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Base Excision Repair Is Limited by Different Proteins in Male Germ Cell Nuclear Extracts Prepared from Young and Old Mice

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The combined observations of elevated DNA repair gene expression, high uracil-DNA glycosylase-initiated base excision repair, and a low spontaneous mutant frequency for a *lacI* transgene in spermatogenic cells from young mice suggest that base excision repair activity is high in spermatogenic cell types. Notably, the spontaneous mutant frequency of the *lacI* transgene is greater in spermatogenic cells obtained from old mice, suggesting that germ line DNA repair activity may decline with age. A paternal age effect in spermatogenic cells is recognized for the human population as well. To determine if male germ cell base excision repair activity changes with age, uracil-DNA glycosylase-initiated base excision repair activity was measured in mixed germ cell (i.e., all spermatogenic cell types in adult testis) nuclear extracts prepared from young, middle-aged, and old mice. Base excision repair activity was also assessed in nuclear extracts from premeiotic, meiotic, and postmeiotic spermatogenic cell types obtained from young mice. Mixed germ cell nuclear extracts exhibited an age-related decrease in base excision repair activity that was restored by addition of apurinic/apyrimidinic (AP) endonuclease. Uracil-DNA glycosylase and DNA ligase were determined to be limiting in mixed germ cell nuclear extracts prepared from young animals. Base excision repair activity was only modestly elevated in pachytene spermatocytes and round spermatids relative to other spermatogenic cells. Thus, germ line short-patch base excision repair activity appears to be relatively constant throughout spermatogenesis in young animals, limited by uracil-DNA glycosylase and DNA ligase in young animals, and limited by AP endonuclease in old animals.

Germ line genomic stability is an important factor in reproductive health, with approximately 20% of genetic diseases attributed to new germ line mutations (12, 13, 46). Several autosomal dominant genetic diseases due to de novo male germ line mutations are associated with increased paternal age (18, 21, 45, 49), suggesting that male germ line genomic stability is compromised with age. Analysis of chromosomes in spermatozoa revealed an increased frequency of aberrations with age (24, 34, 41, 54), providing additional evidence that male germ line genomic stability decreases with age. Similarly in mice, the spontaneous mutant frequency in a *lacI* transgene recovered from pachytene spermatocytes, round spermatids, and epididymal spermatozoa obtained from old animals was approximately 10-fold higher than the mutant frequency observed for young mice (60).

In contrast to the decline in germ line genetic integrity with old age, the germ line DNA of young mice appears to be well maintained. Compared to somatic tissues, a lower spontaneous mutant frequency in the male germ line for a *lacI* transgene has been reported for fish (65), mice (32, 60), and rats (16). The murine mitotically proliferating male germ cell pool is comprised of primitive type A spermatogonia, which sequentially give rise to mitotically active, type A spermatogonia, intermediate spermatogonia, and type B spermatogonia (3). Type B spermatogonia generate a population of primary spermatocytes that proceed through meiosis and become haploid round spermatids (5) and then undergo complex morphological and molecular transformations to generate spermatozoa (3).

The spontaneous mutant frequency in a *lacI* transgene was analyzed by using specific spermatogenic cell types and found to decline between primitive type A spermatogonia and type B spermatogonia (60). The lower mutant frequency was maintained throughout the remainder of spermatogenesis. These observations suggest that mechanisms operate to provide spermatozoa with a low mutant frequency.

The specific-locus test and analysis of embryonic malformations have been used to indirectly study germ line mutagenesis in mice (19, 44, 47, 52). These studies revealed a differential susceptibility to induced mutagenesis. For example, many chemicals have been shown to exert their most potent mutagenic germ line effects on postmeiotic cell types (reviewed in

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reference 53). The mechanism(s) mediating the apparent differential mutability has not been described.

DNA base excision repair (BER) may be one of the mechanisms regulating germ line genomic stability because this repair pathway is largely responsible for ameliorating spontaneous base damage (33). Consistent with this are the observations that high levels of DNA BER gene transcripts are detected in the testis (1, 11, 17, 25, 36, 59, 61, 66). Specifically, male germ cells appear to be the source of the highest observed expression of several base excision repair genes, including those for Xrcc-1 (59, 61), DNA polymerase β (β-pol) (1, 25), and DNA ligase III (11, 36). Peak expression of these repair genes generally occurs in pachytene spermatocytes and round spermatids.

