Mitochondrial protein extracts from normal and immortalized mammalian somatic cells catalyze homologous recombination of plasmid DNA substrates. Mitochondrial homologous recombination activity required exogenous adenosine triphosphate, although substantial activity remained when non-hydrolyzable analogs were used instead. There was no requirement for added nucleoside triphosphates, and the reaction was not inhibited by diepoxyadenosine triphosphate or aphidicolin. The majority of recombinant plasmid molecules result from a conservative process, indicating that nuclease-mediated strand-anneling is not responsible for the mitochondrial homologous recombination activity. Affinity-purified anti-recA antibodies inhibited the reaction, suggesting that activity is dependent on a mammalian mitochondrial homolog of the bacterial strand-transferase protein. The presence of homologous recombination activity within mammalian mitochondrial extracts suggests that this process is involved in mitochondrial DNA repair.

Mitochondrial DNA comprise nearly 1% of total cellular DNA in most mammalian somatic cells (1). Encoded on the ~16-kilobase mammalian mitochondrial genome are 13 respiratory chain polypeptides, 2 ribosomal RNAs, and 22 tRNA molecules (2). Abundant evidence indicates that mutations in mitochondrial DNA contribute to human disease. Sporadic mitochondrial deletions are associated with the Kearns Sayre syndrome (3), while point mutations within this DNA cause Leber’s hereditary optic neuropathy (4), Pearson’s syndrome (5), as well as other disorders (6). Recently, autosomal dominant and recessive mitochondrial myopathies associated with multiple deletions of mitochondrial DNA have been described (7–9).

Mutations in mitochondrial DNA are important for several additional reasons as well. First, a number of studies have shown that as human beings age, mutations accumulate within their mitochondrial DNA (10–11). Second, it has been reported that the brains of patients with Parkinsonism harbor elevated levels of mitochondrial DNA mutations (12). These observations support the hypothesis that many features of human aging may be a consequence of accumulated mitochondrial DNA mutations (13, 14). Finally, it is known that chemotherapeutic agents such as cisplatin (15) and bleomycin (16) damage the mitochondrial genome. It is believed that some cases of organ-specific toxicity associated with these agents result from damage to mitochondrial DNA (17).

Although DNA repair of the nuclear genome of eukaryotes has been extensively studied (18), much less is known about DNA repair in mitochondria. Attempts to measure nucleotide excision repair in mitochondria failed to detect activity (19). Furthermore, mitochondrial DNA mutates at rates greater than does nuclear DNA (20). These observations have prompted speculation that mammalian mitochondria lack DNA repair (1, 13).

Recently, however, a number of reports have concluded that mammalian mitochondria possess base excision repair activity (21–23). Similar studies have shown that mitochondria can repair DNA inter-strand cross-links caused by cisplatin (24), as well as DNA damage caused by bleomycin (25), and the reactive oxygen species-generating compound alloxan (26). These results suggest that while mammalian mitochondria lack nucleotide excision repair activity, they are likely to possess a number of distinct DNA repair pathways.

The idea that one of these mammalian mitochondrial DNA repair pathways may involve homologous DNA recombination (HR)

is plausible for several reasons. First, as mentioned above, cisplatin inter-strand DNA cross-links are repaired within the mammalian mitochondria (24). While the reaction mechanism by which these cross-links are repaired in mammalian mitochondria is unknown, in both prokaryotes, and in yeast nuclei, repair of these DNA lesions is accomplished via homologous recombination (27–30). Second, a number of nuclear-encoded yeast genes have been shown to be involved in mitochondrial recombination and repair (31, 32). Finally, studies of DNA repair in plant chloroplasts have shown that a homolog of the bacterial recA gene is involved in DNA repair in that organelle (39–36).

We set out to test the hypothesis that recombinational DNA repair occurs within the mammalian mitochondria. Due to the unavailability of mammalian mitochondrial transformation protocols, a biochemical approach was pursued (37–41). Subcellular fractions highly enriched in mitochondria were isolated and protein extracts from this material shown to catalyze cell-free HR. The majority of recombinant clones analyzed are the products of a conservative recombination reaction, strongly suggesting that authentic strand-transferase activity, rather than a nonspecific nuclease-mediated process is responsible for their creation. This hypothesis is supported by the observation that pretreatment of extracts with affinity-purified anti-recA antibody reduced homologous recombination activity by approximately 90%. These data indicate that mitochondria pos-
assess the enzymatic machinery needed to catalyze HR, and suggest that recombinational DNA repair may occur within the mitochondria of mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Cells**—HT1080 cells (derived from a spontaneous human fibrosarcoma) were obtained from the American Type Culture Collection (Rockville, MD). Human dermal fibroblasts, obtained from a punch biopsy of a normal individual (wild-type, WT), and from an individual afflicted with pyruvate dehydrogenase deficiency, were kindly provided by Dr. Robert O'Dea (University of Minnesota).

