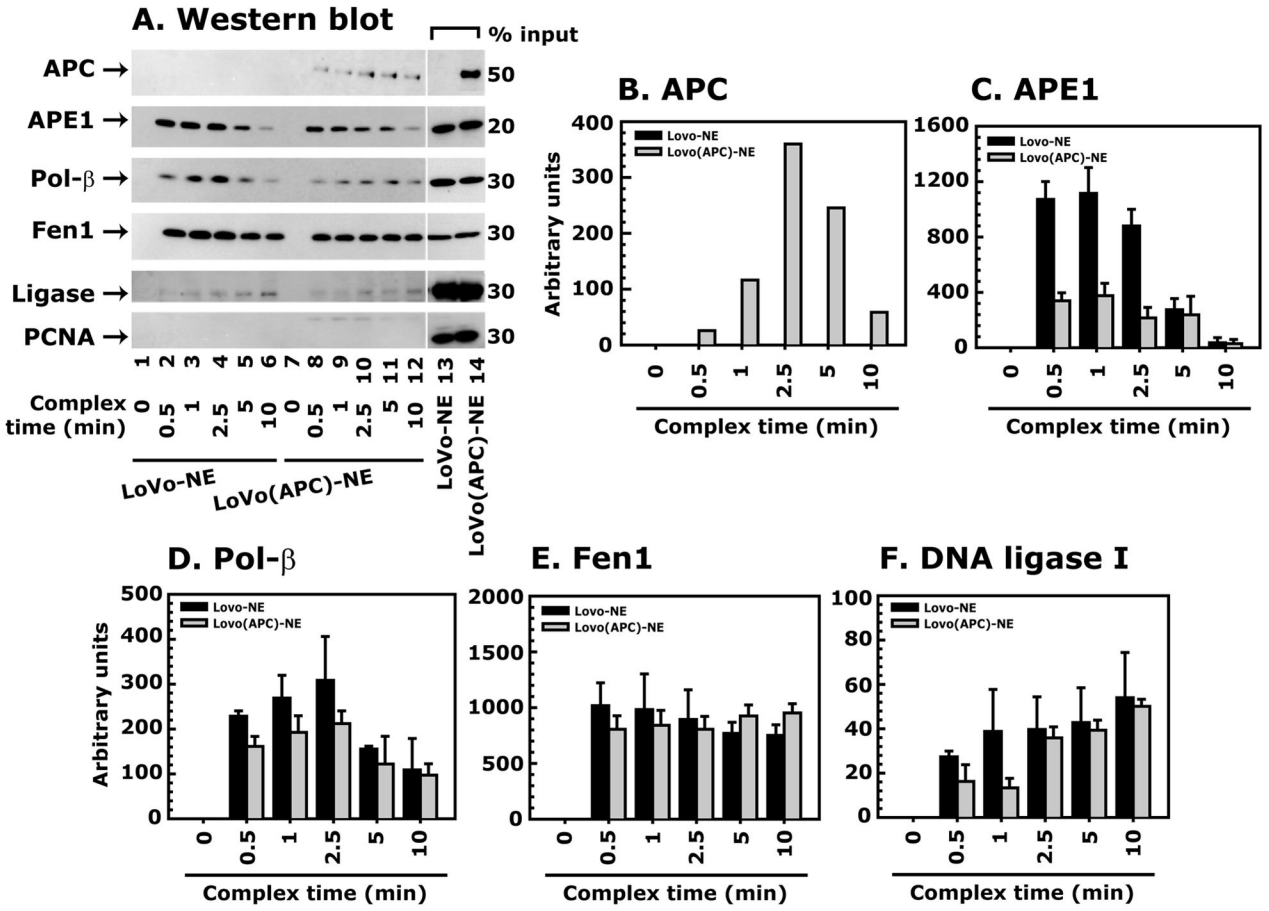

FIG. 1. Time-dependent assembly of DNA repair proteins in a multiprotein BER complex onto biotinylated/F-DNA beads