Western blot analyses have supported the RNA data showing greater levels of DNA ligases I and III, Xrcc-1, Ape/Ref-1, and β-pol proteins in mixed germ cell (MGC, i.e., all spermatogenic cell types in adult testis) nuclear extracts than in adult somatic tissues (26, 48). Furthermore, we have reported higher levels of uracil-DNA glycosylase-initiated base excision repair (UDG-BER) activity in MGC nuclear extracts obtained from young adult mice compared to extracts from brain, liver, enriched preparations of prepubertal Sertoli cells, thymocytes, and small intestine (26). Together, these data support the hypothesis that UDG-BER activity is greater in spermatogenic cells than in somatic tissues.

Two major BER pathways, based on the number of nucleotides incorporated during repair, are recognized. Short-patch BER is characterized by incorporation of a single nucleotide into the DNA strand undergoing repair in a β-pol-dependent manner (55, 56). During long-patch BER, 2 to 6 nucleotides are typically incorporated into the DNA strand undergoing repair in a PCNA-dependent manner (20, 31, 40). While substantial biochemical characterization of these pathways has been realized, there are few reports documenting the repair activities in mammalian tissues or primary cell types.

The studies described herein were performed to determine if DNA repair activity varies among spermatogenic cell types and whether DNA repair activity in spermatogenic cells obtained from old animals is diminished. Such changes could at least partially account for the mutagenesis observations described above. Modest variation in UDG-BER was detected among enriched populations of spermatogenic cell types obtained from young mice. In vitro UDG-BER activity in MGC nuclear extracts prepared from old mice was significantly lower than in MGC nuclear extracts from young adult mice. A significant reduction in the relative abundance of Ape/Ref-1 in MGC nuclear extracts obtained from old mice coincided with the decreased UDG-BER activity, and addition of purified recombinant APE/REF-1 restored the activity in germ cell nuclear extracts prepared from old mice. In contrast, addition of purified uracil-DNA glycosylase and DNA ligase III each elevated the UDG-BER activity in nuclear extracts prepared from MGCs obtained from young animals. Finally, short-patch and long-patch BER pathways were found to function in murine spermatogenic cell nuclear extracts.

MATERIALS AND METHODS

Animals. Male CD1 mice (6, 8, 30, and 60 days old) were obtained from Charles River, and $B6D2F_1$ male mice (3, 16, and 28 months old) were obtained from the National Institute on Aging (NIA). Mice heterozygous for *Apex* were obtained from in-house breedings. *Apex* heterozygous knockout mice were originally developed using 129-derived embryonic stem cells (35), but were backcrossed into C57BL/6J mice for at least five generations before the studies described herein. All animals were housed in an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility and fed standard mouse lab chow and water ad libitum. Mice were specific pathogen free.

At the appropriate ages, mice were humanely euthanized, and then testes were rapidly removed and used immediately for MGC preparations or enriched spermatogenic cell type and Sertoli cell isolations. Thymuses were removed from 6-day-old mice and used immediately for thymocyte isolation. All procedures were performed in accordance with federal and institutional guidelines for animal welfare.

MGC preparations, spermatogenic cell types, Sertoli cells, and thymocyte isolations. Testes were collected from three to four adult male $B6D2F_1$ or *Apex* heterozygous knockout mice and used to prepare MGCs as described previously (4, 5, 51). The composition of the MGC preparations from adult mice was approximately 4% spermatogonia, 22% spermatocytes, 71% spermatids, and <3% Sertoli cells (3). Specific populations of spermatogenic cell types and Sertoli cells were prepared from male CD1 mice of the appropriate ages using a Sta Put gradient system as described previously (4, 51).

Primitive type A spermatogonia and prepubertal Sertoli cells were recovered from mice 6 days old, type A and type B spermatogonia were from mice 8 days old, and pachytene spermatocytes and round spermatids were from mice 30 and >60 days old. Purities of recovered spermatogonia and Sertoli cells were ≥85% by morphological criteria. Purities of pachytene spermatocytes and round spermatids were ≥95%. Thymuses were obtained from sets of 50 6-day-old male CD1 mice and were teased apart in homogenization buffer as previously described (26). Cells were dissociated by gentle passage through a 16-gauge needle, and thymocytes were enriched by passage through Nytex mesh.