**Recombination Substrates**—The substrate molecules pSV2neoDL (Fig. 1C) and pSV2neoDR (Fig. 1D) have been described (38). Both plasmid DNA molecules functional ampicillin-resistance gene, and a non-functional neomycin phosphotransferase (neo) gene. The inactivating mutations in pSV2neoDL and pSV2neoDR are non-overlapping deletions (of 248 and 283 base pairs, respectively) within the coding region of the neo gene. These alleles do not spontaneously revert, and generation of a functional neo gene is dependent on inter-plasmid recombination (38). The single-stranded circular molecule ssneoDR (42) harbors the neo gene from pSV2neoDR inserted into the multiple cloning site of M13mp18 (Fig. 1A).

**Isolation of Mitochondria**—Crude mitochondria preparations were prepared from livers removed from Fisher 344 female rats, or from cultured cells, as described (43). This material was further purified by centrifugation through a Percoll gradient (44).

**Preparation of Protein Extracts**—Purified mitochondria were lysed by hypo-osmotic shock, and the membrane fraction removed by centrifugation for 30 min at 70,000 rpm in a Beckman TL100/3 rotor. Protein concentration was determined using the Bradford assay (45). Nuclear protein extracts were prepared as outlined by Jessler and Berg (41).

**Homologous DNA Recombination Assay**—DNA recombination substrates (500 ng of each substrate) were incubated with protein extracts for 30 min using buffer conditions previously described (41), unless otherwise indicated. Briefly, the DNA substrates were incubated with 5 μg of mitochondrial protein extract in a buffer containing 60 mM sodium chloride, 2 mM 2-mercaptoethanol, 2 mM potassium chloride, 12 mM Tris hydrochloride (pH 7.4), 1 mM ATP, 0.1 mM each deoxynucleoside triphosphate (dNTP), 2.5 mM spermidine, 2% glycerol, and 0.2 mM hydrochloride (pH 7.4), 1 mM ATP, 0.1 mM each deoxynucleoside chloride, 2 mM 2-mercaptoethanol, 2 mM potassium chloride, 12 mM Tris chloride. After the incubation period, DNA was extracted with phenol:chloroform (1:1), ethanol precipitated, resuspended in water, and used to transform DH10B Escherichia coli via electroporation (Cell-Portar, Life Technologies, Inc.). The transformed bacteria were then plated onto LB-agar plates containing either ampicillin, or kanamycin and ampicillin. Since both pSV2neoDL and pSV2neoDR contain a functional ampicillin-resistance gene, the number of ampicillin-resistant colonies served as a plasmid recovery/transformation control. (Note that in experiments using ssneoDR and pSV2neoDL, only the latter molecule contained an ampicillin-resistance gene. Nevertheless, the number of ampicillin-resistant colonies serves as an effective plasmid recovery/transformation control in these experiments as well.)

**Isolation of Mitochondria**—Crude mitochondria preparations were separately incubated with mitochondrial protein extracts, as above, and pooled prior to bacterial transformation. The recombination frequency obtained from these latter experiments (calculated as above) was subtracted from that obtained in the co-incubation experiments to obtain a corrected HR frequency value. When no kanamycin-resistant colonies were observed in these control experiments, the background HR frequency was presented as less than that calculated had one kanamycin-resistant colony been recovered. With the exception of those experiments involving the use of two double-stranded linearized plasmid molecules, the “background” HR frequency was always at least 10-fold lower than that obtained when the DNA substrates were co-incubated with mitochondrial extracts. An exception was seen in the case when pSV2neoDL and pSV2neoDR, which had both been linearized with SalI, were used. Under these conditions, the background HR frequency was essentially equivalent to that seen when the two plasmids were co-incubated with mitochondrial extracts. As discussed below, it is likely that the recombination mechanism involved in the formation of recombinant plasmids when this particular substrate combination is used is distinct from the pathway that occurs when at least one of the substrates is circular (see Fig. 3, and Table II).

