Repair of DNA damage in a mitochondrial lysate of Xenopus laevis oocytes

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ABSTRACT

We examined DNA repair activities of a mitochondrial lysate derived from Xenopus laevis oocytes. Plasmid DNA, exposed to HCl, H2O2 or UV light, was used as the substrate for the in vitro repair reaction. DNA synthesis in the lysate was stimulated 2–8-fold by such lesions, indicating the presence of excision repair activities. This repair DNA synthesis was not affected by aphidicolin, but was sensitive to N-ethylmaleimide. Thus the mitochondrial DNA polymerase, i.e., pol γ is indeed involved in the reaction. Actual repair of the depurinated DNA was demonstrated by using the polymerase chain reaction (PCR), where the amount of the amplified DNA fragment increased significantly if the depurinated template was incubated in the lysate prior to the PCR. UV-irradiated DNA, on the other hand, restored its ability as a PCR template only if the repair reaction was carried out under the light. Therefore, in this system, UV-induced damage is repaired mainly by photoreactivation. These results show that mitochondria of Xenopus oocytes possess excision repair as well as photolyase activities, and that the in vitro repair system described here should be useful for further molecular characterization of such DNA repair machinery.

INTRODUCTION

The animal cell contains several hundreds of mitochondria, each having multiple copies of circular DNA. This DNA carries 37 essential genes including those for the components of the electron-transport chain, and thus its stable maintenance is important for survival of the cell. However, unlike the nuclear DNA, the mitochondrial genome is not protected by histones from various genotoxic agents. Furthermore, the oxidative environment inherent to this organelle creates very unfavorable conditions for the stability of DNA (1). Therefore it is reasonable to assume that mitochondria have some effective means of repairing DNA damage frequently generated in their genome. Defects in such mechanisms may be the cause of sequence alterations like those found in patients afflicted with mitochondrial encephalomyopathy or in normal adults of advanced age (2,3).

Because of their compartmentalized structure, mitochondria are presumed to have their own repair machinery distinct from that of the nucleus. Although early studies did not reveal the activity to repair pyrimidine dimers in mitochondria (4,5), several repair-related enzymes such as AP endonuclease, UV endonuclease, uracil DNA glycosylase and methyl transferase have been isolated and characterized (6–8). It has further been reported that mitochondria have the ability to remove DNA damage generated by alkylation or oxidation (8–10). More recently, a homologue of the Escherichia coli MutS protein has been purified from yeast mitochondria, suggesting that a mismatch repair pathway is also operative (11). Despite all these findings, systematic studies of each repair pathway as a whole have not been fully explored. We decided to initiate such studies employing an in vitro repair system derived from isolated mitochondria.

Oocytes of Xenopus laevis are well suited for the source of mitochondria, because they have an extremely large number of this organelle that accumulates up to 10^7 per oocyte in the course of oogenesis (12–14), and are available in large quantities throughout the year. Furthermore it was shown that a lysate of oocyte mitochondria contains the activity of replicating mitochondrial DNA (15). Thus such an in vitro system may also be useful for biochemical studies of the DNA repair machinery. In this paper, we provide evidence that a similarly prepared lysate of mitochondria in fact possesses the ability to repair various types of DNA lesions such as those caused by depurination, oxidation or UV-irradiation.

MATERIALS AND METHODS

Preparation of the mitochondrial lysate

Ovaries were removed from five mature females of X.laevis. They were rinsed in OR-2 medium (5 mM HEPES pH 7.6, 1 mM Na2HPO4, 83 mM NaCl, 2.5 mM KCl, 1 mM MgCl2 and 1 mM CaCl2), and gently homogenized in the Dounce homogenizer using five strokes of the loose-fitting pestle. The homogenization buffer, which was used at 9 ml per 1 g of ovaries, contained 10 mM Tris–HCl pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 15% (w/w) sucrose. Mitochondria were obtained from the homogenate as described by Brun et al. (16) with minor modifications. Thus the oocyte homogenate was cleared by
Preparation of a crude oocyte extract depleted of mitochondrial proteins

An oocyte extract, which contains nuclear and cytosolic material but is depleted of mitochondrial proteins, was prepared as follows. The details of the procedure will be published elsewhere (Ryoji et al., manuscript in preparation). Briefly, ovaries were treated with 300 µg/ml of collagenase for 16 h at 20°C to separate oocytes from follicle cells. Oocytes were washed, transferred to the extraction buffer (25 mM HEPES pH 7.5, 50 mM KCl, 2.5 mM MgCl₂, 1 mM dithiothreitol and 2 mM β-mercaptoethanol), and concentrated by filtration to 10 mg protein/ml. It was aliquoted and stored at −85°C.

