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Double strand break rejoining by mammalian mitochondrial extracts

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ABSTRACT

DNA end-joining was measured by incubating linearized plasmid DNA with mitochondrial protein extracts. A spectrum of end-joined molecules ranging from re-circularized monomer to dimer and higher molecular weight forms was observed. The DNA end-joining reaction required ATP and Mg^{2+}, and was inhibited by sodium chloride. Both cohesive- and blunt-ended DNA molecules were end-joined, although the former were more efficient substrates. Molecular analysis of re-joined molecules revealed that >95% of the linearized DNA were precisely end-joined. The few imprecisely end-joined molecules recovered, sustained deletions that spanned direct repeat sequences. The deletions observed are strikingly similar to those present in mitochondrial genomes of patients with Kearns–Sayre or Pearson syndromes, certain ophthalmic myopathies and the aged. These results suggest that mammalian mitochondria possess a DNA double strand break repair activity similar to that seen in the nucleus, and that this repair pathway may play a role in the generation of mitochondrial DNA deletions associated with a number of human pathologies.

INTRODUCTION

Mammalian mitochondrial DNA (mtDNA) accumulates mutations at a 10-fold higher rate than nuclear DNA (1,2). Interest in the integrity of mtDNA has greatly increased since the observation that specific mtDNA mutations are associated with several human diseases (3). Deletions in the mitochondrial genome are detected in the brains of aged humans (4,5), in neurodegenerative disorders such as Kearns–Sayre syndrome (6,7), Pearson syndrome (8), some myopathies (9) and chronic external ophthalmoplegia (10). Similar mtDNA deletions are seen in tissues of aged rodents (11,12). It is unclear whether this increased incidence in mtDNA damage is due to increased susceptibility to damage and/or lack of appropriate repair pathways (13,14).

Recent reports have begun to shed light on DNA repair pathways in the mitochondria (15). Base excision repair pathway has been well characterized in the organelle (16,17). Lesions caused by alkylating agents are efficiently removed, suggesting the presence of an alkyltransferase-dependent repair mechanism (18–21). There is no evidence for the removal of lesions such as bulky adducts indicating the absence of a nucleotide excision repair mechanism in the mitochondria (20,22,23). However, DNA damage caused by 4-nitroquinoline, which creates complex lesions thought to be removed via the nucleotide excision repair pathway, is repaired in the mitochondria (24). Other types of damage such as that induced by bleomycin, which generates lesions such as abasic sites, single strand and double strand breaks are also removed from mtDNA (25,26). Intrastrand cisplatin adducts are repaired with a greater efficiency in the mitochondria compared to the repair in nuclear DNA, while interstand cross links are repaired at similar rates in both mitochondria and nucleus (20). The exact mechanism of cisplatin-adduct removal is unknown but it is thought to involve a recombination event in addition to an excision mechanism (27).

Genetic and biochemical evidence suggest that distinct DNA repair pathways function in the nucleus and the mitochondria. In the case of xeroderma pigmentosa complementation group D cells, the repair of N-methyl purines is deficient in the nucleus but proficient in the mitochondria (28). Mitochondrial-specific forms of some of nuclear DNA repair enzymes have been identified, such as methyl transferase (18), uracil-DNA glycosylase (29,30), apurinic/apyrimidinic endonuclease (31), ultraviolet endonuclease (32) and oxidative damage endonuclease (33). It has been shown that nuclear and mitochondrial forms of uracil-DNA glycosylase are encoded by the same gene. The mitochondrial form of the protein is translated from an alternately spliced mRNA that encodes an N-terminal mitochondrial targeting domain. The catalytic domains of the nuclear and mitochondrial forms of this enzyme are identical (34). A recent paper presented immunological evidence suggesting that *Xenopus laevis* possesses a mitochondrial form of the previously identified nuclear DNA ligase III. The authors concluded that this protein is involved in base excision repair in the mitochondria of frogs and speculated a similar role for this gene in other vertebrates (35).

