Reconstitution of the DNA base excision-repair pathway

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Background: The base excision-repair pathway is the major cellular defence mechanism against spontaneous DNA damage. The enzymes involved have been highly conserved during evolution. Base excision-repair has been reproduced previously with crude cell-free extracts of bacterial or human origin. To further our understanding of base excision-repair, we have attempted to reconstitute the pathway in vitro using purified enzymes.

Results: We report here the successful reconstitution of the base excision-repair pathway with five purified enzymes from Escherichia coli: uracil-DNA glycosylase, a representative of the DNA glycosylases that remove various lesions from DNA; the AP endonuclease IV that specifically cleaves at abasic sites; RecJ protein which excises a 5' terminal deoxyribose-phosphate residue; DNA polymerase I; and DNA ligase. The reaction proceeds with high efficiency in the absence of additional factors in the reconstituted system. Four of the enzymes are absolutely required for completion of the repair reaction. An unusual feature we have discovered is that the pathway branches after enzymatic incision at an abasic DNA site. RecJ protein is required for the major reaction, which involves replacement of only a single nucleotide at the damaged site; in its absence, an alternative pathway is observed, with generation of longer repair patches by the 5' nuclease function of DNA polymerase I.

Conclusions: Repair of uracil in DNA is achieved by a very short-patch excision-repair process involving five different enzymes. No additional protein factors seem to be required. There is a minor, back-up pathway that uses replication factors to generate longer repair patches.

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Background

Three different excision-repair pathways are universally present in living organisms, and constitute the main cellular strategies for removing lesions and pre-mutagenic errors from DNA. Strand-specific mismatch repair provides protection against occasional rare errors that occur during DNA replication and have escaped proof-reading mechanisms. Nucleotide excision-repair (NER) is responsible for correction of damage that causes major helix distortion, in particular the dipyrimidine adducts generated on exposure of cells to sunlight. Base excision-repair (BER) is the main mechanism for removing 'spontaneous' DNA lesions that are caused by hydrolysis, oxidation or exposure to reactive small molecules such as S-adenosylmethionine [1]. Some of these DNA lesions, including deaminated cytosine residues and abasic sites, cause very little helix distortion but are mutagenic if not repaired.

In general, there is little overlap between the three excision-repair pathways, both with regard to the enzymes involved and the structural alterations in DNA that they recognize. For this reason, there are no cellular back-up mechanisms for the activities that initiate either the mismatch correction or the NER pathway. Loss of these functions therefore has drastic consequences, such as hypermutation and increased cancer frequency in man [2]. On the other hand, duplication of functions and limited overlap with other forms of excision-repair are characteristic of the BER pathway. This may reflect the fact that, in the absence of exposure to large doses of external DNA-damaging agents, the most frequent DNA lesions are those handled by BER, and consequently cellular back-up mechanisms are frequently employed in this essential process. Thus, it has not been possible to construct an Escherichia coli mutant totally deficient in repair of DNA abasic sites [3], implying that continuous correction of spontaneous DNA lesions is required for viability.

The BER pathway is initiated by hydrolytic removal of a base either by one of the several DNA glycosylases that recognize different types of lesions or by non-enzymatic base loss. The pathway proceeds by incision at the abasic site, generation of a gap, repair synthesis and ligation. The entire reaction has been reproduced in vitro with extracts from E. coli [4], Saccharomyces cerevisiae [5], Xenopus laevis [6-8] and human cells [4]. Marked heterogeneity of repair patch sizes has been observed [4,8,9], indicating that there may be more than one route of excision and gap-filling. Using radiolabelled precursors for DNA synthesis and detailed restriction enzyme cleavage, the length of the repaired tract after excision of an uracil residue has been found to be limited to a single nucleotide in most repair events, in both E. coli and human cells, but longer tracts have also been observed [4]. Similarly, most of the repair tracts generated at apurinic sites by fractionated X. laevis cell extracts were very short and dependent on DNA polymerase β, though longer patches of about four nucleotides were also produced in a proliferating cell nuclear antigen (PCNA)-dependent process [8]. In the present work, we have investigated the BER process by employing five
purified enzymes from *E. coli* instead of cell extracts to reconstitute the pathway.