Panel A is a Western blot analysis showing levels of basic BER proteins present in multiprotein complex bound to F-DNA. The multiprotein BER complex was allowed to assemble in a time-dependent manner (0–10 min) from nuclear extracts of control (lanes 1–6) and wild-type APC-overexpressed (lanes 7–12) LoVo cells. The wild-type APC (310 kDa, lanes 7–12), APE1 (37-kDa; lanes 1–12), Pol-β (39-kDa; lanes 1–12), Fen1 (42-kDa; lanes 1–12), DNA ligase I (125-kDa; lanes 1–12) and PCNA (36-kDa; lanes 1–12) protein levels are shown. Corresponding endogenous levels of these proteins are shown in lanes 13 and 14 of the same panel. The percent input of nuclear extracts is shown on the right hand side of the autoradiogram. Immunoblots are representative of three independent experiments. **Panel B** shows the quantitative analysis of the APC protein present in the BER complex bound to F-DNA isolated at different time intervals. Data are representative of two independent experiments. **Panels C, D, E and F** show the quantitative analysis of APE1, Pol-β, Fen1 and DNA ligase I protein levels, respectively, present in the BER complex bound to F-DNA isolated at different time intervals. Data are mean ± SE of three different experiments.

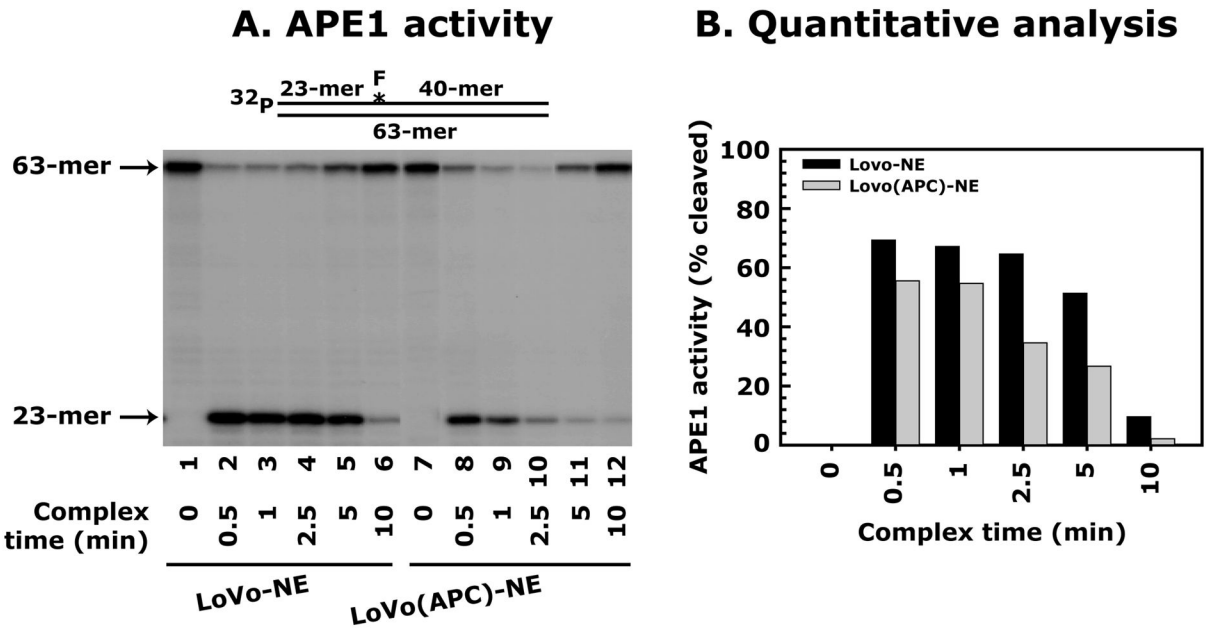

FIG. 2. Analysis of APE1 activity of multiprotein BER complex isolated from the nuclear extract of LoVo cells