Repair of lesions induced by bleomycin and cisplatin suggests the existence of a double strand break repair (DSBR) pathway in the mitochondria. In the mammalian nucleus, DSBR occurs by either end-joining or by homologous recombination pathways (36). Considerable evidence suggests that non-homologous end-joining is the predominant mode of nuclear DSBR (37–42). Based on the evidence that DNA repair pathways in the mitochondria are analogous yet distinct from those in the nucleus, it is reasonable to propose the existence of mitochondrial-specific DSBR. Our lab has detected homologous recombination activity in mammalian mitochondrial protein extracts (43). This observation,

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coupled with data indicating that mtDNA deletions and duplications occur in patients with Kearns–Sayre syndrome and chronic external ophthalmoplegia, suggests that unequal homologous recombination may occur within the mitochondrial genome (44,45). Alternatively, since non-homologous DNA end-joining is also associated with the generation of deletions that span direct repeat sequences (40), it is conceivable that mammalian mtDNA deletions arise via a non-homologous DNA end-joining pathway that functions within the mammalian mitochondria.

We therefore investigated the ability of mitochondrial protein extracts to catalyze the repair of DNA double strand breaks by end-joining. A cell-free reaction based on the repair of double strand breaks introduced into plasmid DNA via restriction enzyme digestion was performed. Using a strategy similar to that pursued by North *et al*. (46) to characterize DSBR in mammalian nuclear extracts, we analyzed the recovered products. In this way, we examined both the efficiency and the precision of the DNA end-joining by the mitochondrial protein extracts. Our results indicate that mitochondrial protein extracts possess DNA end-joining activity. End-joining is highly precise in the case of DNA with cohesive ends while blunt-ended DNA rejoins with reduced efficiency and precision. Molecular characterization of the imprecisely repaired products revealed deletions spanning two direct repeats in all cases. These deletions are similar to those observed in the mtDNA of certain pathological states as well as in aging cells.

MATERIALS AND METHODS

Isolation of mitochondria

Mitochondria were prepared from livers of either Fischer 344 rats or mice of a variety of different strains and their purity checked as described (47). Cells were homogenized and the filtrate centrifuged three times at 1000 *g* for 10 min at 4°C, to remove unbroken cells, cell debris and nuclei. Mitochondria were collected from the supernatant by centrifugation at 20 000 *g* for 20 min. The mitochondria were washed three to four times to obtain a crude mitochondrial pellet. In some cases, this was further subjected to density gradient centrifugation on 30% percoll. The purity of the preparation was confirmed by assaying for the mitochondrial marker enzyme, cytochrome c oxidase.

Electroporation of intact mitochondria

Intact mitochondria obtained from mouse liver were suspended in 0.33 M sucrose and electroporations carried out as described (48). Briefly, 5 µg of pUC118 plasmid linearized with different restriction enzymes was added to 100 µl of 100 mg/ml mitochondrial suspension. Electroporation was carried out using a Gibco BRL Gene pulser at a field strength of 2.44 kV/cm.

Preparation of mitochondrial protein extracts

Isolated mitochondria were lysed by hypo-osmotic shock and proteins separated from the membrane fraction by centrifugation at 70 000 r.p.m. for 30 min in a Beckman TL 100/3 rotor at 4°C. The resulting material was dialyzed against buffer containing 50 mM Tris, pH 7.5, 0.1 mM EDTA, 10 mM β-mercaptoethanol, 10 mM phenylmethylsulfonyl fluoride and 10% glycerol. Protein concentration was determined using the Bradford assay (49). The release of mitochondrial contents was confirmed by assaying for malate dehydrogenase activity. Cytochrome c oxidase and malate dehydrogenase enzyme assays were carried out in a Beckman spectrophotometer according to methods previously described (50).

End-joining reactions

Circular pUC118 plasmid DNA was linearized using restriction endonucleases (New England Biolabs, Beverly, MA). An aliquot of 1 µg of linearized DNA was incubated with 10 µg of the mitochondrial protein extract in 70 mM Tris, pH 7.5, buffer in the presence of 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP, in a total volume of 25 µl. The reaction was carried out either at 14°C for 8–12 h or at 37°C for 30 min. Samples were then extracted with phenol–chloroform and precipitated with ethanol. An aliquot of the sample was run on an agarose gel to check for DNA recovery.

DNA adenylation

DNA adenylation reactions were carried out as previously described (51). An aliquot of 5 µg of the mitochondrial protein extract was incubated with 0.5 µCi [α-^{32}P]ATP in the presence of 60 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT and 50 µg/ml BSA in a 20 µl reaction volume. The reaction was carried out at room temperature for 15 min and terminated by boiling in the presence of SDS-loading buffer. The adenylated product was separated on a 10% SDS–PAGE, fixed in 10% acetic acid and exposed to PhosphorImager screen.

