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Mitochondrial DNA Repair Pathways

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It has long been held that there is no DNA repair in mitochondria. Early observations suggested that the reason for the observed accumulation of DNA damage in mitochondrial DNA is that DNA lesions are not removed. This is in contrast to the very efficient repair that is seen in the nuclear DNA. Mitochondrial DNA does not code for any DNA repair proteins, but it has been observed that a number of repair factors can be found in mitochondrial extracts. Most of these participate in the base excision DNA repair pathway which is responsible for the removal of simple lesions in DNA. Recent work has shown that there is efficient base excision repair in mammalian mitochondria and there are also indications of the presence of more complex repair processes. Thus, an active field of mitochondrial DNA repair is emerging. An understanding of the DNA repair processes in mammalian mitochondria is an important current challenge and it is likely to lead to clarification of the etiology of the common mutations and deletions that are found in mitochondria, and which are thought to cause various human disorders and to play a role in the aging phenotype.

KEY WORDS: Mitochondrial DNA; DNA; aging.

NUCLEAR DNA REPAIR PROCESSES

Living cells are constantly exposed to environmental agents and endogenous processes that inflict damage in DNA. Several complex enzymic mechanisms have evolved to repair DNA lesions, and lately there has been tremendous progress toward greater understanding of the mechanisms involved. There are several major pathways of DNA repair and the particular pathway used depends, in part, upon the type of DNA damage that is being removed. An overview of the major pathways is shown in Fig. 1. Most types of cellular stress or damage induce a large spectrum of DNA lesions. Endogenous metabolic processes generate oxygen radicals yielding oxidized bases that are removed from the DNA mainly by the base excision repair (BER) pathway. UV exposure generates two major lesions, or adducts, in the DNA: the pyrimidine dimer (PD) and the 6-4 photoproduct (6-4 PP). Both of these adducts and other bulky lesions are removed by the nucleotide excision repair (NER) pathway.

NER is deficient in the human genetic disorder xeroderma pigmentosum (XP). This condition involves hyperpigmentation of the skin and the continuous development of skin cancers. In XP there are seven different complementation groups representing different genes involved in the disorder. These genes have now been cloned and characterized, leading to a clearer understanding of the NER process in humans. The XP genes together with several other repair factors participate in the NER pathway and it as been possible to reconstitute this process *in vitro*. This pathway has been discussed in some recent reviews (Friedberg, 1996; Wood, 1996, 1997). In the last 10 years evidence has accumulated that the repair process differs significantly in efficiency between different regions of the genome. DNA repair occurs preferentially in genes and, particularly, those that are actively transcribed. The active component of the genome is only about 1% of the total DNA, but it appears to be much more efficiently repaired than the rest of the genome, which is largely inactive. A component of this repair is

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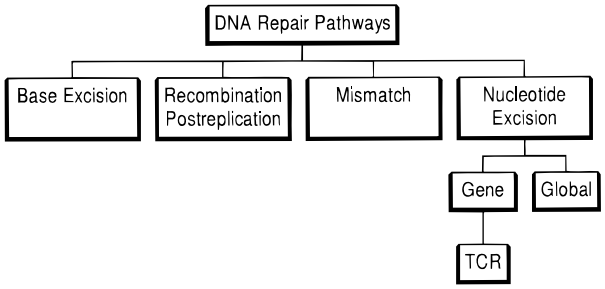

Fig. 1. Major DNA repair pathways in mammalian cells.

directly linked to the basal transcription process. This pathway is also termed transcription-coupled DNA repair (TCR) or "strand-specific" DNA repair, since the transcribed strand of the active genes is preferentially repaired. The molecular link between DNA repair and transcription involves the basal transcription factor TFIIH which, among its nine components, contains products of at least two DNA repair genes, *XPD* and *XPB* (Friedberg, 1996)

One of the most prevalent forms of cellular stress is the constant exposure to reactive species of oxygen or radicals that cause protein and DNA damage. These come from exogenous sources but are even more abundant as products of endogenous reactions, such as cellular metabolism. A large number of DNA base modifications caused by oxidative stress have been detected in various mammalian cells and have been found at higher levels in cancer cells (Wiseman *et al.*, 1995). It is not clear which of these many modifications are of the most biological relevance, but the one that has been most widely studied is 8-hydroxydeoxyguanosine (8-oxo-dG). This is partly due to the availability of techniques to measure this modification at high sensitivity. 8-oxo-dG is a mutagenic lesion, not a blocking lesion. It will often adapt the *syn* conformation (Kouchakdjian *et al.*, 1991) and mispair with adenine during DNA replication and transcription. Most DNA polymerases fail to recognize this mismatch, resulting in G $\rightarrow$ T transversions (Shibutani *et al.*, 1991). The mitochondrial DNA polymerase γ is no exception (Pinz *et al.*, 1995).