**Results**

**Requirement for a DNA glycosylase in the in vitro reaction**

Uracil residues in DNA can be generated by cytosine deamination and are repaired efficiently *in vivo* by a BER process initiated by uracil-DNA glycosylase [10]. Double-stranded oligonucleotides containing a centrally placed G-U base pair, surrounded by appropriate restriction enzyme sites, have been employed as substrates for *in vitro* reactions [4]. In previous work with *E. coli* extracts, the dUMP residue was replaced efficiently by a dCMP residue. No incorporation of newly synthesized material occurred on the 5' side of the non-conventional residue, whereas small amounts of exchanged residues were observed on the 3' side. These results were independent of the base composition of the oligonucleotide and the sequence adjacent to the uracil residue.

We have used a similar strategy in introductory experiments with cell extracts, but to minimize problems of non-specific degradation and turnover of the terminal residues of the oligonucleotides, the terminal residues were joined by phosphorothioate bonds (Fig. 1). Repair synthesis of substrates was assessed in an [α-32P]dCTP-containing reaction mixture, using denaturing gel electrophoresis and autoradiography for analysis of the oligonucleotide product. Incorporation of radioactive material was completely dependent on the presence of a dUMP residue in the double-stranded oligonucleotide substrate (Fig. 2a). An extract of an ung strain, deficient in uracil-DNA glycosylase, showed no detectable repair of the uracil-containing oligonucleotide, whereas an isogenic ung+ strain was clearly repair-proficient (Fig. 2b).

Comparison of the two repair-proficient strains NH5033 (sbcB, recB, endA) and W3110 indicates that neither exonuclease I, RecBCD nuclease nor endonuclease I are involved in the repair process. When the double-stranded substrate was recovered from the reaction mixture and analyzed by HpaII cleavage prior to denaturing gel electrophoresis, 80-90% of the incorporated 32P-dCMP was found at the previous position of the dUMP residue rather than on its 3' side, as estimated from the amount of incorporation in the 12-nucleotide-long fragment.
In agreement with previous results [4], no detectable incorporation of radioactive material occurred on the 5' side of the dUMP residue (data not shown). The presence of a U rather than a C residue within the HpaII recognition site makes the oligonucleotide refractory to restriction enzyme cleavage. As >95% of the oligonucleotide substrate had been converted to a form susceptible to HpaII cleavage, the in vitro repair reaction had proceeded virtually to completion (Fig. 2c). These data with oligonucleotides containing terminal phosphorothioate bonds are in agreement with previous results [4] and demonstrate more clearly the absolute dependence of the reaction on the ung gene product.

Using low concentrations of the five *E. coli* enzymes — uracil-DNA glycosylase, endonuclease IV, RecJ protein, DNA polymerase I and DNA ligase — >90% replacement of the dUMP residue with dCMP was achieved with a double-stranded DNA substrate (Fig. 3). The substrate for this experiment had the same sequence as the oligonucleotide in Fig. 1a, but lacked terminal phosphorothioate bonds and contained a 5'32P residue in the upper strand. At the end of the repair reaction, the oligonucleotide duplex was recovered, deproteinized, divided into two aliquots, one of which was treated with HpaII, and subject to denaturing gel electrophoresis and autoradiography. Repair was detected as conversion of the oligonucleotide to a full-length form susceptible to HpaII digestion (Fig. 3, lanes 4 and 9). In the experiments with cell extracts (Fig. 2), ~2 pmoles of uracil-containing oligonucleotide duplex were repaired in 20 minutes at 37°C, so in the reconstituted repair system the lowest concentrations of each enzyme that would allow a similar level of excision-repair were determined and used.

When all five enzymes were present, full-length oligonucleotide was regenerated and was cleaved by HpaII to yield a 12-mer, demonstrating replacement of uracil by cytosine and completion of the BER reaction. If uracil-DNA glycosylase was excluded from the reaction mixture, no repair occurred, as judged from the resistance of the oligonucleotide to HpaII digestion (Fig. 3, lane 6). This result is in agreement with the experiment using an ung− cell extract (Fig. 2b). Similar data were obtained when the AP endonuclease was excluded from the
reaction mixture. In this case, reactions were stopped by NaBH₄ treatment [4] to stabilize the abasic site during subsequent analysis. A very slight mobility shift of the 30-mer was observed, caused by conversion of an internal dUMP residue to an abasic site (Fig. 3, lanes 2 and 7). In the absence of DNA polymerase I, cleavage of the uracil-containing substrate occurred on the 5’ side of the dUMP residue by the concerted action of uracil-DNA glycosylase and endonuclease IV to generate a 5’-32P-labelled 11-mer (Fig. 3, lane 3), but rejoining did not occur as E. coli DNA ligase shows little or no activity at an incised abasic site. These results demonstrate that the expected reaction intermediates could be isolated, and that the crude cell extract used previously for the BER in vitro reaction could be efficiently replaced with purified enzymes.