Bacterial transformation

Electroporated intact mitochondria were resuspended in 100 µl DNase buffer (0.33 M sucrose, 2 mM magnesium acetate, 10 mM Tris–HCl, pH 7.5) and incubated with 20 µg/ml DNase I for 1 h at room temperature to digest non-internalized DNA. DNase I was inactivated at 65°C for 10 min in the presence of EDTA and treated with 1 mg/ml proteinase K for 4 h at 37°C. Mitochondria were lysed with SDS and the internalized DNA extracted with phenol–chloroform, precipitated with 0.1 vol of 3 M sodium acetate, 3 vol of 100% ethanol and 20 ng of carrier yeast tRNA at –20°C overnight. The DNA pellet obtained was resuspended in TE buffer. Equal amounts of DNA recovered from intact mitochondria or following end-joining reactions in the presence of purified mitochondrial protein extracts were electroporated into electrocompetent *Escherichia coli* DH10B. Transformants were recovered on an LB plate containing ampicillin, IPTG and 5-bromo, 5-chloro, 3-indoyl, β-D-galactoside (X-gal) (52).

Molecular characterization of end-joined products

To visualize the end-joined molecules, linearized DNA exposed to mitochondrial protein extract was treated with proteinase K at 37°C for 30 min and electrophoretically separated on a 0.8% agarose gel in Tris–borate–EDTA buffer at 0.55 V/cm for 12–15 h (52). In some cases, the samples run on the agarose gel were transferred onto nitrocellulose membrane and probed with radioactive pUC118 (specific activity 5×10^6 c.p.m./µg DNA). Bacterial transformants harboring imprecisely end-joined plasmids were recovered as white colonies on an ampicillin plate containing IPTG and X-gal. Plasmid DNA was isolated by alkaline lysis method (52), characterized by restriction mapping and sequenced using a Perkin-Elmer automated DNA sequencer (Microchemical Facility, University of Minnesota).

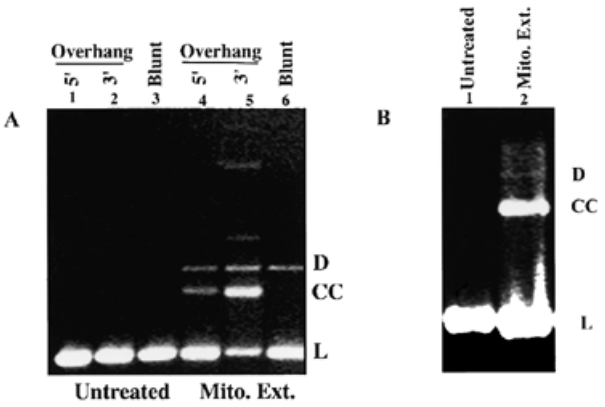

Figure 1. Analysis of the products of DNA end-joining reactions. DNA end-joining reactions were carried out at 4°C for 12 h (**A**) or at 37°C for 30 min (**B**) as described in the Materials and Methods. Samples loaded on a 0.8% agarose gel, electrophoretically resolved, and stained with ethidium bromide. L and CC indicate the mobility of linear and closed circular monomer DNA, respectively; D indicates the mobility of linear dimer DNA.

