Abasic sites (apurinic/apyrimidinic, AP sites) are the most common DNA lesions generated by both spontaneous and induced base loss. In a previous study we have shown that circular plasmid molecules containing multiple AP sites are efficiently repaired by Chinese hamster extracts in an in vitro repair assay. An average patch size of 6.6 nucleotides for a single AP site was calculated. To define the exact repair patch, a circular DNA duplex with a single AP site was constructed. The repair synthesis carried out by hamster and human cell extracts was characterized by restriction endonuclease analysis of the area containing the lesion. The results indicate that, besides the repair events involving the incorporation of a single nucleotide at the lesion site, the existence of backup systems (5). In a previous study (6) we have shown that circular plasmid molecules containing multiple AP sites are efficiently repaired by Chinese hamster cell extracts in an in vitro repair assay with an estimated repair patch of 6.6 nucleotides for a single AP site, suggesting that tracts longer than one nucleotide are synthesized at the lesion site. The analysis of repair of AP sites in a reconstituted repair system of Xenopus laevis oocytes has provided very convincing evidence that AP sites are repaired not only via a DNA polymerase β-dependent system but also by a PCNA-dependent system (7). Therefore, DNA polymerase δ or ε seems to function in repair of this ubiquitous form of DNA damage. Accordingly, evidence has been recently presented that DNA polymerase δ is required for base excision repair of DNA methylation damage in Saccharomyces cerevisiae (8).

To investigate whether mammalian cells repair abasic sites via alternative repair pathways a circular DNA duplex molecule was constructed with a single AP site in a defined sequence and the repair patch at the lesion was measured in both hamster and human cell-free repair assays.

We provide the first evidence that, in addition to the single nucleotide insertion pathway, a PCNA-dependent longer patch repair system is active in mammalian cells.

MATERIALS AND METHODS

Construction of DNA Molecules Containing a Single AP Site—An oligonucleotide that contains a single uracil, 5'-GATCCTCTAGAGUC-GACCTGCA-3', and a control oligonucleotide, 5'-GATCCTCTAGATC-GACCTGCA-3', were prepared by automated DNA synthesis. Closed circular DNA was produced by priming single-stranded (+) pGEM-3Zf DNA (Promega) with a 30-fold molar excess of uracil-containing or control oligonucleotide and incubating with T4 DNA polymerase holoenzyme, single-stranded DNA-binding protein and T4 DNA ligase (Boehringer Mannheim). Closed circular DNA duplex molecules were purified by cesium chloride equilibrium centrifugation. The single uracil-containing plasmid, pGEM-U, and the control oligonucleotide-containing plasmid, pGEM-T, were stored at −80 °C. The characterization of the substrate was performed on duplex DNA molecules synthesized by using a 5'-2P-labeled oligonucleotide 30 ng of pGEM-U or pGEM-T DNA were then incubated with E. coli uracil-DNA glycosylase.
(1 ng, specific activity: 3.1 × 10⁶ units/mg) at 37 °C for 30 min, followed by digestion with E. coli endonuclease III (0.45 μg, specific activity: 3.8 × 10⁶ units/mg) at 37 °C for 30 min when required. The incubation buffer for both enzymes was 7 mM Hepes-KOH (pH 7.8), 5 mM 2-mercaptoethanol, 2 mM EDTA disodium salt, and 35 mM NaCl. The uracil-DNA glycosylase and the endonuclease III, purified to apparent homo-
geneity as described in (9, 10), were kindly provided by S. Boiteux, Institute Gustave Roussy, Villejuif, France. For the repair experiments, pGEM-U DNA was incubated with uracil-DNA glycosylase immediately before the repair reactions to generate the plasmid pGEM-X, which contains a single abasic site at a known position of its genome. The plasmid pGEM-T was also treated with uracil-DNA glycosylase before incubation with cell extracts.

Plasmid DNA—Plasmid pAT153 (3.7 kb) was UV-irradiated to produce an average of 15 (cytodilute + 6-4) photo products/circle as described previously (6). Plasmid pBR322 (4.3 kb) was used as a control.