When a non-radiolabelled substrate (Fig. 1a) was used in an [α-32P]dCTP-containing reaction mixture with the same amounts of reagent enzymes as in Figure 3, replacement of the dUMP with a dCMP residue was visualized directly (Fig. 4, lane 6). In agreement with the data in Fig. 3, no incorporation of radioactive material occurred in reaction mixtures lacking uracil-DNA glycosylase, endonuclease IV or DNA polymerase I (Fig. 4, lanes 1–3). In the absence of DNA ligase, a heterogeneous array of radioactive large fragments were observed, most likely generated by strand displacement and gap-filling by DNA polymerase I (Fig. 4, lane 4). In the absence of RecJ protein, a larger amount of radioactive material was incorporated into the repaired 30-mer (Fig. 4, lane 5), even more than in the fully repaired substrate from the complete reaction mixture (Fig. 4, lane 6; compare Fig. 3, lanes 4 and 9). This effect may be dependent on excision of dRp residues as part of small oligonucleotides by Pol I and generation of larger repair patches.

Role of RecJ protein in minimizing the size of repair patches

In order to assess further the potential role of RecJ protein in counteracting extensive gap-filling by DNA polymerase I, the product of the reconstituted repair reaction was analyzed by HpaII cleavage and two different concentrations of DNA polymerase I were used in parallel reaction mixtures. At a relatively high concentration of DNA polymerase I (0.5 unit), a larger amount of repair synthesis was observed in the absence than in the presence of RecJ protein (Fig. 5a). The increased repair synthesis in the absence of RecJ protein was almost entirely due to incorporation at sites on the 3’ side of the dUMP residue rather than at this residue, as seen from the distribution of incorporated radioactive material between the two HpaII fragments (Fig. 5a). This is consistent with the increased strand displacement, removal of dRp as part of a small oligonucleotide by DNA polymerase I, and longer repair patches generated in the absence of RecJ protein. At a 10-fold lower concentration of DNA polymerase I (0.05 unit), the results were different in that RecJ protein now promoted DNA repair synthesis (Fig. 5b, lanes 1 and 3). In the presence of RecJ protein, the amount of repair synthesis was similar with low and high concentrations of DNA polymerase I (Fig. 5a, lane 2 and Fig. 5b, lane 3) and repair occurred mainly by one-nucleotide replacement, as demonstrated by digestion of the product with HpaII (Figs. 5a and 5b, lane 4).

The 5’→3’ exonuclease function of DNA polymerase I is known to be strongly but not completely inhibited by a base-free deoxyribose-phosphate residue at the 5’ terminus [12], so the removal of dRp apparently proceeded poorly in the absence of a separate excision function such as the RecJ protein. In fact, the small amount of resynthesis observed in the absence of RecJ protein at a low DNA polymerase I concentration (Fig. 5b) most likely represented removal of a small proportion of 5’-terminal dRp residues by non-enzymatic β-elimination, rather than by the 5’→3’ exonuclease activity of DNA polymerase I, to generate small amounts of single-nucleotide patches (Fig. 5b, lane 2). The increased efficiency of generation of one-nucleotide patches in the
The uracil-containing oligonucleotide duplex (Fig. 1a) was incubated in a reaction mixture containing the enzymes necessary to reconstitute the reaction. RecJ protein was present or absent, as indicated. One half of each mixture was treated with HpaII, as indicated, prior to analysis by gel electrophoresis. (a) Reactions at a moderately high concentration (0.5 unit) of DNA polymerase I. (b) Reactions at a low concentration (0.05 unit) of DNA polymerase I.

The presence of RecJ protein (Fig. 5b, lane 4) also shows that DNA polymerase I at a low concentration more readily fills a one-nucleotide gap with a 5'-terminal nucleotide residue than a gap with a remaining dRP residue at the 5' end. Thus, a single-nucleotide gap rather than an 'overhang' or 'flap' structure with a single 5' displaced dRP residue is the most likely reaction intermediate in this very short patch repair process.