Nuclear extracts. Nuclear extracts were prepared from MGCs, enriched spermatogenic cell types, Sertoli cells, and thymocytes as described by Widen and Wilson (63) with modification, as described previously (26). Briefly, a known amount of luciferase protein (Roche Molecular Biochemicals, Indianapolis, Ind.) was added by including it in the buffer used to resuspend pelleted nuclei for homogenization. Protein concentrations were determined using the Bradford assay (7) with immunoglobulin (Ig) as a standard (Bio-Rad, Hercules, Calif.). Nuclear extracts were routinely diluted to 10 mg/ml, separated into single-use aliquots, and stored at −80°C until use.

Prior to use in repair assays or Western analyses, luciferase assays were performed by adding 2 μl of nuclear extract to 400 μl of luciferase buffer (60 mM Tris-acetate [pH 7.5], 2.5 mM EDTA, 12 mM magnesium acetate, 60 mM dithiothreitol, 5 mM ATP, 0.075% bovine serum albumin, and 150 μM luciferin). Relative light units were measured on a Lumat LB 9501 (Berthold) luminometer and compared to a luciferase standard curve (14, 15). The amount of luciferase recovered was used to correct for differences in protein recovered between samples (26).

Short-patch BER assay. The UDG-BER assay was performed as previously described (26, 55). Briefly, 3 pmol of a 51-mer oligonucleotide containing a single G:U mismatch and a 5′ fluorescein label on the U-containing strand (Integrated DNA Technologies) was combined with nuclear extract and incubated at 37°C for 10 min in reaction buffer (100 mM Tris-HCl [pH 7.5], 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM ATP, 0.5 mM NAD, 20 μM each dATP, dGTP, and dTTP, 5 mM ditrisphosphocreatine, 10 U of creatine phosphokinase, 20 nM unlabeled dCTP, and 20 μCi of [α-^{33}P]dCTP [3,000 Ci/mmol]). Ten micrograms of nuclear extract prepared from MGCs and purified populations of spermatogenic cell types and 40 μg of nuclear extract prepared from somatic cell types were used for UDG-BER assays. Reactions were stopped by placing the mixes on ice and the addition of 4.5 μl of stop solution (50 mM EDTA, 0.3 M NaCl, and 80% formamide) and then subjected to denaturing polyacrylamide gel electrophoresis (PAGE).

Oligonucleotide standards encompassing the linear range of fluorescent quantification were run simultaneously on each gel. Fluorescent visualization and quantification of oligonucleotide recovery were performed on a ChemiImage 4400 (Alpha Innotech), and radionucleotide incorporation was measured using a GS-363 Molecular Imager System (Bio-Rad). Various purified BER proteins, *Escherichia coli* UDG (Life Technologies, Grand Island, N.Y.), human β-pol, and human APE/REF-1 (Trevigen, Gaithersburg, Md.) and murine DNA ligases I and IIIβ (A. Tomkinson, University of Texas Health Science Center Institute of Biotechnology, San Antonio, Tex.) were added independently to nuclear extracts in a volume of 1 μl to determine which activity could enhance overall UDG-BER activity.

Long-patch BER assay. Three different long-patch BER substrates were prepared containing a G:U mismatch, a natural apurinic/apyrimidinic (AP) site, and a reduced AP site. Covalently closed circular DNA (cccDNA) was prepared as previously described (39, 40). cccDNA containing a reduced AP site was prepared by treating uracil-containing cccDNA with uracil-DNA glycosylase (10 U/μg of DNA) for 1 h at 37°C in the presence of $NaBH_4$. Substrate DNA was ^{32}P labeled as previously described (39).