Base excision repair of oxidative DNA damage is initiated by DNA glycosylases, a class of enzymes that recognize and remove damaged bases from DNA by hydrolytic cleavage of the base-sugar bond leaving an abasic site (AP site) (Lindahl, 1993). Several DNA glycosylases that recognize and process oxidation products of purines and pyrimidines have been identified in human cells. The human homolog of the bacterial endonuclease III (hNTH1) has been cloned and purified to homogeneity (Aspinwall *et al.*, 1997; Hilbert *et al.*, 1997). It has a structural and functional homology to the bacterial enzyme and possesses a DNA glycosylase activity against a variety of oxidized pyrimidines. Two human genes, hOGG1 and hOGG2, have been cloned. The gene products recognize and process 8-oxo-dG in DNA (Radicella *et al.*, 1997; Roldán-Arjona *et al.*, 1997; Rosenquist *et al.*, 1997). There are at least two pathways for further processing of the arising AP site (Matsumoto *et al.*, 1994; Frosina *et al.*, 1996). One of these is catalyzed by the AP endonuclease, HAP1 (Ape, Apex, Ref1), which cleaves the phosphodiester bond immediately 5′ to the AP site and generates 5′-sugar-phosphate and 3′-OH ends as it nicks DNA (Demple and Harrison, 1994). Removal of the 5′-terminal deoxyribose phosphate residue results in a single nucleotide gap that is then filled by a DNA polymerase and sealed by DNA ligase (Dianov and Lindahl, 1994; Kubota *et al.*, 1996; Singhal *et al.*, 1995). In mammalian cells, DNA polymerase β (pol β) is the major polymerase involved in the single-nucleotide excision patch repair pathway (Singhal *et al.*, 1995; Sobol *et al.*, 1996). It was recently demonstrated that DNA pol β has an intrinsic AP lyase activity that removes 5′-sugar-phosphate by β-elimination and then pol β fills the single-nucleotide gap (Matsumoto and Kim, 1995; Piersen *et al.*, 1996). The hNTH glycosylase and the glycosylases OGG1 and OGG2 also have intrinsic AP lyase activity, which introduces DNA strand breaks 3′ to the baseless sugar (Radicella *et al.*, 1997). An alternative, long-patch BER pathway has been reported (Matsumoto *et al.*, 1994; Frosina *et al.*, 1996). In addition to a DNA glycosylase and AP endonuclease, this pathway also involves flap endonuclease (FEN1), proliferating cell nuclear antigen (PCNA), DNA polymerase δ, and DNA ligase. Neither of these enzymes can remove a 5′ sugar phosphate and generate a one-nucleotide gap. DNA polymerase first adds several nucleotides to the 3′ end of the nick and exposes the 5′ sugar phosphate as part of a single-stranded flap structure. This flap structure is recognized and excised by FEN1 and DNA is finally ligated by DNA ligase (Klungland and Lindahl, 1997). These repair events result in a 2–7 nucleotide long-repair patch. In this reaction, PCNA and probably replication factor C (RF-C), assist in loading the DNA polymerase onto the DNA and also stimulate the endonuclease FEN1 (Li *et al.*, 1995).

MITOCHONDRIAL DNA REPAIR

Progress in the field of mitochondrial DNA (mtDNA) repair has been affected by the observation more than 20 years ago that there was no removal of UV-induced pyrimidine dimers in mtDNA (Clayton *et al.*, 1974). This observation led to the more general notion, not sustained by further data, that there was no DNA repair activity in mitochondria. This general conclusion has been corroborated by several studies, which have concluded that mitochondria appear to accumulate DNA damage. Mitochondria are where oxidative phosphorylation occurs and, consequently, a large number of reactive oxygen species are generated here. Mitochondrial DNA (mtDNA) is not protected by histones and lies in close proximity to the free-radical producing electron transport chain. Early work indicated that mtDNA carried high steady state levels of oxidative damage (Richter, 1992). More recent work indicates that full length genomes—at least in normal young animals—do not carry high levels of damage (Suter, 1999; Anson 1999). MtDNA that contains oxidative damage is confined to a fragmented subset of the total complement (Suter, 1999). Whether this damaged subset is confined to certain mitochondria within cells or perhaps even to a subset of cells has not yet been determined. However, the ability of mitochondria to maintain full length, damage free DNA despite being the primary cellular source of reactive oxygen species argues that mitochondria would be able to repair oxidative DNA damage.