A 5' terminal dRP residue at a strand interruption in DNA may be removed in a β-elimination process rather than by hydrolysis. Such events are promoted efficiently by the E. coli Fpg protein [15], and at a slower rate by a variety of basic proteins and polyamines [16,17]. Both hydrolysis and β-elimination events generate identical one-nucleotide gaps in DNA, so they would seem equally useful strategies in this regard, although in the latter process the product excised is an unsaturated aldehyde form of dRP. We have been able to substitute the RecJ protein with the same amount of Fpg protein in reconstitution experiments such as that shown in Fig. 5 to obtain similar results (data not shown). Thus, the separate dRP excision function required for the one-nucleotide replacement pathway can be carried out by at least two different enzymes with equivalent results.

In the alternative pathway, a 'patch-and-cut' strategy is used, involving strand displacement of the 5' terminus with the dRP residue. This is followed by incision due to the structure-specific 5' nuclease activity of DNA polymerase I [20], which cuts at the branch point to release a single-stranded displaced oligonucleotide. Results with E. coli [4] and X. laevis [7,8] extracts indicate that such repair patches are usually only two to five nucleotides long, but occasionally much longer displaced regions are generated [21]. Another minor back-up pathway could involve cutting on both sides of an intact or incised abasic site by the UvrABC nuclease; a gap about thirteen nucleotides long would be generated, which would be repaired by the NER pathway. The UvrABC enzyme can slowly incise abasic sites [22], although it is much less efficient than AP endonuclease, and this pathway could be of relevance if the dRP-excision activity is low.

In view of the size heterogeneity of repair patches, average estimates based on overall incorporation of radioactive precursors are of limited value, and more detailed analysis of the DNA product is required. Similar observations of repair patch heterogeneity have been made with either covalently closed DNA circles [8] or double-stranded oligonucleotides [4] as substrates. The occurrence of an alternative pathway dependent on strand displacement by DNA polymerase I explains an early study by Verly et al. [23], in which apurinic sites in DNA could be repaired by incubation with the major E. coli AP endonuclease (exonuclease III), DNA polymerase I and T4 DNA ligase. This alternative pathway, which generates an 'overhang' structure of single-stranded DNA [24] that could interact with related DNA sequences, also offers a satisfactory explanation for the hyper-recombinogenic phenotype of E. coli mutants defective in dUTPase, the 5' nuclease activity of DNA polymerase I or DNA ligase, all of which would result in increased or longer-lasting 'overhang' reaction intermediates [24,25].

Discussion

A general model for the BER process (Fig. 6) may be proposed on the basis of the results described above and recent results obtained with cell extracts and partially purified components [4,8]. This model is similar to previous schemes [18,19], but the existence of a branched pathway and the events associated with removal of the deoxyribose-phosphate residue at an incised abasic site are new features. The main route, which has been reproduced with purified enzymes in the present study, involves generation of a single nucleotide gap as reaction intermediate and appears to be a 'cut-and-patch' process. After DNA glycosylase and AP endonuclease have acted, the terminal dRP residue is excised. The AP lyase activity of endonuclease III, which may occasionally promote β-elimination at intrinsic abasic sites, and most 5'→3' exonucleases of E. coli, are unable to release a 5'-terminal dRP residue in free form to generate a one-nucleotide gap, but enzymes such as RecJ and Fpg proteins possess the necessary activity.

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Fig. 6. Scheme for the branched BER pathway. The major, very short-patch pathway, reconstituted in the present work with purified enzymes, is shown in the left branch. A minor pathway, resulting in replacement of several nucleotides, is shown in the right branch. Newly incorporated residues are indicated with bold, yellow-highlighted bases. See text for details.

The E. coli enzymes active in the reconstituted pathway have direct counterparts in human cells, and the main features of the reaction would be expected to be retained in all living cells. Thus, uracil-DNA glycosylase is one of the most highly conserved proteins between E. coli and man [26]; human AP endonuclease [27] has biochemical properties very similar to those of E. coli endonuclease IV; human DNA polymerase β can replace E. coli DNA polymerase I in vivo [28]; and, similarly, a DNA ligase-deficient E. coli strain can be complemented with the catalytic domain of a human DNA ligase [29]. Mammalian cells also contain a 50kD activity that
References

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Materials and methods

Reagent enzymes

The following E. coli enzymes were purified from enzyme-overproducing strains as described: uracil-DNA glycosylase [33], endonuclease IV [34], RecJ protein [13,14] and Fpg protein [15]. E.coli DNA polymerase I, DNA ligase, exonuclease III and T4 polyribonucleotide kinase were obtained from Boehringer-Mannheim. Restriction endonuclease HpaII was purchased from New England Biolabs, Inc.