Long-patch BER was conducted as previously described (6) with a few modifications. Briefly, 100 ng of cccDNA (50 fmol of reduced AP:G) was incubated with 5 μg of nuclear extract for 5, 10, 20, and 40 min at 25°C in a reaction buffer (20 mM HEPES, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM each of the four deoxynucleoside triphosphates [dNTPs], 2 mM ATP, 2 mM NAD, 40 mM phosphocreatine, and 1 U of creatine phophokinase) in a total reaction volume of 20 μl. Repair reactions were stopped by addition of sodium dodecyl sulfate (SDS) to a final concentration of 0.4%. Samples were incubated with 2 μg of proteinase K for 20 min at 37°C, and the DNA was recovered after phenol-chloroform extraction by ethanol precipitation. DNA was digested with *Hin*fI as previously described (40), and repair products were analyzed by gel electrophoresis through 20% polyacrylamide and 8 M urea and detected by autoradiography. Quantitative analysis was performed with a Fuji BAS1000.

Western blot analysis. Nuclear extracts prepared from enriched populations of spermatogenic cell types (50 μg) and MGCs (50 μg) obtained from young (3-month-old), middle-aged (16-month-old), and old (28-month-old) mice were fractionated on SDS–10% PAGE gels (acrylamide-bisacrylamide, 29:1), followed by electroblotting onto Trans-Blot transfer medium (Bio-Rad). To facilitate detection of specific antigens, blots were cut into three sections based on molecular mass.

Rabbit polyclonal anti-ligase I and -ligase III antibodies (A. Tomkinson, University of Texas Health Science Center at San Antonio, San Antonio, Tex.) were used to detect DNA ligases I and III, respectively. Xrcc1 was detected with rabbit anti-human XRCC1 (hXRCC1) polyclonal antibody (Serotec, Raleigh, N.C.). Rabbit anti-human APE/REF-1 (hAPE/REF-1; Novus Biologicals, Littleton, Colo.) and rabbit polyclonal anti-β-pol (S. Wilson, NIEHS, Research Triangle Park, N.C.) were used to detect Ape/Ref-1 and β-pol, respectively. Purified β-pol and APE/REF-1 (Trevigen, Gaithersburg, Md.) and DNA ligases I and III (A. Tomkinson) were included as standards and controls.

Primary antibody was followed by horseradish peroxidase-conjugated goat anti-rabbit Ig antibody (Pierce, Rockford, Ill.), and signal was generated with enhanced chemiluminescence (ECL; Pierce, Rockford, Ill.). Intensity of visualized bands was measured using a ChemiImager 4400 (Alpha Innotech, San Leandro, Calif.) as an integrated density value.

Statistical analysis. The data were analyzed using analysis of variance. For spermatogenic cell types for which all pairs of means were compared, the Duncan's new multiple range test was used. For analyses in which fewer than all pairs of means were compared, the Bonferroni adjustment was used. *P* values are presented from analysis of log-transformed data, whereas means and standard errors computed from untransformed data are presented. *P* values of <0.05 were considered significant.

RESULTS

In vitro BER activity in spermatogenic cells. To assess UDG-BER activity for specific spermatogenic cell types, nuclear extracts were prepared from premeiotic cell types (enriched populations each of primitive type A, type A, and type B spermatogonia), a meiotic cell type (pachytene spermatocytes), and a postmeiotic cell type (round spermatids) obtained from appropriately aged CD1 mice (a strain commonly used for obtaining defined populations of spermatogenic cell types). Two products can potentially be detected in the UDG-BER assay. One product, a 51-mer, represents complete repair, while the second product, a 23-mer, represents repair through incorporation of [^{33}P]dCTP without ligation. In every assay, the 23-mer represented less than 5% of the counts. Consequently, the data are presented as completely repaired oligonucleotide based on counts detected for the 51-mer.

No significant differences were detected in UDG-BER activity between nuclear extracts prepared from primitive type A spermatogonia, type A spermatogonia, and type B spermatogonia (Fig. 1). Nuclear extracts prepared from pachytene spermatocytes (obtained from 30- and >60-day-old mice) displayed a very modest 1.4-fold-higher UDG-BER activity than nuclear extracts prepared from MGCs obtained from 3- to 6-month-old CD1 mice and 1.6-fold-higher UDG-BER activity than spermatogonia. Pachytene spermatocytes exhibited a modest but significant 1.2-fold-higher UDG-BER activity than nuclear extracts prepared from round spermatids. Round spermatid nuclear extracts prepared from 30- and >60-day-old mice exhibited a modest 1.3-fold-higher UDG-BER activity than nuclear extracts prepared from spermatogonia. While these differences are small, statistical analyses revealed that the differences were significant, and thus they are reported here. The UDG-BER activity of nuclear extracts prepared from prepubertal Sertoli cells, a somatic cell type present in the testis, and thymocytes was similar ($P < 0.05$) and lower than that in nuclear extracts prepared from enriched spermatogenic cell types ($P < 0.05$; Fig. 1).