In Vitro Repair Synthesis—Extracts from CHO-9 and HeLa cells were prepared as described (6). Repair reactions were carried out with 150 μg of cell extract protein in reaction buffer containing 20 μM dGTP, dCTP, dATP, 8 μM dTTP, and 2 μCi of [32P]dTTP (3000 Ci/mmol). When [32P]dTTP (3000 Ci/mmol) was added to the repair reactions, the concentration of dATP was lowered to 8 μM. In the experiments carried out with human cell extracts the repair reaction buffer contained 10 μM dGTP, dCTP, dTTP, and dATP and 5 μCi of [32P]dTTP (3000 Ci/mmol) and 50 μM dCTP (3000 Ci/mmol). After 3 h at 30°C the DNA was recovered and digested with the appropriate restriction enzymes (40 units for 5 h at 37°C). The digestion products were resolved on 7% top/20% bottom polyacrylamide gels. Purified antibody retained the ability to recognize PCNA as determined by Western blotting.

Preparation of Anti-PCNA Antibody—A polyclonal monospecific anti-PCNA antibody termed 3009, was raised by immunization of a rabbit with keyhole limpet hemocyanin, as described elsewhere. Antibody was purified from whole serum essentially as described by (11). Briefly, 1 ml of immune serum was incubated overnight at 4°C with 1 ml of protein G-Sepharose beads (Pharamacia Biotech Inc.) in phosphate-buffered saline (142 mM NaCl, 7.2 mM Na2HPO4, 3.26 mM NaH2PO4). Beads were loaded onto a 1-ml column that was washed with 10 column volumes of phosphate-buffered saline, then antibody was eluted in 100 mM glycine HCl (pH 2.5) and 0.5-ml fractions neutralized with 50 μl of 2 M Tris-HCl (pH 8.0). Peak antibody fraction was determined by Bradford assays and purity verified on Coomassie-stained polyacrylamide gels. Purified antibody retained the ability to recognize PCNA as determined by Western blotting.

Inhibition of Repair Synthesis by Anti-PCNA Antibodies—Protein extracts from CHO-9 cells were preincubated for 20 min at 30°C with the indicated amounts (0.15 to 4.6 μg) of anti-PCNA polyclonal antibody. Plasmid DNA was then added in the repair reaction, which was allowed to proceed for 3 h at 30°C.

RESULTS

Synthesis of Duplex DNA Containing a Single AP Site—Single-stranded (+) pGEM-3zf DNA was primed with 5′-end [32P]labeled uracil-containing oligonucleotide or control oligonucleotide and DNA was synthesized in vitro on this primed template (Fig. 1). As shown in Fig. 2, in both cases closed circular molecules were obtained with an efficiency close to 100% (Fig. 2A, A and B, lane 1). When the duplex DNA molecules containing the single uracil residue, pGEM-U, were digested with uracil-DNA glycosylase followed by endonuclease III, a complete conversion to Form II was observed (Fig. 2A, lane 4), confirming that these duplex molecules are homogeneous closed circular molecules all containing a single uracil residue. Both pGEM-U and pGEM-T DNA were not significantly cleaved (conversion Form I to Form II <5%) by incubation with uracil-DNA glycosylase (Fig. 2A, lane 2 and Fig. 2B, lane 4) or endonuclease III (Fig. 2A, lane 3 and Fig. 2B, lane 5) alone. pGEM-T and pGEM-X duplex molecules were further characterized by restriction endonuclease analysis (Fig. 2, B and C). As expected from the correct insertion of the primer oligonucleotide, the duplex molecules contained a single BamHI (Fig. 2B, lane 2, and Fig. 2C, lane 7) and a single Acl site (Fig. 2B, lane 3, and Fig. 2C, lane 8). In the case of pGEM-X (Fig. 2C), the presence of the AP site within the recognition sequence of Acl caused a partial resistance to cleavage by this restriction enzyme (Fig. 2C, lane 8). Interestingly, the presence of a methoxyamine-modified AP site in the same sequence leads to a complete inhibition of the cleavage of the lesion-containing strand (data not shown).