Panel A shows the effect of APC on the APE1 activity of multiprotein complex isolated from the nuclear extract of control (multiprotein complex isolated at varying time intervals of 0–10 min; lane 1–6) and wild-type APC-overexpressed (lane 7–12) LoVo cells. The reaction mixture containing ^{32}P-F-DNA (2.5 nM) was incubated for 2.5 min at 37°C. Lane 1 shows ^{32}P-labeled 63-mer F-DNA and Lane 2 shows the 23-mer product after APE1 incision. **Panel B** shows the quantitative analysis of APE1 activity of the assembled BER complex onto F- DNA at different time intervals. Data are representative of two independent experiments.

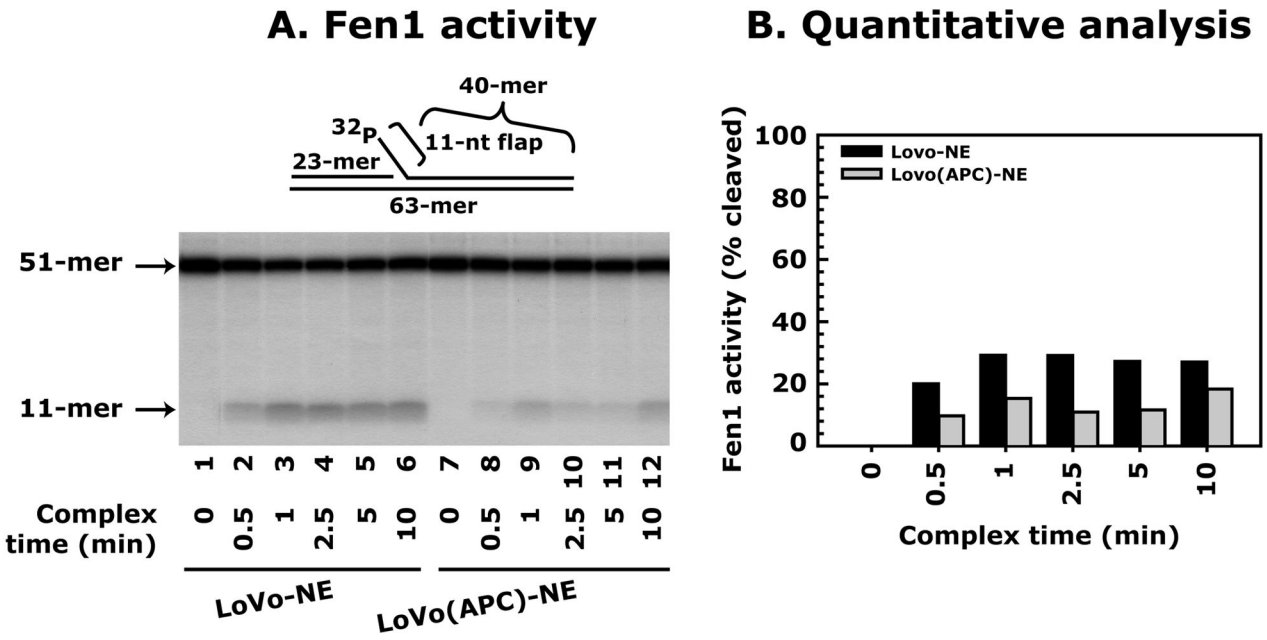

FIG. 3. Analysis of Fen1 activity of multiprotein BER complex isolated from the nuclear extract of control and wild-type APC-overexpressed LoVo cells

Panel A shows the effect of APC on the Fen1 activity of the multiprotein BER complex isolated from the nuclear extract of control and wild-type APC-overexpressed LoVo cells (multiprotein complex isolated at varying time intervals of 0–10 min; lanes 1–6 and 7–12, respectively). The reaction mixture containing ^{32}P-flap-DNA substrate (2.5 nM) was incubated for 15 min at 37°C with the purified complex. Lane 1 shows ^{32}P-labeled 51-mer F-DNA and lane 2 shows 11-mer product after Fen1 activity. **Panel B** shows the quantitative analysis of Fen1 activity of the BER complex. Data are representative of two independent experiments.