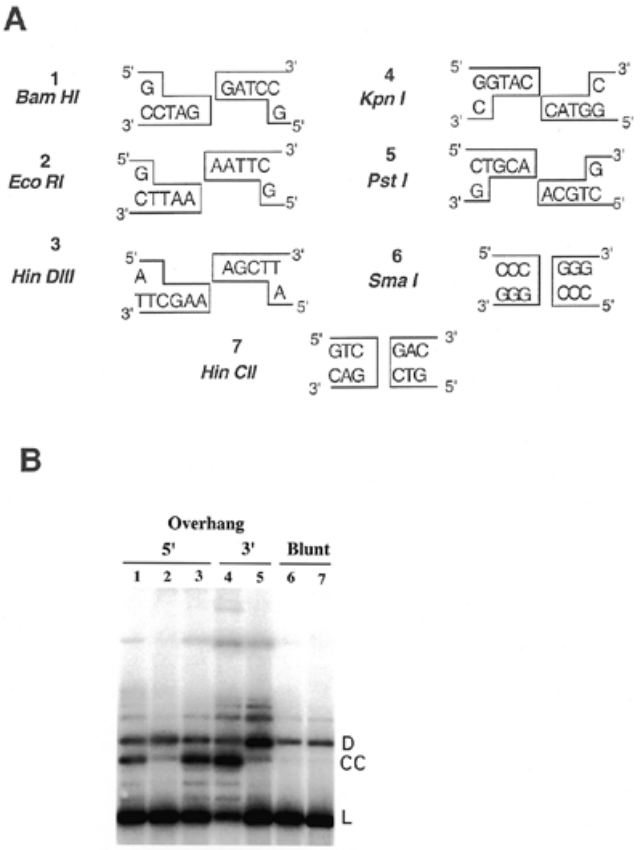

Figure 2. DNA end-joining activity is not sequence dependent. (**A**) Sequences at the cleavage site of different restriction enzymes. (**B**) End-joining reactions were performed as in Figure 1, and a Southern blot-hybridization performed using pUC118 end-labeled with [α-^{32}P]dATP. DNA was linearized with the following enzymes: lane 1, *Bam*HI; lane 2, *Eco*RI; lane 3, *Hin*dIII; lane 4, *Kpn*I; lane 5, *Pst*I; lane 6, *Sma*I, lane 7, *Hin*cII.

RESULTS

End-joining activity of mitochondrial extracts

The ability of mitochondrial protein extracts to promote end-joining of linearized substrates was assessed. pUC118 plasmid DNA linearized with *Bam*HI, *Kpn*I or *Hin*cII to generate either 5′ cohesive overhang, 3′ cohesive overhang or blunt-ended DNA (1 μg), respectively, was incubated with mitochondrial protein extracts (10 μg) at 14°C. Following incubation, DNA was subjected to agarose gel electrophoresis and stained with ethidium bromide. Untreated linear DNA are shown in Figure 1A, lanes 1–3. Incubation of linear DNA with cohesive ends with mitochondrial protein extracts, yielded molecules ranging from closed circular (CC) and linear dimer (D) to other higher molecular weight forms (Fig. 1A, lanes 4 and 5). DNA with blunt ends end-joined to a relatively lower extent compared to DNA with cohesive ends and the products were mainly dimers (Fig. 1A, lane 6). The results presented in Figure 1A were obtained from experiments in which linearized DNA was incubated with mitochondrial protein extracts for 12 h at 14°C. However, we have determined that the end-joining reaction is >50% completed in the first 4 h. We further observed that the pattern of products formed does not change with time (data not shown). The pattern of product formation was also independent of the protein concentration over the range of 0.1–1.0 mg/ml.

The product formed following incubation of linearized DNA with mitochondrial extracts could either be due to a simple ligation reaction or due to DNA end-joining which requires accessory proteins apart from a ligase. To distinguish between the two reactions, end-joining was carried out at 37°C, since most known mammalian ligases are inactive *in vitro* at physiological temperature and need accessory proteins for their activity (53). As linearized DNA with 3′ overhangs were better substrates for end-joining by the mitochondrial extracts at 14°C, this substrate was used to measure end-joining reaction at physiological temperature. Figure 1B shows the pattern of end-joined products formed following incubation of linearized DNA with mitochondrial extracts for 30 min at 37°C: lane 1 shows untreated linearized DNA. Following incubation with mitochondrial protein extract rejoined products were formed (Fig. 1B, lane 2). The products formed corresponded to mainly closed circular and dimer forms. Longer incubation times did not lead to more product formation but instead, the substrate became non-ligatable, suggesting phosphatase or nuclease in the extract is active at this temperature (data not shown). The above results indicate that the mitochondrial extracts prepared from rat liver catalyze end-joining of linearized DNA substrates. Similar end-joining reaction was also detected in mitochondrial protein extracts prepared from other mammalian sources such as human fibroblast cells (HT1080) and Chinese hamster V79 cells (data not shown).