Cell extracts and oligonucleotide substrates

E. coli NH5033 (recB, shB, endA) was obtained from S.C. West, and E. coli BD10 (ung, thyA, deoC) and its ung+ parent strain were from B. Weiss. Cell extracts (~10 mg ml⁻¹ protein) were prepared by the lysozyme-EDTA method [35] and stored as aliquots at −80°C. Single-stranded oligonucleotides were prepared on a commercial DNA synthesizer. Double-stranded oligonucleotides were made by mixing single-stranded ones in equimolar amounts, as described [4]. Formation of the appropriate double-stranded molecules was verified by gel electrophoresis under non-denaturing conditions prior to use. Double-stranded oligonucleotides, 5'-32P-labelled in one strand, were made by incubation of single-stranded oligonucleotides with T4 polynucleotide kinase and [y-32P]ATP, followed by annealing with a complementary strand and gel purification.

BER reactions

Standard reaction mixtures with purified DNA repair enzymes (50 μl) contained 0.1 M Tris-Cl (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM NAD, 20 μM each of dATP, dGTP and TTP, 2 μM dCTP including 2 μCi [α-32P]dCTP (3000 Ci mmol⁻¹, Amersham), 5 ng uracil-DNA glycosylase, 5 ng endonuclease IV, 50 ng RecJ protein, 0.05–0.5 units of DNA polymerase I, and 0.2 units DNA ligase. The components were mixed at 0°C and reactions were initiated by addition of 2 pmoles duplex oligonucleotide and transfer of the reaction mixture to 37°C. Thus, the ratio in reaction mixtures between dCTP molecules and uracil residues in oligonucleotide form was 50:1. After 20 min at 37°C, reaction mixtures were chilled, and 5 μl 3 M NaCl containing 0.1 μg ml⁻¹ tRNA and 50 μl of phenol-chloroform (1:1) were added. After agitation and centrifugation, the aqueous phase was recovered and supplemented with 3 volumes of ethanol. The precipitate was recovered by centrifugation, washed twice with ethanol, and dried under vacuum.

For restriction enzyme analysis, the duplex oligonucleotide was redissolved in 50 μl 10 mM Tris-Cl (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol and digested with 50 units HpaII for 60 minutes at 37°C. Reactions were stopped by phenol-chloroform extraction in 0.3 M NaCl followed by ethanol precipitation. The dried precipitate was dissolved in 10 μl 80 % formamide, 0.1 % xylene cyanol, 0.1 % bromophenol blue. After heating at 90°C for 2 minutes, the material was loaded onto a 15 % polyacrylamide gel containing 7 M urea in 90 mM Tris-borate/2 mM EDTA (pH 8.8) and electrophoresed at 30 mA, followed by drying of the gel and autoradiography. Band intensities on autoradiograms were quantified with an LKB Ultrascan XL scanning laser densitometer. Reactions containing crude cell extracts instead of purified enzymes were carried out in a similar reaction mixture as described [4], and were analyzed as above.

Conclusions

The main pathway of the DNA base excision-repair process results in replacement of a single nucleotide residue and has been reproduced at high efficiency with a mixture of five different enzymes: a DNA glycosylase to excise the damaged base, an AP endonuclease for incision at the abasic site, a deoxyribophosphodiesterase for excision of the 5' terminal deoxyribophosphate residue, a DNA polymerase for gap-filling, and a DNA ligase for rejoicing. The results with the five purified enzymes are similar to those obtained with crude cell extracts, indicating that no additional enzymes or accessory protein factors are required for the reaction. However, a minor alternative pathway also exists and generates longer repair patches. This route is initiated in the same way by a DNA glycosylase and an AP endonuclease, but subsequent DNA strand displacement and resynthesis would be expected to occur in a way closely related to the later stages of NER or lagging-strand DNA replication, and to require similar activities. The arrangement provides increased versatility for this major repair process, which is used to counteract spontaneous cellular DNA damage.

References