**Plasmid DNA Analysis**—Kanamycin-resistant bacterial colonies were picked with a sterile toothpick and grown overnight at 37°C in non-selective media. Plasmid DNA was isolated, and subjected to restriction endonuclease digestion and gel electrophoresis using standard methods (46).

**Cytochrome-c Oxidase Assay**—Cytochrome-c oxidase activity was measured in a Gilford Response spectrophotometer as described previously (47).

**Materials**—Nucleosides and analogs, aphidicolin, and all other chemicals used were obtained from Sigma. Restriction enzymes were from New England Biolabs (Beverly, MA).

**RESULTS**

**Homologous Recombination Assay**—In contrast to yeast and plants (48), introduction of genetic material into the mitochondria of mammalian cells has not been described. Therefore, in order to measure HR in mammalian mitochondria, a direct, biochemical approach was utilized. This strategy has been used previously to show that crude nuclear extracts (37–41) or a high molecular weight protein complex (49) from mammalian cells catalyze HR. In brief, this assay is based upon the following observations. Co-incubation of two DNA substrates that separately encode defective heteroalleles of a selectable marker with a protein extract followed by transformation of this material into recombination-deficient E. coli results in the generation of resistant bacterial colonies. These colonies harbor recombinant molecules encoding a functional marker gene. In some of the experiments described in this report, we have used two covalently closed circular double-stranded molecules, pSV2neoDL and pSV2neoDR, while in other experiments, the pSV2neoDL molecule, which had been linearized with the restriction enzyme SalI (which cuts within the neomycin phosphotransferase gene, see Fig. 1B) was co-incubated with the single-stranded circular molecule ssneoDR (see Fig. 1A). In all cases, the HR frequency was determined by measuring the ratio of homologous recombinant molecules (kanamycin-resistant) to non-recombinant molecules (ampicillin-resistant) as described under “Experimental Procedures.”

**HR and Cytochrome-c Oxidase Activity in Rat Liver Mitochondrial Protein Extracts**—Crude mitochondria were purified from rat liver (43), and centrifuged through a Percoll gradient as described (44). Four fractions (numbered 1 to 4 from the top of the gradient to the bottom of the gradient) were isolated from the Percoll gradient and processed as described under “Experimental Procedures.” The soluble fraction was characterized with respect to HR activity and the membrane pellet was tested for cytochrome-c oxidase activity, a mitochondrial marker enzyme. Specific activity values obtained from the analysis of the
We nevertheless measured HR activity in four independent experiments in which the DNA substrates were separately incubated with mitochondrial extracts prepared from immortalized cells. Two independent arguments render this hypothesis untenable. First, we performed mixing experiments on nuclear protein extracts prepared from normal and immortalized cells, and found no evidence for a HR inhibitor in the former. Second, the specific activity of HR in mitochondrial extracts was essentially equal to that of nuclear extracts prepared from immortalized cells. It does not seem likely that (presumably) minor contamination of the mitochondrial extract by nuclear proteins could provide such robust HR activity.

**Molecular Analysis of Products of HR**—While homologous recombination is classically thought to occur via double-strand break repair (DSBR, see Ref. 52 for a review), an alternate pathway, based on sequential nuclease and re-annealing activity, termed single-strand annealing (SSA), has been shown to represent a major pathway for cellular recombination in mammalian nuclei (53–55) and Xenopus laevis oocytes (56), and also occurs in yeast (57, 58). A major distinguishing feature of the SSA and DSBR HR pathways is that in the former, homologous recombination is non-conservative, whereas in the latter, conservative recombination events occur. One would predict therefore that bacterial colonies containing plasmids with a single neomycin phosphotransferase gene result from SSA recombination, while DSBR-mediated recombination events will generate bacteria that harbor two neomycin phosphotransferase alleles (see Fig. 3).