Preparation of plasmid DNA carrying various lesions

Partially depurinated DNA was obtained by treatment of supercoiled plasmid pUC9 with 2–10 mM HCl for 30 min at 20°C. The solution was neutralized with 1 M Tris–HCl pH 7.6, and DNA was recovered by ethanol precipitation. Nicked, open circular DNA was prepared in a 110 µl mixture containing 10 µg of supercoiled pUC9 DNA, indicated quantities of DNase I, 50 mM Tris–HCl pH 7.6, 85 mM KCl, 3 mM MgCl₂, 50 µM CaCl₂, 5.5 µg bovine serum albumin and 5% (w/w) glycerol. The mixture was incubated for 2 min at 24°C. DNA was purified by phenol–chloroform extraction. Oxygen free radical-induced lesions were generated in supercoiled pUC9 DNA by incubation with 3.2 mM H₂O₂ and 50 µM CuSO₄ for 2 min at 37°C as described (18). UV-induced damage was formed by irradiating supercoiled pUC9 DNA (94 ng/µl) under the short-wavelength UV lamp to a fluence of 900 J/m².

DNA repair reactions in vitro

The reaction mixture (11.7 µl) for excision repair consisted of 10 ng pUC9 DNA, 40 µg mitochondrial lysate proteins, 2–10 µCi [α-³²P]dCTP, 10 µM dNTP but dCTP, 50 mM HEPES pH 7.5, 5 mM MgCl₂, 3 mM ATP, 30 mM creatin phosphate, 0.12 µg creatin kinase and 1.5 mM β-mercaptoethanol. Unless otherwise stated, it was incubated for 2 h at 37°C. To assess the effects, if any, of such nicks on DNA synthesis, supercoiled DNA was treated with 500 IU of T4 DNA polymerase according to the standard protocol (17). The size of the amplified fragment was expected to be 889 bp.

RESULTS

DNA synthesis induced by abasic sites

We first studied whether DNA synthesis for excision repair can be seen in the lysate of mitochondria. Figure 1A shows a time course of [α-³²P]dCTP incorporation into depurinated pUC9 DNA (lanes 7–9). Undamaged DNA was used as a control (lanes 1–3). Although DNA synthesis continued for at least 4 h in both cases, depurinated DNA displayed a much higher incorporation. A Southern analysis performed in parallel confirmed that the template DNA was present in about equal quantities in all the cases (Fig. 1B). Note in this figure that the radioactivity contributed by the DNA synthesis was negligibly small in comparison with the hybridization signals of the Southern analysis. Therefore, the densities of DNA bands can be taken directly as the quantities of the template DNA.

We noticed that the depurinated DNA, though it was initially supercoiled, was gradually converted to the nicked circular form during storage (compare lanes 1 and 7 in Fig. 1B). To assess the effects, if any, of such nicks on DNA synthesis, supercoiled DNA was briefly digested with DNase I, and DNA which contained the open circular form to the same extent as seen in the depurinated DNA was used as a template for DNA synthesis (compare lanes 4 and 7 in Fig. 1B). A quantitative analysis of the DNA bands in Figure 1A showed that the depurinated DNA incorporated the radioactivity eight times as much as the undamaged control did (Fig. 1C). On the other hand, the presence of nicks per se did not increase DNA synthesis.
whether the covalently closed, relaxed ones (form Ir). Therefore it is not clear if circular molecules include both nicked circles (form II) and a population of supercoiled ones. It should be noted that the open α measure of the excision repair reaction in vitro

As seen in Figure 1A, DNA molecules which incorporated [α-32P]dCTP were mostly open circles with only a minor population of supercoiled ones. It should be noted that the open circular molecules include both nicked circles (form II) and covalently closed, relaxed ones (form Ir). Therefore it is not clear whether the excision repair reaction described here proceeded efficiently to the final ligation step. To know the extent of the repair reaction in this system, we electrophoresed the radioactive DNA molecules in a buffer containing 15 µg/ml of chloroquine phosphate. It is known that, in the presence of this concentration of chloroquine phosphate, covalently closed but relaxed circles (form Ir) migrate ahead of the other types of molecules, whereas nicked circles (form II) move as the slowest species (19; see arrows in Fig. 2). It is evident that the majority of the radioactive DNA molecules carried nicks or gaps, and thus the repair reaction was not completed in most DNA molecules. Nevertheless, once the repair patches were sealed, DNA molecules became supercoiled rapidly as evidenced by the faint bands corresponding to the fully supercoiled form, i.e., form I.