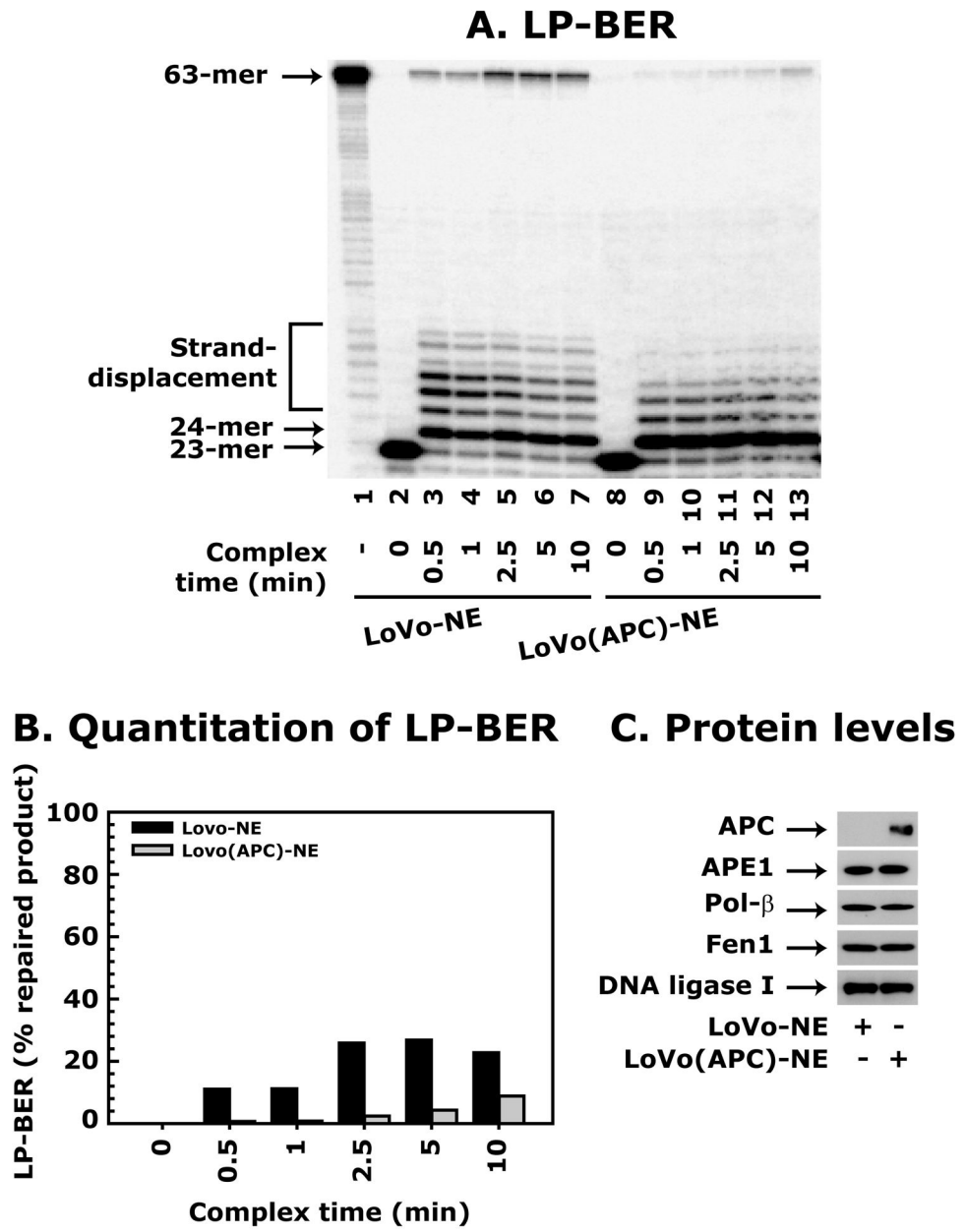

FIG. 4. Analysis of LP-BER activity of multiprotein BER complex isolated from nuclear extract of control and wild-type APC-overexpressed LoVo cells