Repair of a Single AP Site by Hamster Cell Extracts—The ability of CHO-9 cell extracts to repair the single AP site was tested in vitro (Fig. 3). pGEM-T (lanes 1, 2, 5, and 6) and pGEM-X (lanes 3, 4, 7, and 8) plasmids, which are identical except for the presence of the AP site, were used as substrates to determine the localization and extent of repair synthesis stimulated by the excision of a single abasic site. The restriction fragments XbaI-HindIII, containing the AP site and 6 nucleotides 5′ to it (A), and SalI-PstI, containing the AP site and 9 nucleotides 3′ to it (B), were selected for this analysis. The incorporation of radiolabeled dTMP and dAMP residues was determined in these two fragments, which contain the same number of Ade and Thy residues. Repair synthesis was exclusively AP site-dependent as shown by the lack of incorporation in the undamaged A and B fragments (lanes 1–2 and 5–6, respectively). In the case of the single AP site containing substrate (lanes 3, 4, 7, and 8), incorporation of radioactively labeled dTMP residues was observed in both fragments A (lane 4) and B (lane 8) with a higher incorporation in fragment B. These data indicate that: (i) the replacement of the AP site was predominantly with a dTMP residue, (ii) repair synthesis extended also 3′ to the lesion, and (iii) no significant repair replication occurred 5′ to the abasic site. The extension of the repair patch exclusively 3′ to the abasic site was confirmed by the incorporation of labeled dAMP residues in fragment B (lane 7) but not in fragment A (lane 3). The background incorporation present over the top half of the gel is likely due to unspecific incorporation at nicks which occur randomly along the plasmid genome (12).

The extent of DNA repair replication around the AP site was further characterized by a panel of restriction enzyme diges-
factor PCNA was used. This antibody was raised against the C-terminal 15 amino acids of human PCNA and was able to inhibit completely SV40 replication in an in vitro assay. The ability of this antibody to efficiently inhibit PCNA-dependent repair synthesis was tested in UV-damaged plasmid DNA (Fig. 5). CHO cell extracts were preincubated with 0.15, 1.5, 3.7, or 4.6 μg of purified anti-PCNA antibody for 30 min at 30 °C prior to repair reactions. As shown in Fig. 5 (right panel), the antibody was able to inhibit UV-induced repair synthesis mediated by hamster extracts (compare lanes 1 and 2 with lanes 3–6), causing a reduction of incorporation almost to background levels.

The role of PCNA in the AP site-induced repair synthesis was investigated. CHO cell extracts were preincubated with 3009 anti-PCNA antibody prior to repair reactions on AP site-containing plasmid DNA. As shown in Fig. 6, the repair synthesis 3′ to the AP site (fragment B) was strongly inhibited by anti-PCNA antibody (compare lane 5 with lanes 6 and 7). In contrast, the 1-nucleotide insertion pathway (fragment A) was not inhibited under these conditions (lanes 1–3). The slight increase in repair synthesis observed in the presence of the antibody (compare lane 1 with lanes 2 and 3) indicates that some competition might exist between the two pathways.

These data demonstrate that two repair pathways are involved in AP site repair in mammalian cells. The single nucleotide gap filling is PCNA-independent, while the resynthesis of...
most besides DNA polymerase(s) (DNA polymerase $\delta$ (4, 18), PCNA-dependent polymerase(s)) (DNA polymerase $\delta$ and/or $\epsilon$) might be involved in AP site repair.