Effects of aphidicolin and N-ethylmaleimide on the excision repair reaction

Eukaryotic cells contain at least five species of DNA polymerase, i.e., pol α, β, γ, δ and ε (see ref. 20 for a review). Among them, pol γ is localized exclusively within the mitochondrion, and participates in replication of the mitochondrial genome (16). The other polymerases are present largely in the nucleus, and are responsible for replication as well as repair of the chromosome. To establish that DNA synthesis described above was carried out by the mitochondrial polymerase but not by nuclear or cytosolic contaminants, we looked at the sensitivity of the DNA synthesis to aphidicolin. This inhibitor blocks pol α, δ and ε but does not affect pol β and γ activities (21–24). For comparison, the excision repair reaction was also performed in a crude extract of oocytes depleted of the mitochondrial fraction. It was found that DNA synthesis in the mitochondrial lysate was refractory to aphidicolin up to a concentration of at least 100 µg/ml (Fig. 3, upper panel). In contrast, DNA synthesis in the crude oocyte extract was inhibited by 50% at 5 µg/ml. Thus aphidicolin-resistant pol β or γ is responsible for the repair DNA synthesis in the lysate of mitochondria.

To rule out the possibility of an involvement of pol β, we analyzed the effect of N-ethylmaleimide (NEM) on DNA synthesis. NEM at a concentration of 2 mM is known to inhibit pol α, γ and δ by >50%, whereas even 10 mM of NEM does not affect pol β (23,24). As shown in Figure 3 (lower panel), DNA synthesis in the mitochondrial lysate was as sensitive to NEM as that in the oocyte extract. We concluded that the excision repair activity described here is indeed carried out by the mitochondrial DNA polymerase, i.e., pol γ, but not by nuclear enzymes possibly contaminating the mitochondrial preparation.

Figure 1. DNA synthesis induced by abasic sites. Depurinated pUC9 DNA was incubated in the lysate of oocyte mitochondria for 0, 2 and 4 h as described in Materials and Methods. DNA was then separated by agarose gel electrophoresis. (A) The agarose gel was dried and subjected to autoradiography to monitor the incorporation of [α-32P]dCTP. Lanes 1–3, undamaged pUC9 DNA was used as a control; lanes 4–6, DNA nicked with 4 × 10^{-6} U of DNase I was used as a control; lanes 7–9, DNA depurinated by exposure to 2 mM HCl was used. I, supercoiled circles; Ir, relaxed but covalently closed circles; II, nicked circles. (B) The same samples were analyzed by Southern blotting. The hybridization probe was 32P-labeled pUC9 DNA. (C) The data shown in (A) were quantified using the Molecular Imager (Bio Rad). Incorporation after 2 and 4 h of incubation was shown.

Figure 2. Conformation of pUC9 DNA after excision repair reaction. The material shown in lane 9 in Figure 1A was electrophoresed in a chloroquine-containing agarose gel to separate form Ir from form II (lane 2). Nonradioactive supercoiled pUC9 was run side by side as a conformational marker (lane 1). After electrophoresis, DNA in the gel was transferred to nylon membrane. Radioactive bands in lane 2 were detected by direct autoradiography, whereas DNA bands in lane 1 were detected by hybridization using 32P-labeled pUC9 as a probe. The position of form Ir was estimated from the data reported previously (19), and no corresponding radioactive bands were detected in lane 2, I, topoisomers of the supercoiled form; III, linear DNA.
Nucleic Acids Research, 1996, Vol. 24, No. 20

Figure 3. Effects of aphidicolin and N-ethylmaleimide on repair DNA synthesis. (Top) Depurinated DNA was incubated in the mitochondrial lysate under the standard conditions except for the addition of aphidicolin at various concentrations. After incubation, DNA was electrophoresed, and the radioactivities in the DNA bands were quantified by densitometric tracing of the autoradiograph of the gel (open circles). The data were normalized against the case of no aphidicolin. For comparison, DNA synthesis in the oocyte extract was analyzed in the same way (solid circles). All the reaction mixtures contained 1 mM exogenous, unlabeled dCTP to keep the dCTP:aphidicolin ratio the same in both cases, since aphidicolin is known to compete with dCTP. The endogenous dCTP concentration estimated by the isotope dilution assay was <0.1 mM in both cases, and thus did not affect the analysis that was performed in the presence of 1 mM exogenous dCTP. (Bottom) Incorporation of [\(\alpha\)-\(32\)P]dCTP was analyzed as those shown in the top panel except that aphidicolin was replaced with NEM.