Panel A shows the effect of APC on the LP-BER activity of multiprotein BER complex isolated from the nuclear extract of control and wild-type APC-overexpressed LoVo cells (complex isolated at varying time intervals of 0–10 min; lanes 3–7 and lanes 9–13, respectively). The reaction mixture containing APE1 precut-^{32}P-F-DNA substrate (2.5 nM) and multiprotein complex was incubated for 60 min at 37°C. Lane-1 shows ^{32}P-63-mer F-DNA and lane-2 shows the 23-mer product. Lane-3 shows Pol-β-mediated 1-nt (24-mer) and strand-displacement (1–7 nt) products. **Panel B** shows the quantitative analysis of LP-BER activity of the BER complex isolated from the nuclear extract of control and wild-type APC-overexpressed LoVo cells. LP-BER activity is expressed as % of repaired (63-mer) product. **Panel C** depicts corresponding protein levels of APC, APE1, Pol-β, Fen1 and DNA ligase I

in LoVo-NE and LoVo(APC)-NE used in LP-BER. Data are representative of two independent experiments.

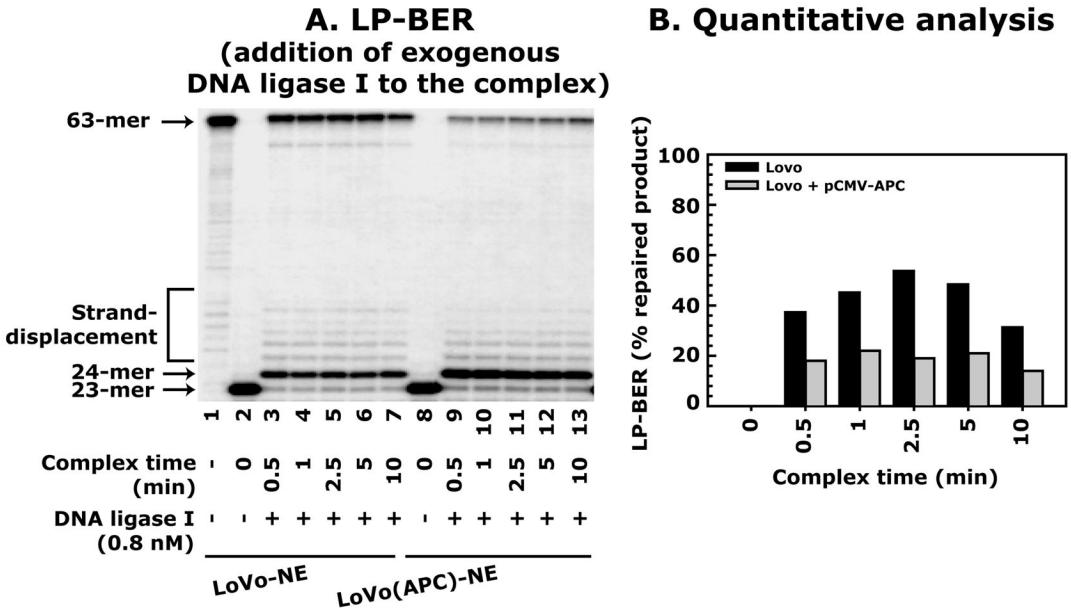

FIG. 5. Effect of supplementation of exogenous DNA ligase I to the multiprotein BER complex isolated from nuclear extract of control and wild-type APC-overexpressed LoVo cells on the LP-BER activity