Repair of a Single AP Site by Human Cell Extracts—To investigate whether the size heterogeneity of repair patches at AP sites was a peculiar feature of hamster cell extracts, the repair of the single abasic site was analyzed by using HeLa cell extracts. In this case, prior to restriction digestions close to the AP site, the repaired pGEM-X duplex molecules were digested with FokI that presents multiple recognition sites on the plasmid genome. In particular, FokI cuts a fragment 1388 bp long, including the AP site where the abasic site is located 122 bp from the 5’ terminus. The extent of both dTMP and dCMP incorporation was analyzed. As shown in Fig. 7, repair synthesis by HeLa cell extracts was observed in the Sall–PstI fragment (A, lanes 2, 4, and 6) as well as in the Hindl–PstI fragment (B, lanes 3, 5, and 7) independently of the labeled nucleotide used. The identical fragments from the control plasmid DNA incorporated no label (data not shown). In the presence of [32P]dTTP (lanes 4 and 5), the higher level of incorporation detected in fragment A as compared with fragment B indicates that the substitution of a single nucleotide at the AP site is the favored pathway in our cell extracts. The detection of a labeled repair patch in the presence of [32P]dTTP (lanes 6 and 7), which is not incorporated at the lesions site, is the formal proof that resynthesis 3’ to the AP site is indeed occurring also in human cells. The level of incorporation of dCMP in the two fragments was proportional to the number of Cyt residues present in the A or B fragments that might be substituted, indicating that the patch of resynthesis 3’ to the lesion is at
endonuclease, APN1, have shown that a failure to rapidly repair AP sites leads to a strong increase in spontaneous mutation frequency (16). This is presumably due to AP sites arising from the action of endogenous DNA damaging agents such as oxygen radicals or alkylating agents. Similarly, HeLa cell transfectants expressing HAP1 antisense RNA and unable to efficiently repair AP sites are hypersensitive to killing by a range of DNA-damaging chemicals and radiation, including oxygen radical-generating agents and alkylating agents (17). Thus, the repair of AP sites is essential for suppression of mutations and, therefore, vital for genome stability. It is not surprising if backup systems are employed for the repair of these very common and potentially harmful DNA lesions.

In this study we have provided clear evidence that natural AP sites are repaired by two distinct pathways by mammalian cell extracts. Besides the repair route which involves a single nucleotide gap filling reaction at the abasic site, a new pathway was identified that is PCNA-dependent and involves the replacement of a short oligonucleotide containing the AP site and 6–13 nucleotides 3’ to it. Similarly, a PCNA-dependent system for repair of AP sites has been previously identified in X. laevis oocytes (7).

How much is the relative contribution of the two pathways to the repair of abasic sites still remains an open question. The generation of a single nucleotide repair patch seems to be the favored route for AP site repair in mammalian cell extracts, although the PCNA-dependent pathway is undoubtedly active on natural AP sites. The short patch BER pathway has been shown to be specific for AP site repair, while the long patch BER is also able to repair AP site analogues like the tetrahydrofuran residues (7). It might well be that in vivo after cell damage the type and rate of AP site production determines whether the long patch BER pathway enters into action as backup system.

In a recent study aimed at identifying the DNA polymerase(s) requirement for BER in mammalian nuclear extract (18), only DNA polymerase β was shown to be required for the gap filling step following uracil excision. However, it is important to notice that in this study a 51-bp synthetic oligonucleotide, e.g. a linear double-stranded molecule, containing a single uracil was used as substrate. Clear evidence has been provided recently that a stable RF-C-PCNA complex can be assembled on circular but not on linear DNA (19). It has been proposed that RF-C, after binding to DNA, might load PCNA onto circular duplex DNA and then act as a clamp to ensure the correct loading of the replicative polymerase at the synthesis site. The mechanism of assembly of polymerase δ or ε auxiliary proteins on DNA would then be compromised on a linear substrate containing the AP site, thus favoring the PCNA-independent, polymerase β-mediated, repair pathway.

The repair of an AP site via 1-nucleotide replacement involves the generation of a single nucleotide gap as a reaction intermediate. DNA polymerase β is able to catalyze both the excision of the 5’-terminal deoxyribose-phosphate at the incised AP site (20) and DNA synthesis to fill the gap. The repair of an AP site via the PCNA-dependent pathway might imply the formation of a flap structure intermediate. A DNA flap is a bifurcated structure composed of a double-stranded DNA and a displaced single-strand. It will be of interest to investigate whether, in the PCNA-dependent pathway, polymerase δε and 5’-3’-exonuclease of FEN-1/DNase IV (21, 22) may function together to replace the damaged bases by nick translation.