To provide additional evidence that the end-joining activity was not dependent on the sequence present at the break-site, experiments were carried out using DNA linearized with various restriction enzymes. Plasmid DNA was linearized using restriction enzymes *Bam*HI, *Eco*RI and *Hin*dIII to generate 5′ overhangs, *Kpn*I and *Pst*I to generate 3′ overhangs and *Sma*I and *Hin*cII to generate blunt-ended DNA (Fig. 2A). Following incubation of the linear DNA with the mitochondrial protein extracts, the products were separated on an agarose gel and analyzed by Southern blotting using a radiolabeled pUC118 probe to increase the sensitivity of detection. As was the case with the data

presented in Figure 1A, DNA with 3′ overhangs (Fig. 2B, lanes 4 and 5) showed relatively greater amounts of product formation compared to DNA with 5′ overhangs (Fig. 2B, lanes 1–3). Of the three substrates with 5′ overhangs, those cut with *Hin*dIII (Fig. 2B, lane 3) and *Bam*HI (Fig. 2B, lane 1) were more efficiently end-joined than was the *Eco*RI-linearized substrate (Fig. 2B, lane 2). The minor differences in the efficiency of the end-joining seen amongst these latter substrates can most likely be attributed to the thermodynamic stability of the cohesive ends present in the substrates. Thus, plasmids linearized with either *Hin*dIII or *Bam*HI, both of which contain GC-rich cohesive ends, are better substrates than plasmids linearized with *Eco*RI, whose cohesive ends lack any G or C bases. DNA with blunt ends were also end-joining by the mitochondrial protein extracts but the efficiency of the reaction was reduced, compared to DNA with cohesive ends.

The cofactor requirements for the end-joining reaction catalyzed by the mitochondrial extracts are summarized in Table 1. Optimum end-joining activity was observed in the presence of Mg^{2+} and ATP. Absence of either of the two cofactors resulted in a complete loss of activity. dATP could not replace ATP in the reaction. Protein extracts pre-treated with heat at either 45 or 90°C for 15 min failed to show end-joining activity. The end-joining reaction was highly sensitive to salt concentration, with 0.1 M NaCl inhibiting the reaction almost completely. This is a characteristic feature of DNA ligase III (54). Interestingly, it has been previously suggested (35) that a mitochondrial form of this enzyme may exist in vertebrates. The sequence of the human DNA ligase III gene reveals an in-frame ATG present upstream from the putative start site for the nuclear form of ligase III. It is conceivable that translation initiated from this 'upstream' AUG would result in the generation of a novel form of DNA ligase III that contained an N-terminal mitochondrial targeting peptide (35). We therefore performed an adenylation reaction on a mitochondrial extract prepared from HT1080 human fibrosarcoma cells. As shown in Figure 3, we detected a mitochondrial ligase with an apparent molecular weight of ~80 kDa. This molecular weight is identical to that of DNA ligase II, which is known to be a proteolytic fragment of DNA ligase III (55,56). We have also obtained additional evidence suggesting that DNA ligase III is responsible for the DNA end-joining activity we detect in mitochondrial protein extracts. We have prepared mitochondrial protein extracts from human fibrosarcoma cells expressing antisense DNA ligase III mRNA. We observed that levels of the 80 kDa DNA ligase protein were reduced by 75% in these extracts. We also observed a 75–80% reduction in the DNA end-joining activity of these extracts (U.L. and C.C., submitted).

Bacterial transformation

It is known that linearized DNA is far less effective than circular DNA at transforming bacteria (57). Therefore, as a means of providing a more quantitative analysis of the relative ability of DNA substrates to undergo end-joining, different linearized DNAs were incubated with mitochondrial protein extracts, purified by phenol–chloroform extraction and ethanol precipitation, and transformed into DH10B *E.coli*. As indicated in Table 2, treatment with mitochondrial protein extract substantially increased the ability of all DNA substrates to transform *E.coli*. Treatment of cohesive-ended DNA led to a 30–100-fold increase in transformation efficiency, while similar treatment of blunt-ended

Figure 3. DNA adenylation of mitochondrial protein extract. Mitochondrial protein extract (5 µg) prepared from HT1080 fibroblast was incubated with 0.5 µCi [α-^{32}P]ATP for 15 min at room temperature. The adenylated products were separated on a 10% SDS–PAGE, fixed with 10% acetic acid and exposed to PhosphorImager screen.