Although UV-induced pyrimidine dimers are not repaired in mitochondria, recent studies clearly show that mitochondria repair their DNA. The DNA repair mechanisms that exist in mitochondria are inferred from the enzymes that have been identified and through the identification of which class of lesions are repaired.

Several observations support that mitochondria are capable of base excision repair. Simple monofunctional alkylating agents are repaired efficiently from mtDNA (Pirsel and Bohr, 1993) and several base excision repair enzymes have been identified in mitochondria. Identification of AP endonucleases in mitochondria suggested that AP sites might be repaired in mitochondria (reviewed in Croteau and Bohr, 1997). Uracil DNA glycosylase was one of the first repair activities detected in mitochondrial extracts (Anderson and Friedberg, 1980). This activity, UDG1, was later purified from human cells by affinity chromatography (Caradonna *et al.*, 1996). It is a 30-kDa protein, encoded by the same gene that encodes the 36-kDa nuclear form of the protein (Nilsen *et al.*, 1997).

Several other repair enzymes have been detected in mammalian mitochondria. The mouse and human homologs of the yeast OGG1, which excises 8-oxo-dG from DNA, have been reported to contain mitochondrial localization sequences (Rosenquist *et al.*, 1997). Whether or not OGG1 and mtODE are the same protein has not yet been determined. Three of the four isoforms of hOGG1, the human homolog of of the yeast OGG1, have been shown in immunohistochemical studies to be present in the mitochondria. It was shown in the same study that the human homolog of the E. *coli* nth gene product, hNTH1, which excises oxidized pyrimidines such as thymine glycol from DNA, is also present in mitochondria (Takao *et al.*, 1998). So too is hMYH, which excises adenine from DNA when it occurs opposite 8-oxo-dG (Takao *et al.*, 1998). Rather than an extensive review of the DNA repair proteins found in mitochondria, we have listed a number of important observations together with the literature reference in Table I.

Studies on mitochondrial DNA damage and repair have traditionally required the purification of mitochondria and mtDNA. As an alternative approach, the gene-specific repair assay (Bohr *et al.*, 1985) was modified to detect various DNA lesions in addition to UV-induced dimers. Bacterial repair enzymes that recognize and cleave the DNA at specific lesions are used. Oxidative lesions can be detected in the entire mitochondrial genome or in parts of it and can be compared to the lesions present in the nuclear DNA from the same biological sample. In addition, strand bias, or transcription-coupled repair (TCR), can be assayed with this approach.

Using the gene-specific repair assay and a variety of DNA-damaging agents, repair of strand breaks and alkali-sensitive sites has been demonstrated in rodent and human mitochondrial DNA (Croteau and Bohr, 1997). With this approach, it is not necessary to isolate or purify the mitochondrial DNA, but instead, the removal of lesions in the mitochondrial DNA is revealed by specifically probing total cellular DNA. This procedure also avoids the oxidation of the mitochondrial DNA, which may occur during the extraction procedure. The steps involved in this experiment are shown in Fig. 2.

The formamidopyrimidine DNA glycosylase (Fpg) enzyme detects 8-oxo-dG and the ring-opened FaPy lesion. It cleaves the DNA at the site of the lesion using its associated endonuclease activity. Repair of

Table I. DNA Repair Mechanisms in Mitochondria

Direct reversal of DNA damage
DNA photolyase activity detected in *Xenopus*[a]
O^6-Ethylguanine transferase[b]
Methyltansferase[c]
Base Excision Repair
Enzyme activities detected: uracil DNA glycosylase[d]
8-oxoG endonuclease[e] class I
and II AP endonucleases[f]
dRP lyase activity[g]
Repair detected of singlet oxygen damage[h]
monofunctional alkylation[i]
4 NQO damage[j] and strand breaks[k]
Mismatch Repair
mut S homolog detected in yeast[l]
Recombination Repair
Cisplatin interstrand crosslinks repaired in rodent cells[m]
In vitro recombination detected in human cells[n]