Neomycin phosphotransferase alleles resulting from DSBR recombination could be present in a number of different combinations. For example, if a simple reciprocal recombination event occurred between pSV2neoDL and pSV2neoDR, the bacteria would harbor one WT allele, and one double-deletion-bearing allele. These two alleles could be present on the same (dimer) molecule, or as two separate (monomer) molecules, depending on the orientation of the crossover event that generated them. Likewise, if a gene conversion event occurred (as

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**Table I**

| Rat liver mitochondrial extracts carry out HR using different recombination substrate | HR frequency (background) |
|--------------------------------|--------------------------|
| Rat liver mitochondrial preparation number | pSV2neoDL (SalI) + ssneoDR | pSV2neoDR + pSV2neoDL |
| 1<sup>b</sup> | 2.6 (0.12) | ND<sup>2</sup> |
| 2 | 6.6 (0.21) | 0.06 (0.005) |
| 3 | 7.2 (0.05) | 0.06 (0.017) |
| 4 | ND<sup>2</sup> | 0.063 (0.003) |
| M<sup>d</sup> | 5.5 ± 1.4 | 0.06 ± 0.001 |

<sup>a</sup> HR frequency is expressed as the number of kanamycin-resistant colonies obtained per 10<sup>6</sup> ampicillin-resistant colonies. (Background HR frequency calculated as described under “Experimental Procedures.”)

<sup>b</sup> Rat liver mitochondrial extracts were prepared on four separate occasions. Each extract was independently tested for HR activity using the substrates indicated.

<sup>c</sup> ND, not done.

<sup>d</sup> M, mean ± S.E.
Directional exonuclease activity (the neomycin phosphotransferase genes are indicated by thick lines), alleles. On the right-hand side is illustrated the SSA homologous dimer molecule or two monomers. In the example illustrated gene evolution of the Holliday junctions occurs (break is extended and one strand invades pSV2neoDR, creating a double-strand break repair (52) mediated homologous recombination between two uncut double-stranded molecules (pSV2neoDL and pSV2neoDR). The neomycin phosphotransferase alleles are indicated by white boxes, and deletions are represented as black triangles. In the example shown, the DSBR reaction has been initiated (a) by the formation of a double-strand break in pSV2neoDR. This double strand break is extended and one strand invades pSV2neoDR, creating a D-loop (b). Branch migration permits the formation of a second Holliday junction formation (c). Gap-filling DNA synthesis (not shown) and resolution of the Holliday junctions occurs (d), generating either a single dimer molecule or two monomers. In the example illustrated gene conversion has resulted in the formation of full-length (WT) and DL alleles. On the right-hand side is illustrated the SSA homologous recombination pathway using SalI-linearized pSV2neoDR and pSV2neoDL. The plasmid molecules are represented by thin lines, while the neomycin phosphotransferase genes are indicated by thick lines. Directional exonuclease activity (a) generates complementary single-stranded ends on each molecule. These regions of complementarity allow annealing to form linear (b) and circular (d) molecules, as illustrated. Gap-filling or ligation (c) completes the formation of a full-length, WT neomycin allele. While it is not shown, four linear molecules may anneal, resulting in the formation of a dimer plasmid molecule containing two WT neomycin alleles.

illustrated in Fig. 3), the bacteria would harbor a WT allele, as well as either one or the other of the two substrate molecules (pSV2neoDL or pSV2neoDR). Once again, these alleles could be present as a dimer molecule or as two monomers. (A fundamental assumption here is that co-transformation of bacteria does not occur. We have verified that, under the conditions employed in our studies, co-transformation rarely occurs. We therefore conclude that bacterial colonies harboring two different plasmids result from transformation by recombinant molecules formed within with the mitochondrial protein extract.)

We performed a homologous recombination experiment (see “Experimental Procedures”) using uncut pSV2neoDL and uncut pSV2neoDR, and analyzed plasmid DNA from kanamycin-resistant bacterial colonies. As the results in Table II indicate, in the majority of cases (49/59, 83%), the recombination event that generated the full-length neomycin allele was conservative. While a number of dimer molecules were identified during this analysis (8/59, 14%), none of these molecules harbored two wild-type alleles of the neomycin phosphotransferase gene. These results strongly suggest that DSBR rather than SSA recombination is being catalyzed by the mitochondrial extracts. Similar conclusions have been reached by others studying nuclear protein extract-mediated HR in both yeast (37) and mammalian (59) systems.