DNA resulted in an ~3-fold increase. Untreated samples did not yield significant number of colonies. For example, DNA linearized with *Hin*dIII yielded 37 colonies, while *Hin*dIII-linearized DNA incubated with mitochondrial protein extracts, yielded 1098 colonies (Table 2). This indicates that the increase in transformation efficiency is a consequence of end-joining reaction by the mitochondrial extracts and not due to a rejoining reaction carried out within the *E.coli*. In all cases, analysis using the *t*-test indicated that these results were statistically significant ($P < 0.02$). In addition, they support the conclusion, suggested by the data presented in Figure 1, that DNA with cohesive ends are better substrate than blunt-ended DNA molecules.

Table 1. Co-factor requirements

Assay conditions	Activity (% control)
(+)ATP, (+)Mg^{2+}	100
42°C, 15 min	ND
90°C, 15 min	ND
(+)0.1 M NaCl	30
(+)0.2 M NaCl	ND
(+)dATP, (+)Mg^{2+}	ND
(+)ATP, (–)Mg^{2+}	ND
(–)ATP, (–)Mg^{2+}	ND

Products of the end-joining reaction were separated on an agarose gel and the ethidium bromide-stained gel quantitated using the Bio-Rad Molecular Analyst program. Values are presented as percentage to the control reaction which yielded maximum activity (75–80%). ND, products not detected either by ethidium bromide staining or <5% by Southern blot analysis.

End-joining within intact mitochondria *in vitro*

In order to confirm that the end-joining observed with mitochondrial protein extracts was indeed due to the action of mitochondrial proteins, we performed the following experiment. Linearized DNA was introduced into isolated, intact mitochondria via

electroporation at a field strength of 2.44 kV/cm (50). Following incubation at 37°C for 4 h, the plasmid DNA was recovered and transformed into *E.coli* DH10B (Materials and Methods). Bacteria were plated, and the number of colonies obtained was counted. As a control, we performed parallel experiments in which linearized DNA was incubated with isolated, intact mitochondria. In these latter experiments, however, the samples were not subjected to electroporation, and thus the DNA did not gain entry into the intact mitochondria. Following 4 h incubation, DNA was recovered and used to transform DH10B bacteria. As shown in Table 2, in experiments in which the linearized DNA gained entry into mitochondria via electroporation we observed an increase in bacterial transformation efficiency, compared to control experiments. Again, we observed that the transformation efficiency was greater for DNA with cohesive ends, relative to blunt-ended substrate. We also performed control experiments in which 200–400 ng of linearized DNA was directly transformed into bacteria. These experiments failed to yield significant numbers of transformants (not shown). These results indicate that DNA end-joining occurs within isolated, intact mitochondria *in vitro*.

Table 2. Enhanced bacterial transformation of linear DNA following incubation in intact mitochondria or with mitochondrial protein extracts

	Mitochondrial protein extract[a]		Intact isolated mitochondria[b]	
	–	+	–	+
*Hin*dIII	37	1098	0	20
(5′ overhang)	(±4)	(±198)		
*Pst*I	34	3413	2	44
(3′ overhang)	(±6)	(±189)		
*Hin*cII	46	129	1	7
(Blunt)	(±7)	(±15)		

DNA linearized with restriction endonucleases was incubated with mitochondrial protein extracts or electroporated into isolated intact mitochondria. The DNA was recovered and transformed into *E.coli* as described in the Materials and Methods.

[a]Values represent the average of three separate experiments, ±S.E. –, linear DNA; +, linear DNA incubated with mitochondrial extracts.

[b]Values represent the average of two experiments. –, linear DNA incubated with intact, isolated mitochondria without electroporation; +, linear DNA incubated with intact, isolated mitochondria following electroporation.

Precision of end-joining

A DNA end-joining reaction can be either precise or may result in the loss of information at the break site. The DNA substrates used above have double strand breaks within the coding region of a LacZ fusion gene. Imprecise rejoining at this break site would lead to a disruption in the LacZ gene. Bacteria harboring such a plasmid would appear white when grown in the presence of IPTG, and X-gal, while bacteria containing a wild-type LacZ gene will appear blue. This strategy has been used previously to analyze the precision of DNA end-joining catalyzed by nuclear protein extracts (46). Analysis of the bacteria recovered from the transformation experiments described above yielded the data presented in Table 3. A greater percentage of white colonies were recovered from DNA substrates having blunt ends (5%) compared to DNA with cohesive ends (0.07–0.85%). Interestingly, there is an inverse correlation between the efficiency with which substrates are end-joined, and the frequency with which imprecise events were detected. Imprecise end-joining is not a result of processing of the linear DNA ends within the *E.coli* cells as no white colonies were recovered when linearized DNA molecules which had not been treated with mitochondrial extracts were transformed into *E.coli*.