[a] Ryoji *et al.*, 1996.
[b] Satoh *et al.*, 1988.
[c] Myers *et al.*, 1988.
[d] Anderson and Friedberg, 1980.
[e] Croteau *et al.*, 1997.
[f] Tomkinson *et al.*, 1988, 1990.
[g] Pinz and Bogenhagen, 1988; Longley *et al.*, 1998.
[h] Taffe *et al.*, 1996; Anson *et al.*, 1998.
[i] Pettepher *et al.*, 1991; LeDoux *et al.*, 1992.
[j] Snyderwine and Bohr, 1992.
[k] Shen *et al.*, 1995, Driggers *et al.*, 1996.
[l] Reenan and Kolodner, 1992.
[m] LeDoux *et al.*, 1992.
[n] Thyagarajan *et al.*, 1996

Fpg-sensitive sites in mitochondrial DNA has been reported for rat cells (Driggers *et al.*, 1993), CHO cells (Taffe *et al.*, 1996) and human cells (Anson *et al.*, 1998). In the study by Taffe *et al.* (1996), acridine orange plus light (AO/light) was used as a method to generate oxidative damage; Fpg protein was used in the gene-specific repair assay to assess repair of Fpg-sensitive sites. The AO/light-induced DNA damage was repaired from both mitochondrial and nuclear DNA sequences. Approximately 65% of the lesions were repaired within 4 h and the repair in the mtDNA was as fast or faster as in the nuclear dihydrofolate reductase (DHFR) gene, which was also assayed in the experiments. In this study, it was also observed that the frequency of lesion induction was no higher in the mitochondrial DNA than in the endogenous nuclear gene. The mechanism of mitochondrial repair was further examined by Anson *et al.* (1998), who assayed the repair of 8-oxo-dG from human mitochondrial DNA using the gene-specific repair assay. Again, the Fpg enzyme was used to detect the lesions. In this case, the cells were exposed to methylene blue plus light before the repair; this treatment has been shown to be highly specific for the induction of 8-oxo-dG (Schneider *et al.*, 1990; Boiteux *et al.*, 1992; Ravanat and Cadet, 1995). Anson *et al*. found no strand bias of the repair and thus determined that the removal of 8-oxo-dG lesions from mtDNA was not via a transcription-coupled pathway. This was further asserted by measuring the repair in different regions of the mitochondrial genome where the transcription rate differs substantially. The repair in different regions of the mitochondrial DNA did not vary, but appeared to be homogeneous (Anson *et al.*, 1998). This is shown in Fig. 3, where it can be seen that the repair of various regions in the mtDNA in human fetal lung fibroblasts is fairly uniform.

The efficient repair of the Fpg-sensitive sites suggested that mitochondria may contain a base excision repair Fpg homolog. Recently, an 8-oxo-dG incising activity has been partially purified and characterized from rat liver mitochondria in this laboratory (Croteau *et al.*, 1997). This enzyme, called MtODE, has properties that are similar to OGG1, but appears to be unique. We have characterized this enzyme and some of its properties; they are listed in Table II. Using different approaches including comparison of the activity colocalized with mitoplasts (mitochondrial inner membrane and matrix) versus with the mitochondrial outer membrane, we are certain that the enzyme is purely mitochondrial and not a nuclear contaminant (Croteau *et al.*, 1997). The enzymic activity and its characteristics resemble those reported for OGG1, but studies so far using antibodies have not identified this enzyme as OGG1. Because of its low abundance, it has, thus far, not been possible to purify sufficient quantities for microsequencing, but this approach is in the works and should provide the final answer about its uniqueness. Recently, we have also purified another mitochondrial DNA repair enzyme from rat liver (R. Stierum, submitted). This enzyme specifically recognizes thymine glycol (TG) lesions in DNA and thus has an activity that resembles the endonuclease III type enzymes that have been found in bacteria and human cells. It recognizes TG lesions in double-stranded DNA, but not 8-oxo-dG or uracil lesions. We are currently characterizing this enzyme activity further.