As a control, we tested the hypothesis that SSA-mediated recombination would generate non-conservative products by performing a recombination experiment using pSV2neoDL and pSV2neoDR that were both linearized with the restriction endonuclease SalI (see Fig. 1). Analysis of 36 kanamycin-resistant colonies revealed that in each case the product of the recombination reaction was non-conservative (i.e. only WT neomycin alleles were recovered). While 33 of these molecules were monomers, 3 were dimers that harbored two full-length neomycin phosphotransferase alleles, such as would be predicted to occur if 4 plasmids (rather than two as illustrated in Fig. 3) annealed together. The results of this control experiment indicate that, as predicted (53–58), SSA-mediated recombination generates non-conservative recombination products, further supporting our conclusion that the HR activity we detect in mitochondrial extracts proceeds via DSBR.

**HR Activity in Mitochondria from Cultured Fibroblasts**—To determine whether mitochondria from human fibroblasts in culture were also capable of catalyzing HR, mitochondrial extracts prepared from a number of cells, including both normal and immortalized fibroblasts, were tested for HR activity (using pSV2neoDL cut with SalI, and sneoDR). As shown in Table III, efficient HR activity was detected in the mitochondrial fraction in normal and immortalized human fibroblasts. While slight differences were seen between the different cell strains, mitochondrial extracts from both normal and transformed cells were quite similar in their ability to catalyze HR. This is most interesting in light of our recent observation that nuclear HR activity levels are 100-fold higher in immortalized cells than in non-immortalized cells, with levels in the latter being essentially undetectable.

**Treatment with Ketone Bodies Partially Rescues Mitochondrial HR Activity in Mutant Fibroblasts**—The conventional view has been that DNA repair is not a significant feature of mitochondrial genetic function. We therefore wanted to provide additional evidence that the activity seen here was truly mitochondrial in origin. Fibroblasts were obtained from an individual afflicted with pyruvate dehydrogenase deficiency. These patients have impaired mitochondrial function, which presumably diminishes the ability of the mitochondria to maintain an optimal inner membrane potential and thereby results in improper transport of proteins into the mitochondria. We postulated that mitochondria from these cells would possess reduced levels of HR and cytochrome-c oxidase activity when compared to wild-type fibroblasts. To test this hypothesis, we prepared mitochondrial extracts from these cells and tested them for cytochrome-c oxidase and HR activity (using pSV2neoDL cut with SalI and single-stranded sneoDR). We found that the levels of cytochrome-c oxidase and HR activity were significantly reduced compared to normal levels (not shown). Patients with pyruvate dehydrogenase deficiency are routinely fed high lipid diets in an effort to improve their mitochondrial function (lipid β-oxidation generates acetyl-CoA, which helps overcome the block to glucose metabolism by feeding acetyl-CoA into the mitochondria). With this in mind, we reasoned that it might be possible to improve mitochondrial function (and thereby enhance levels of mitochondrial HR activity) by supplementing the culture media of pyruvate dehydrogenase-deficient cells with high levels of the ketone body 3-hydroxybutyrate. Mitochondrial extracts were prepared from 3-hydroxybutyrate-supplemented cells and similarly analyzed. Results from two experiments demonstrated that cytochrome-c oxidase activity in...
these mitochondria was increased 4.6-fold, thereby showing that mitochondrial function was improved in these cells (Fig. 4). When HR activity was examined in these extracts, we found that 3-hydroxybutyrate enhanced the specific HR activity in these extracts by 3.3-fold when compared to mitochondrial extracts prepared from cells not supplemented with 3-hydroxybutyrate (Fig. 4). Interestingly, while the 3-hydroxybutyrate supplement enhanced HR activities to levels slightly greater than those seen in normal cells, the cytochrome-c oxidase activity levels, although substantially elevated, remained significantly below those seen in normal cells (not shown). This is most likely due to the fact that the cytochrome c-oxidase protein contains multiple, nuclear-encoded subunits that must each traverse the mitochondrial inner membrane.

Characterization of Mitochondrial HR Activity—We characterized the mitochondrial extract HR activity with respect to nucleotide co-factors and the reaction conditions required for maximal activity (substrate plasmids pSV2neoDL, SaII, and single-stranded sneoDR were used). These experiments (Table IV) indicate that added ATP was essential for HR activity, while there was no requirement for exogenous deoxyxynucleoside triphosphates. Heat treatment of the extract at 65 °C for 15 min completely destroyed activity. We also determined that HR activity was responsive to the amount of added extract, with a peak of activity at 5 μg of protein, and that maximal activity was detected following a 30-min incubation with extracts (not shown). The inability of either aphidicolin or deoxyxynosines triphosphate to inhibit the reaction (data not shown) indicated that no previously identified DNA polymerase was involved in the reaction.