DNA synthesis induced by other types of damage

Plasmid pUC9 DNA exposed to H\(2\)O\(2\) was incubated in the lysate of mitochondria. The oxidative damage presumably includes strand breaks and altered bases like 8-hydroxyguanine and thymine glycol (25). Since we noticed that the open circular form of DNA increased in quantity from 12 to 36\% upon oxidation of DNA, we prepared a control DNA sample which was intentionally nicked with DNase I to the extent where a similar percentage of the open circular DNA was present. This DNA was compared with the oxidized one in regard to their abilities to induce DNA synthesis. As shown in Figure 4, DNase I-induced nicks alone did not significantly stimulate DNA synthesis, whereas the template carrying oxidative damage caused a 4-fold increase in incorporation. This indicates that oxidative damage can also be repaired through an excision repair pathway in Xenopus mitochondria.

In contrast with the abasic sites and the oxidative damage, UV-induced lesions did not increase DNA synthesis significantly over that caused by the undamaged DNA (Fig. 5). A stimulation of DNA synthesis became detectable only when the fluence of irradiation was raised to a level of 1800 J/m\(^2\). On the other hand, we observed an ~10-fold stimulation even at 900 J/m\(^2\) if DNA was incubated in the crude oocyte extract (data not shown).

Restoration of DNA bases by the repair reaction

The damage-dependent DNA synthesis described above does not necessarily infer that the lesion is actually repaired. We wanted to see an actual reduction of the lesions upon incubation in the lysate. It was shown that the movement of *Taq* DNA polymerase on the template is arrested when it encounters damaged bases. Thus several investigators reported that the PCR product increases in quantity if the damaged template is subjected to a repair reaction prior to the PCR (see, e.g., ref. 26). We carried out such an assay to examine whether depurinated or UV-irradiated DNA is actually restored from the damage when incubated with the mitochondrial lysate. To determine how much of the template should be used for the PCR analysis, various amounts of undamaged plasmid DNA were subjected to PCR. The 889 bp PCR product increased linearly if no more than 1 pg of the template was used (Fig. 6, upper panel). Thus we took an aliquot containing 1 pg of DNA from the repair reaction mixture, and then used it for PCR (Fig. 6, lower panel). When the template that
had been depurinated with 3 mM HCl was subjected directly to PCR, the amount of the amplified fragment was only 20–30% of the control. On the other hand, prior incubation of the same template in the lysate resulted in an ∼70% increase in the amount of the 889 bp fragment. Therefore some of the abasic sites are repaired in the mitochondrial lysate. If the template was exposed to 10 mM HCl, the repair was not effective enough to be detected by this method.

UV-damaged DNA was also subjected to the same analysis (Fig. 7). The lesions that severely impeded PCR were repaired almost completely in the light reaction, but not in the dark reaction. Thus the photolyase activity, rather than excision repair, is the major mechanism in *Xenopus* mitochondria to repair UV-induced lesions.

**DISCUSSION**

The damage-dependent DNA synthesis we observed is an indication of excision repair activities of *Xenopus* oocyte mitochondria. We tested three types of DNA damaging agents, i.e., HCl, H2O2 and UV-light, and all of them generated lesions that caused considerable increases of DNA synthesis. In the case of HCl-mediated, depurinated lesions, the PCR analysis revealed that the restoration of DNA bases was only partial when the damaged DNA was incubated in the lysate for 4 h. This result is due in part to the fact that the excision repair reaction did not proceed efficiently to the completion of gap filling and subsequent ligation of the nick (Fig. 2). Although the damage itself might be excised, the remaining gap, if not sealed completely, would impede the movement of Taq DNA polymerase. It should be mentioned that the same PCR analysis cannot be applied to the oxidative damage, since lesions like 8-hydroxyguanine allow improper base pairings, and do not effectively block Taq DNA polymerase (27). These data provide the first direct evidence of the excision repair activity of mitochondria. To further characterize the nature of such reactions in detail, it would be necessary to introduce more defined damage at specific sites in the template DNA.

According to the present studies, DNA synthesis in response to UV-damage was relatively low compared with that observed in the crude extract of oocytes. This is consistent with the fact that the PCR analysis did not reveal any restoration of the UV-induced damage if the reaction was performed in the dark. However, the damage was repaired by photoreactivation, though the complete repair required an extended incubation time (6 h) and a larger quantity of the lysate (80 μg protein). Incubation under the standard conditions (4 h, 40 μg protein) resulted in only ∼15% of restoration (data not shown). It remains to be seen whether the
mitochondrial photolyase described here differs from that observed in the nucleus.

We hope that further studies in this system will uncover a number of features of the mitochondrial repair machinery distinct from that of the nucleus.

REFERENCES