Table 3. Precision of end-joining

Samples	Total colonies counted	White colonies (% total)
5′ Overhang	3295	28 (0.85)
3′ Overhang	3050	2 (0.07)
Blunt	388	18 (4.60)

Linearized DNA incubated with mitochondrial protein extracts was electroporated into DH10B *E.coli*. The transformed bacteria were plated on LB-ampicillin plates containing IPTG and X-gal and the total number of tranformants and the number of white colonies counted.

Analysis of deletions

To gain insight into the molecular mechanism of imprecise end-joining, additional analysis of plasmid DNA recovered from white bacterial colonies was performed. Restriction endonuclease digestion revealed that, as expected, all of these clones had lost the recognition site for the restriction enzyme with which they were originally linearized. In addition, in many cases, the DNA had clearly undergone a deletion. Sequence analysis of a number of independently derived clones was performed, indicating that in all cases the deletion spanned two direct repeats. As Figure 4 indicates, direct repeats were either perfect or imperfect with a single base mismatch. The direct repeat sequences varied from 3 to 5 bp in length, and the deletions generated were between 8 and 218 bp in length. Of the five deletions characterized, three had perfect direct repeats flanking the deletion region while in two cases the direct repeats had single base pair mismatches. In all these cases, one copy of the direct repeat and the intervening sequence between the direct repeats was lost during the end-joining reaction. These deletions bear a striking similarity to those known to occur within the mammalian mtDNA *in vivo* (see below).

DISCUSSION

The results presented above provide compelling evidence that mitochondrial protein extracts prepared from mammalian cells possess DNA end-joining activity. Both cohesive and blunt-ended DNA substrates are rejoined, albeit the latter with much lower efficiency. Irrespective of which DNA substrate was used, the majority of recovered products were precisely repaired. Analysis of imprecisely repaired products revealed the presence of deletions that spanned direct repeat sequences.

This DNA end-joining activity does not result from nuclear contamination. First, the mitochondria used in these studies have been highly purified on a percoll gradient. Only that portion of the gradient possessing mitochondrial marker enzyme activity (cytochrome c oxidase and malate dehydrogenase) contained detectable DNA end-joining activity (data not shown), strongly indicating that this activity is indeed mitochondrial in origin. While a minor nuclear contamination cannot be ruled out, if this were the case, one would expect that the specific activity of DNA end-joining within the nucleus would greatly exceed the activity detected within the mitochondrial fraction. The specific activity of DNA

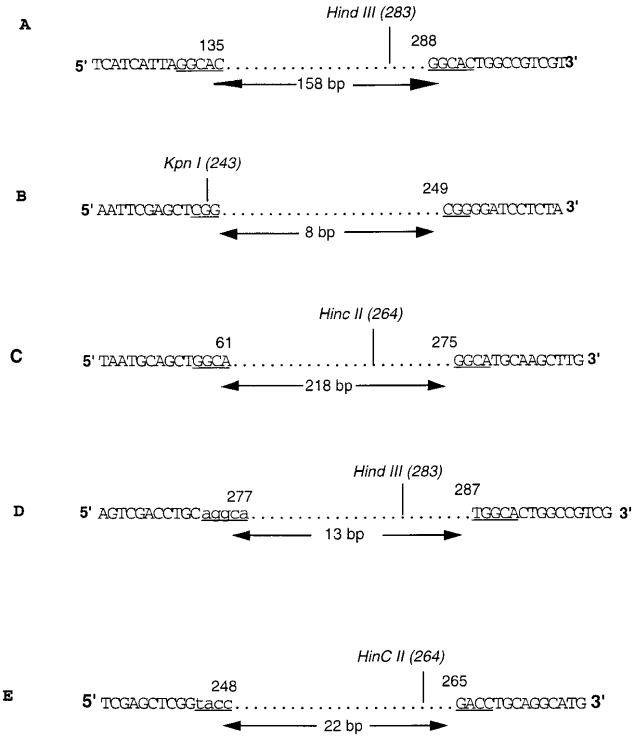

Figure 4. Sequence analysis of imprecisely end-joined plasmid DNA. DNA end-joining reactions were performed and deletion-bearing plasmids identified as described in Materials and Methods. The DNA sequence spanning the deletion was determined. The direct repeat sequences are underlined and the direct repeat sequence lost is represented in lower case (in case of imperfect direct repeats). With perfect direct repeats it is impossible to know which direct repeat is lost and which is retained.