It seems likely that ATP hydrolysis plays an important role in the biological activity of recA (reviewed in Ref. 60). However, it has been established that in vitro recA-mediated strand transfer can occur in the presence of non-hydrolyzable ATP analogs (61, 62). We therefore tested whether the non-hydrolyzable ATP analogs, ATPγS and AMP-PNP, could replace ATP in the reaction. As shown in Table IV, HR activity was diminished, although not abolished, when either of these analogs were substituted for ATP. While only about 50–60% of maximal activity levels were observed, these results strongly suggest that, like recA, the mammalian mitochondrial recombinase protein requires ATP binding, but not necessarily hydrolysis to catalyze the strand transferase reaction. In contrast, nuclear protein extracts prepared from immortalized

| TABLE III |
| --- |
| Mitochondrial extracts prepared from different sources catalyze HR |

| Source of HR extract | HR frequency (background) |
| --- | --- |
| Rat liver | 5.5 (0.1) |
| HT1080 | 1.1 (<0.06) |
| HDF | 2.3 (<2.0) |

a HR frequency is expressed as the number of kanamycin-resistant colonies obtained per 10^5 ampicillin-resistant colonies. (Background HR frequency calculated as described under “Experimental Procedures.”)

b HDF normal human diploid fibroblasts.

c COX and HR activities were determined as indicated in the Materials and Methods section. COX activity is expressed as relative units. HR activity is expressed as the number of kanamycin-resistant colonies obtained per 10^5 ampicillin-resistant colonies.

| TABLE IV |
| --- |
| Characterization of HR activity from rat liver mitochondrial protein extracts |

| Assay conditions | HR frequency (background) | % of control ± S.E. |
| --- | --- | --- |
| 5.0 μg of protein (n = 6) | 7.3 (0.04) | 100 ± 25% |
| Heat treatment (65 °C for 15 min) | 6.9 (0.06) <1% |
| (-)ATP (n = 5) | 0.07 <1% |
| (-)dATP (n = 5) | 7.1 (0.5) 97 ± 31% |
| (-)ATP, (+)AMP-PNP (n = 4) | 4.0 (0.07) 55 ± 10% |
| (-)ATP, (+)AMP-PNP (n = 2) | 4.8 (<0.03) 66% |

a HR frequency is expressed as kanamycin-resistant colonies per 10^5 ampicillin-resistant colonies.

b Control refers to the HR frequency obtained with 5 μg of protein.

c HR frequency was not significantly elevated above background levels.

---

**FIG. 4.** HR and cytochrome-c oxidase in pyruvate dehydrogenase-deficient cells. Mitochondrial extracts were prepared from pyruvate dehydrogenase-deficient human diploid fibroblasts cultured either in the absence (open bars) or presence (closed bars) of 3-hydroxybutyrate. The extracts were tested for HR activity (HR) and cytochrome-c oxidase (COX) activity. The results shown are the average of two experiments. HR frequencies (kanamycin-resistant colonies obtained per 10^5 ampicillin-resistant colonies) and background frequencies (parentheses) were as follows: hydroxybutyrate absent, 0.7 (0.1); hydroxybutyrate present, 2.3 (<0.1).

**TABLE II**

Analysis of recombinant plasmid DNA molecules

| monomer | WT | WT/DR | WT/DD |
| --- | --- | --- | --- |
| Dimer | (WT + DL) | (WT + DR) | (WT + DD) |
| n* | 10 (17) | 4 (7) | 4 (7) |
| % | 29 (49) | 9 (15) | 3 (5) |

* n indicates the number of individual plasmids of each type recovered, while % indicates the percentage of the total represented by each type.
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Fig. 5. Anti-recA antibody inhibits mitochondrial HR activity. Affinity-purified anti-recA antibody (anti-recA) or nonspecific rabbit sera was preincubated with mitochondrial extracts for 30 min prior to conducting a standard HR assay (see “Experimental Procedures”). White bars indicate HR frequency (kanamycin-resistant colonies per 10^7 ampicillin-resistant colonies) present following pretreatment with antibody, black bars indicate HR activity present following pretreatment with heat-inactivated antibody. The standard deviation is indicated by the error bars. HR frequencies and background frequencies (parentheses) were as follows: anti-recA antibody, 0.84 (1.13); heat-treated anti-recA antibody, 8.3 (0.2); nonspecific antibody, 7.8 (0.17); heat-treated nonspecific antibody, 7.8 (0.06).

affinity-purified immunoglobulin destroyed this inhibitory activity, yet had no effect on the control antiserum (Fig. 5).