A polymerase extension assay has been utilized to evaluate H_2O_2 damage induction and removal (Yakes and Van Houten, 1997). Both nuclear and mitochondrial sequences were examined. This assay is not measuring a specific lesion, but rather any damage

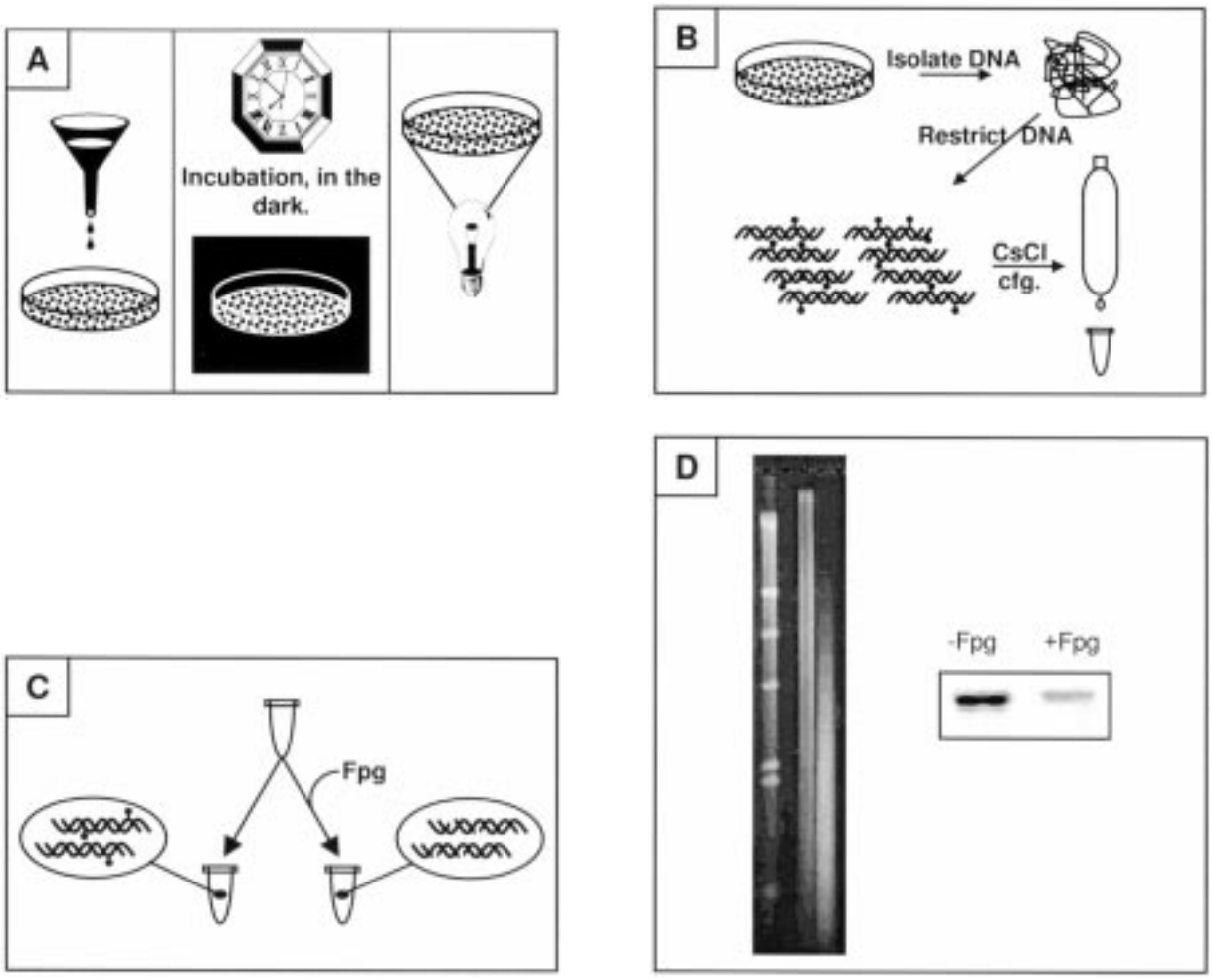

Fig. 2. This cartoon illustrates the gene-specific repair assay, using photoactivated methylene blue as the damaging agent and Fpg as the damage recognition enzyme. Other agents and enzymes are frequently used. Panel (A), cells are preincubated with methylene blue and then exposed to light. Panel (B), DNA is isolated and restricted. If bromodeoxyuridine has been included in the repair medium, CsCl centrifugation is used to separate replicated and nonreplicated DNA. The DNA lesions, 8-oxo guanines, are shown. Panel (C), DNA from each sample is divided into two identical aliquots, one of which is treated with Fpg to create nicks at the sites of damage. Panel (D), The DNA is run on a denaturing, alkaline agarose gel, transferred to nylon, and probed for mitochondrial DNA or any other sequence of interest. Band volumes are quantitated, and the Poisson equation used to calculate the average amount of damage contained in each restriction fragment.