In conclusion, these observations lead to a redifinition of the model of BER in mammalian cells. As proposed by Dianov and Lindahl (23) for E. coli cells, this excision process is likely to be a branched pathway in which, besides the very short patch pathway likely mediated by DNA polymerase β, another sys-
tem enters into the scenario. This repair system results in the replacement of several nucleotides by a PCNA-dependent polymerase, DNA polymerase δ or ε.

**Acknowledgments**—We thank S. Picksley for early work on the development of the anti-PCNA antibody, S. Boiteux for useful suggestions, and M. Bignami for valuable comments on the manuscript.

**REFERENCES**

1. Doetsch, P. W., and Cunningham, R. P. (1990) *Mutat. Res.* 236, 173–201
2. Robson, C. N., and Hickson, I. D. (1991) *Nucleic Acids Res.* 19, 5519–5523
3. Demple, B., Herman, T., and Chen, D. S. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 11450–11454
4. Dianov, G., Price, A., and Lindahl, T. (1992) *Mol. Cell. Biol.* 12, 1605–1612
5. Dianov, G., Sedgwick, B., Daly, G., Olsson, M., Lovett, S., and Lindahl, T. (1994) *Nucleic Acids Res.* 22, 993–998
6. Frosina, G., Fortini, P., Rossi, O., Abbondandolo, A., and Dogliotti, E. (1994) *Biochem. J.* 304, 699–705
7. Matsumoto, Y., Kim, K., and Bogenhagen, D. F. (1994) *Mol. Cell. Biol.* 14, 6187–6197
8. Blank, A., Kim, B., and Loeb, L. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 9047–9051
9. Graves, R. J., Felzenszwalb, I., Laval, J., and O’Connor, T. R. (1992) *J. Biol. Chem.* 267, 14429–14435
10. Dizdaroglu, M., Laval, J., and Boiteux, S. (1993) *Biochemistry* 32, 12105–12111
11. Harlow, E., and Lane, D. P. (1988) *Antibodies: A Laboratory Manual*, p. 310, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. Biggerstaff, M., Robins, P., Coverley, D., and Wood, R. D. (1991) *Mutat. Res.* 254, 217–224
13. Sagher, D., and Strauss, B. (1983) *Biochemistry* 22, 4518–4526
14. Loeb, L. A. (1985) *Cell* 40, 483–484
15. Zhou, W., and Doetsch, P. W. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 6601–6605
16. Ramotar, D., Popoff, S. C., Gralla, E. B., and Demple, B. (1991) *Mol. Cell. Biol.* 11, 4537–4544
17. Walker, L. J., Craig, R. B., Harris, A. L., and Hickson, I. D. (1994) *Nucleic Acids Res.* 22, 4884–4889
18. Singhal, R. K., Prasad, R., and Wilson, S. H. (1995) *J. Biol. Chem.* 270, 949–957
19. Podust, L. M., Podust, V. N., Soga, J. M., and Hubscher, U. (1995) *Mol. Cell. Biol.* 15, 3072–3081
20. Matsumoto, Y., and Kim, K. (1995) *Science* 269, 699–701
21. Harrington, J. J., and Lieber, M. R. (1994) *EMBO J.* 13, 1235–1246
22. Robins, P., Pappin, D. J. C., Wood, R. D., and Lindahl, T. (1994) *J. Biol. Chem.* 269, 28535–28538
23. Dianov, G., and Lindahl, T. (1994) *Curr. Biol.* 4, 1069–1076
Two Pathways for Base Excision Repair in Mammalian Cells
Guido Frosina, Paola Fortini, Ottavio Rossi, Fabio Carrozzino, Giuseppina Raspaglio, Lynne S. Cox, David P. Lane, Angelo Abbondandolo and Eugenia Dogliotti

J. Biol. Chem. 1996, 271:9573-9578.
doi: 10.1074/jbc.271.16.9573

Access the most updated version of this article at http://www.jbc.org/content/271/16/9573

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 22 references, 12 of which can be accessed free at http://www.jbc.org/content/271/16/9573.full.html#ref-list-1