DISCUSSION

Previous results have shown that mammalian mitochondria can repair cisplatin inter-strand DNA cross-links (24). The data presented in this report, combined with the knowledge that inter-strand DNA cross-links formed in bacteria (27–29) and yeast nuclear DNA (30) are most likely repaired via homologous recombination strongly suggest that mammalian mitochondria utilize a homologous recombination-based process to repair damaged DNA. However, while HR is known to occur in yeast mitochondria, genetic recombination has not been reported in mammalian mitochondria (see below). This apparent paradox suggests that either earlier reports failing to measure detectable levels of genetic recombination in mammalian mitochondria are in error, or that our interpretation of the results presented above is incorrect. We believe that a third possibility exists: that mammalian mitochondria possess a recombinational DNA repair pathway that specifically repairs DNA, yet is not associated with genetic recombination. Before presenting the argument that supports this hypothesis, we will first consider two possibilities: 1) that the HR activity we describe in this report results from contaminating nuclear HR proteins present in the mitochondrial extracts, and 2) that the recombinant molecules detected in the HR assays described above are the consequence of nonspecific enzymatic activity within these same extracts.

The HR Activity in Mitochondrial Extracts Is Not Due to Contaminating Nuclear Proteins—We and others have shown that rat liver nuclear protein extracts do not catalyze HR (50, 51), yet mitochondrial extracts prepared from this tissue possess robust HR activity. We performed mixing experiments and determined that the absence of HR activity in nuclear protein extracts prepared from normal fibroblasts was not due to the presence of an inhibitor of recombination. Studies on pyruvate dehydrogenase-deficient fibroblasts showed that including 3-hydroxybutyrate in the growth media enhanced mitochondrial function and increased the specific activity of the mitochondrial-marker enzyme cytochrome c-oxidase. Mitochondrial HR activity in these extracts was enhanced to a similar degree. The presence of 3-hydroxybutyrate in the growth media provides an alternate metabolic pathway by which pyruvate dehydrogenase-deficient cells can generate ATP. This additional ATP restores the inner mitochondrial membrane potential, thereby facilitating protein transport (including nuclear-encoded cytochrome c-oxidase subunits, and the mitochondrial HR protein) into the mitochondrial matrix within the mutant cells. It is unlikely that treatment of cells with 3-hydroxybutyrate would increase the concentration of a non-mitochondrial protein in mitochondrial extracts. Finally, we have provided pharmacological evidence that the nuclear and mitochondrial activities are distinct. While HR activity in nuclear protein extracts prepared from immortalized fibroblasts was completely inhibited when non-hydrolyzable analogs replace ATP, HR activity in mitochondrial extracts prepared from these cells was only somewhat diminished. This combination of genetic, biochemical, and pharmacological evidence makes it extremely unlikely that the HR activity present in mitochondrial extracts is the result of contaminating nuclear proteins.

Authentic Homologous Recombination Is Responsible for the Generation of Recombinant Molecules in Mammalian Mitochondrial Extracts—The experiments described in this report were performed using crude mitochondrial protein extracts. This leaves open the possibility that nonspecific enzymatic activities within the extracts are in some way responsible for generating the recombinant molecules observed. While it is possible that limited nuclease, followed by annealing activity could convert the substrate pair of pSV2neoDL (SalI cut) plus single-stranded ssneoDR into recombinant molecules, it is far more difficult to see how a similar activity could convert the two covalently closed double-stranded circular molecules pSV2neoDR and pSV2neoDL into recombinant products. However, as Table I indicates, both of these substrate pairs are converted to recombinant products with high efficiency by mitochondrial extracts (i.e. at frequencies comparable to those with which nuclear protein extracts prepared from immortalized mammalian somatic cells recombine these substrates). In addition, were nuclease and annealing activity responsible for the recombinant products, it is difficult to understand why ATP binding, but not ATP hydrolysis was required for HR activity. Third, the nuclease/annealing pathway, referred to as SSA, is expected to produce non-conservative recombination products (52–58), yet the majority of recombinant products produced by incubation with mitochondrial extracts were conservative (83%). (This value of 83% may underestimate the number of conservative events. Since there is no selective pressure for maintenance of non-functional neomycin phosphotransferase alleles, it is possible that in some cases the non-selected allele has been lost, or is not present at sufficiently high concentration to be detected via restriction analysis.) We performed a control experiment in which both the double-stranded circular molecules were linearized with SalI and determined that, when substrates well suited to the SSA recombination pathway were utilized (see Fig. 3), 100% non-conservative recombination products (36/36) are recovered.