that blocks the progression of the polymerase. The investigators observed that as the concentration of H_2O_2 increased, it induced more damage into mitochondrial DNA than nuclear DNA, as assessed by the inhibition of the polymerase extension (Yakes and Van Houten, 1997). In contrast, Taffe *et al.* (1996) found no difference in the formation of Fpg-sensitive sites in the mitochondrial and in the nuclear DNA using acridine orange. Interestingly, complex lesions such as cisplatin interstrand crosslinks, which are thought to be repaired via a recombination repair pathway, are removed from hamster mtDNA (LeDoux *et al.*, 1992). This would suggest that mitochondria might possess recombinational activities. In support of this, an *in vitro* assay has demonstrated recombination between plasmids using human mitochondrial protein extracts (Thyagarajan *et al.*, 1996). We also detected the removal of 4-nitroquinoline (4NQO) lesions from mtDNA (Snyderwine and Bohr, 1992). 4NQO is generally thought to be removed via NER pathways. However, NER as it exists in the nucleus, does not exist in mitochondria. Whether any NER proteins play roles in mitochondrial repair remains to be explored and is currently a very challenging proposition.

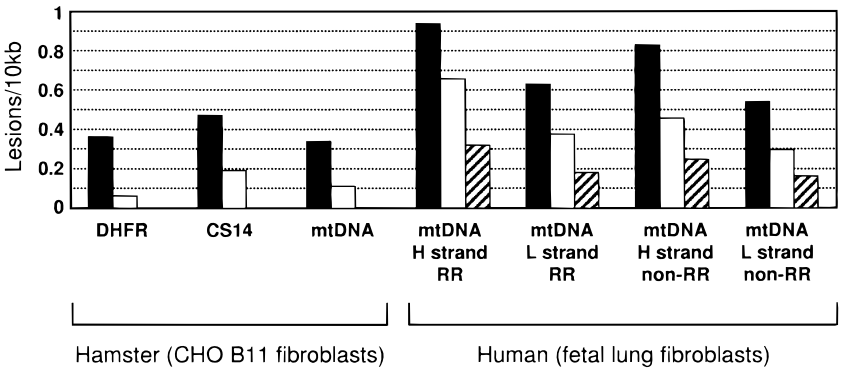

Fig. 3. Although different agents were used to induce the damage and initial levels were quite different, the initial rate at which lesions are repaired seems remarkably constant. Here, damage induced by photoactivated acridine orange (CHO cells) or photoactivated methylene blue (human cells) is repaired. In each case, between 0.2 and 0.4 lesions per 10 kb were repaired in the initial 4-h period. Solid bars, initial damage; open bars, damage remaining after 4 h of repair: striped bars, damage remaining after 20 h of repair. "RR," ribosomal region. Based on data presented in Taffe *et al.* (1996) and Anson *et al.* (1998).

REPAIR OF MITOCHONDRIAL DNA *in vitro*

Some of the studies mentioned above are done in cell culture and thus resemble the *in vivo* methods. Much of the recent progress in the mechanistic studies on DNA repair has been done utilizing *in vitro* approaches. Such experiments are done using purified proteins in simple reactions involving a very limited number of components (Pinz and Bogenhagen, 1998; Longley *et al.*, 1998). This is also being done now in the area of mitochondrial study where the function of mitochondrial repair enzyme are tested in a reconstituted base excision repair pathway based on knowledge about the nuclear processes (Longley *et al.*, 1998; Pinz and Bogenhagen, 1998). Mitochondrial base excision repair has been reconstituted with purified enzymes, mitochondrial uracil glycosylase, AP endonuclease, DNA polymerase, and ligase. This work has demonstrated that the mitochondrial DNA polymerase γ can participate in the base excision repair pathway in the reconstituted system where it appears to have a deoxyribose phosphatase activity resembling the one of β-polymerase (Copeland *et al.*, 1997). This approach is bound to provide us with much molecular insight in the future about the precise mode of action of some of the involved enzymes.