Panel A shows the effect of supplementation of DNA ligase I to the reaction mixture containing BER complex isolated from the control (lanes 3–7) and the APC-overexpressed (lanes 9–13) nuclear extract of LoVo cells. The reaction mixture containing APE1 precut-^{32}P-F-DNA substrate (2.5 nM), multiprotein complex and DNA ligase I (0.8 nM) was incubated for 60 min at 37°C. Lane-1 shows uncut ^{32}P-63-mer F-DNA, lane 2 shows the 23-mer product after APE1-incision activity and lane 3 shows Pol-β-mediated 1-nt addition to the 23-mer incision product. The strand-displacement products are converted into the repair products. **Panel B** shows the quantitative analysis of LP-BER activity of BER complex in presence of exogenous DNA ligase I. Data are representative of two independent experiments.

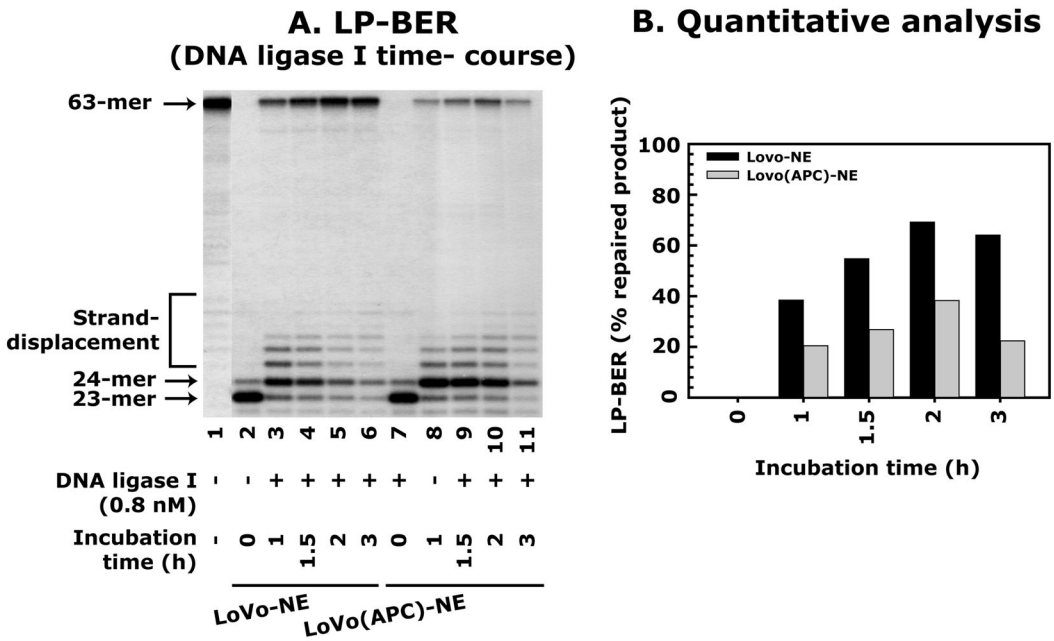

FIG. 6. Effect of increased incubation time on LP-BER activity after supplementation of DNA ligase I to the multiprotein BER complex isolated from nuclear extract of control and wild-type APC-overexpressed LoVo cells

Panel A shows the time-dependent effect of supplementation of DNA ligase I on LP-BER activity of BER complex isolated from the nuclear extract of control (complex assembled on DNA for 10 min; lanes 3–6) and wild-type APC-overexpressed (complex assembled on DNA for 10 min; lanes 8–11) LoVo cells. The reaction mixture containing precut ^{32}P-F-DNA substrate (2.5 nM), multiprotein complex and DNA ligase I (0.8 nM) was incubated for varying time intervals 0–3 h at 37°C. Lane 1 shows uncut-^{32}P-63-mer F-DNA, lane 2 shows the 23-mer product after APE1-incision activity and lane 3 shows Pol-β-mediated 24-mer (1-nt) and strand-displacement synthesis products. **Panel B** shows the quantitative analysis of LP-BER activity of the BER complex in a time-dependent manner in the presence of exogenous DNA ligase I. Data are representative of two independent experiments.