We showed that anti-recA antibodies inhibited the HR activity present in mitochondrial extracts. This antibody inhibition data, coupled with the observation that this HR activity functions in the presence of non-hydrolyzable ATP analogs is consistent with the interpretation that a mammalian mitochondrial homolog of the bacterial recA protein (which can also form joint molecules in the presence of non-hydrolyzable ATP analogs (61, 62)) is responsible for the HR activity we have described. This hypothesis is intriguing since a number of results (39–36) have described a plant chloroplast recombinational DNA repair pathway that is dependent on a plastid recA homolog.

The observation that neither DNA synthesis inhibitors nor the absence of dNTPs reduced the frequency of HR suggests that the recombination events catalyzed by the extract are...
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unlikely to proceed beyond the stage of strand-transfer and Holliday junction branch migration (see Fig. 3, DSBR step c). Subsequent enzymatic events associated with the HR reaction, such as filling in of single-stranded gaps by DNA polymerase, DNA ligation, and resolution of Holliday junctions, are likely to be mediated by the *E. coli* host used in the transfection. Other investigators, studying HR activity in extracts prepared from yeast or mammalian nuclei have reached similar conclusions (37, 59). The observation that recA-deficient *E. coli* can repair transformed heteroduplex DNA formed by annealing linearized, denatured pSV2neoDR and pSV2neoDL (63) provides strong evidence that recombination-deficient bacteria are capable of completing HR reactions initiated by the mitochondrial extracts.

Recombinational Repair versus Genetic Recombination in Mammalian Mitochondria—Based on the arguments presented above, we have proposed that mammalian cells use homologous recombinational to repair damage to the mitochondrial genome. While this hypothesis appears quite reasonable, particularly in light of evidence that an analogous repair pathway functions in lower eukaryotes (31, 32), there remains the issue of why previous reports failed to detect mitochondrial genetic exchange in somatic cell hybrids (64). We believe that the level of nucleotide divergence that exists between the respective mitochondrial genomes of these species provides an answer. Studies in bacterial, as well as yeast and mammalian nuclear genomes have shown that cellular mismatch repair activity dramatically reduces the frequency of homologous recombination between genes that have diverged (65–67). For example, conjugal recombination between *E. coli* and *Salmonella typhimurium*, whose genomes differ by 20%, is nearly undetectable, however, inactivation of bacterial mismatch repair genes can enhance this HR frequency by 50–3000-fold (65). The human and mouse mitochondrial genomes differ by nearly 25% (2, 68), while comparison of a ∼550-base pair region of the mouse and hamster mitochondrial 16 S rDNA genes suggests there is approximately 18% non-identity between mitochondrial DNA in these two species (69). Based on the recent identification of a yeast mitochondrial mismatch repair gene *MSH1* (70), it seems reasonable to propose that mammalian mitochondria possess similar activity. If this were the case, mitochondrial mismatch repair activity would inhibit mitochondrial DNA recombination, providing an explanation for the failure to detect such activity in somatic cell hybrids (64).

A second, more intriguing possibility may also explain the failure to detect genetic exchange within the mammalian mitochondrial compartment. A yeast nuclear recessive mutation for providing us with the affinity-purified protein required for this repair process in mammalian mitochondria. We thank Dr. A. Jagendorf (Cornell University) for providing us with the affinity-purified recA antibody. Drs. Norman Sladek, Robert O'Dea, and Tim Walseth provided assistance with the cytchrome-c oxidase experiments. We thank Drs. Tim Walseth, Stan Thayer, Robert O'Dea, Greg Connell, and Cecilia Warner for helpful editorial suggestions.

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