Table II. Oxidative Damage-Specific Endonuclease from Rat Liver Mitochondria

Physical characterization
Small protein, 25–30 kD
KCl optimum 75 m*M*
Resistant to EDTA
Substrates
Double-stranded DNA with 8-oxo-dG or abasic sites
8-oxo-dG in single-stranded DNA and FapyG are not substrates
Preferred base pair is 8-oxo-dG C
8-oxo-dG A base pairs are poor substrates
Cleavage Mechanism
Generates single strand break 3′ to lesion
3′ cleavage product is an unsaturated sugar residue
5′ cleavage product is a phosphate group
Proposed catalytic mechanism is β-elimination

The use of reconstituted systems, in which a preconceived mechanistic notion is tested, has its limitations. It can also be argued that these systems do not reflect the *in vivo* repair process. We are now developing alternative approaches using whole mitochondrial protein extracts to study the repair of defined DNA lesions. In the study of mammalian, nuclear DNA repair, this approach has proved extremely useful. Damaged plasmid is added to a cell extract and the repair incorporation into the plasmid is then measured after the repair incubation period. With this approach the repair of a wide variety of lesions can be assessed and the measurements resemble the repair situation better as it occurs *in vivo* (Wood, 1996). While these experiments have been carried out for nuclear repair over the last decade, it has still not been done in mitochondrial extracts. In one report (Ryoji *et al.*, 1996), this was attempted, but not optimized, and no specific lesions were studied. It has been a challenge

to get this experiment to work in the mammalian mitochondrial extracts. One problem has been the abundant presence of the mitochondrial endonuclease G in the mitochondrial extracts, since this enzyme will digest DNA containing strings of guanine.

We have recently been able to measure repair *in vitro* in mitochondrial extracts and we are now getting stable repair incorporation into plasmids containing single uracil lesions, which are known to be substrates for base excision repair (Stierum *et al.*, 1999.) This approach lends itself to many important experiments where different DNA lesions are assayed and where biochemical mechanisms of mitochondrial repair are explored.

AGING

Several investigators have reported accumulation of 8-oxo-dG in DNA with age but there are conflicting reports as to the degree of accumulation. One of the controversies in the study of oxidative DNA damage concerns the amount of 8-oxo-dG present in mtDNA. Although there appears to be a consensus about an increase of damage with age, the amounts of oxidative base modifications measured by various methods (LC/MS, HPLC, and enzymatic) do not agree with one another (Collins *et al.*, 1997). There is a need to make concerted efforts to measure oxidative lesions and their repair under identical conditions and in the same biological system using different methods to assess the same changes.

The mitochondrial theory of aging postulates that organisms age due to the accumulation of DNA damage and mutations in the multiple mitochondrial genomes, leading to mitochondrial dysfunction. Among the many types of DNA damage, 8-oxo-dG has received the most attention due to its mutagenicity and because of the possible correlation between its accumulation and pathological processes like cancer, degenerative diseases, and aging. Although 8-oxo-dG accumulation with age in the mtDNA has been well documented, very little is known about its processing and no pubished study has yet examined whether mitochondrial DNA repair changes with age. We have assessed the age-related changes in the potential for mitochondrial DNA repair by measuring endonucleolytic activity toward an 8-oxo-dG-containing substrate. This activity is, as discussed above, due to the presence of MtODE. Mitochondria obtained from rat heart and liver were used to measure the activity of this enzyme that specifically cleaves 8-oxo-dG-containing duplex oligonucleotides. We find that this activity is higher in 12 and 23-month-old than in 6-month-old rats, in both liver and heart extracts (Souza-Pinto *et al.*, 1999). These mitochondrial extracts also cleave oligonucleotides containing a U:A mispair, at the uracil position, reflecting the combined action of mitochondrial uracil DNA glycosylase (mtUDG) and mt AP endonucleases. The mtUDG activity did not change with age in liver mitochondria, but there was a small increase in activity from 6 to 23 months in rat heart extracts, after normalization by citrate synthase activity. Endonuclease G activity, measured by a plasmid relaxation assay, did not show any age-associated change in liver, but there was a significant decrease from 6 to 23 months in heart mitochondria. Our results suggest that the mitochondrial capacity to repair 8-oxo-dG, the main oxidative base damage shown to accumulate with age in mtDNA, does not decrease, but rather increases with age. The specific increase in 8-oxo-dG endonuclease activity, rather than a general upregulation of DNA repair in mitochondria, suggests the possibility of an induction of this repair pathway with age.

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