end-joining (defined as the amount of DNA end-joining observed using the agarose gel electrophoresis assay per µg of protein) obtained for the mitochondrial extracts is essentially equivalent to that seen in mammalian nuclear fractions (not shown). Second, in experiments using isolated, intact mitochondria we also detected significant DNA end-joining activity. Since end-joining was dependent upon electroporation, we can conclude that the end-joining reaction occurs within the interior of intact mitochondria, and therefore cannot be due to the actions of any nuclear proteins that may be contaminating the mitochondrial preparation. We are therefore confident that the DNA end-joining activity we describe herein is of mitochondrial origin.

While a majority of the recovered plasmid products of this mitochondrial extract-catalyzed end-joining activity were precise, a number of clones had undergone deletions. Molecular characterization of these deletions revealed a striking similarity to deletions observed within the mtDNA of humans and aged rodents. A majority of the mtDNA deletions generated *in vivo* span direct DNA repeat sequences 5–13 bases in length, allowing for single base mismatches between the repeats (11,12,44,58). In all cases we examined, the *in vitro* generated plasmid DNA deletions were flanked by direct repeats, either perfect or imperfect. The length of the deletions we observed are somewhat smaller than those typically observed in mammalian mtDNA (mtDNA deletions observed *in vivo* range in length from 2 to 7 kb; 59,60). However, substantially larger deletions would not be recovered if they included the plasmid origin of bacterial DNA replication or the ampicillin resistance gene.

The sensitivity of the end-joining reaction to low levels of NaCl and temperature indicates that the mitochondrial form of DNA ligase resembles the nuclear DNA ligase II/III (61,62). The apparent molecular weight of the DNA ligase detected in mammalian mitochondrial protein extracts is identical to that of DNA ligase II, which is a known proteolytic fragment derived from the DNA ligase III gene product. Interestingly, Pinz and Bogenhagen have found that mitochondrial protein extracts prepared from *X.laevis* contain a ligase protein immunologically related to human DNA ligase III (35). Finally, we have recently determined that expression of DNA ligase III antisense mRNA in human fibroblast cells resulted in a significant decrease in mitochondrial ligase levels (U.L. and C.C., submitted). Based on these data, we have tentatively concluded that a novel form of DNA ligase III is responsible for the mitochondrial end-joining activity described in this report.

Our data suggest that two distinct DNA end-joining pathways may function within the mitochondria of mammalian cells. We noted an inverse correlation between the efficiency of mitochondrial extract-catalyzed DNA end-joining, and the frequency with which plasmids bearing deletions were recovered. For example, blunt-ended plasmids, which were inefficiently end-joined, yielded a relatively high percentage (4.6%) of imprecise products. Conversely, cohesive-ended plasmids were more efficiently end-joined, and produced fewer deletion-bearing products (0.07–0.85%). These data are consistent with the hypothesis that compatible ends are efficiently end-joined via one mechanism, whereas non-compatible ends are joined together through a separate pathway. This latter pathway results in the formation of deletions that span direct repeats.

Interestingly, genetic evidence from the yeast *Saccharomyces cerevisiae* suggests that eukaryotes possess at least two distinct nuclear DNA end-joining pathways. The first is dependent on the yeast Ku-70 homolog HDF1, and is precise, while the other is HDF1-independent, and utilizes short stretches of DNA sequences identity between the re-joined molecules (63). Inactivation of the HDF1 gene leads to error-prone repair of linearized DNA (63). Analysis of the mis-repaired DNA in these mutant clones indicated that re-joining was associated with deletions spanning short direct repeat sequences. It is tempting, therefore, to speculate that a defect in mtDNA DSBR could play a causal role in the generation of multiple mtDNA deletions that are observed in patients suffering from genetic disorder autosomal dominant progressive external ophthalmoplegia (64–67).

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