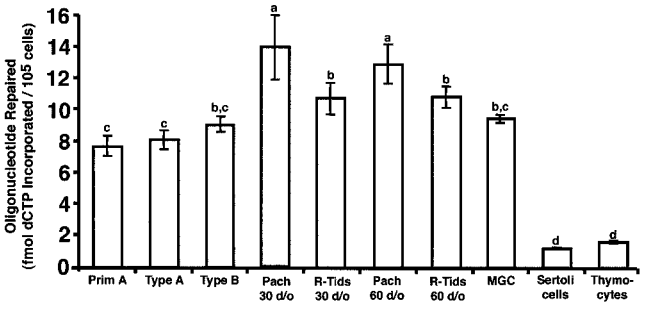

FIG. 1. UDG-BER activities for nuclear extracts prepared from enriched populations of spermatogenic cell types obtained from CD1 mice. Results are presented as mean ± standard error of the mean (SEM) of three replicate assays for each of three independent nuclear extract preparations. Cell types exhibiting similar UDG-BER activity are indicated by having the same letter. Prim A, primitive type A spermatogonia; Type A, type A spermatogonia; Type B, type B spermatogonia; Pach., pachytene spermatocytes; R-Tids, round spermatids.

In vitro BER activity in MGCs. Nuclear extracts were prepared from MGCs isolated from neonatal (6- and 8-day-old), young adult (3-month-old), middle-aged (16-month-old), and old (28-month-old) male $B6D2F_1$ mice and used to assess UDG-BER activity relative to age (Fig. 2). We have previously demonstrated that UDG-BER is similar in C57BL/6J, $B6D2F_1$, and CD1 mice (26). $B6D2F_1$ mice were used to assess the relationship between age and UDG-BER because they are maintained by the NIA for such studies, while CD1 mice are not maintained for aging studies.

UDG-BER activity was reduced 25% ($P < 0.05$) in nuclear extracts prepared from middle-aged mice and 53% ($P < 0.05$) in old mice compared to nuclear extracts prepared from neonatal and young adult mice (Fig. 2). While UDG-BER activity was significantly reduced in nuclear extracts prepared from 16- and 28-month-old mice, the activity of MGC nuclear extracts from 16-month-old mice was 5.8-fold and 4.3-fold higher than that in Sertoli cell and thymocyte nuclear extracts, respectively. Activity in MGC nuclear extracts from 28-month-old mice was

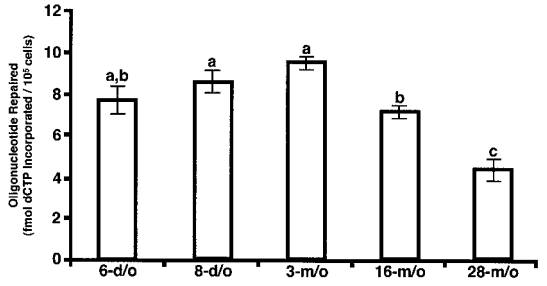

FIG. 2. UDG-BER activities for MGC nuclear extracts prepared from 6-day-old (6-d/o), 8-day-old, 3-month-old (3-m/o), 16-month-old, and 28-month-old male B6D2F$_1$ mice. Results are presented as means ± SEM of three replicate assays for each of three independent nuclear extract preparations. MGC nuclear extracts from different-aged animals with similar UDG-BER activity are indicated by having the same letter.

3.6-fold and 2.7-fold higher than that in Sertoli cell and thymocyte nuclear extracts, respectively (Fig. 1 and 2).

Nuclear extracts prepared from MGCs isolated from young adult, middle-aged, and old male B6D2F$_1$ mice were also used to assess long-patch BER activity. Repair of G:U mismatch on a cccDNA substrate was not detected using MGC nuclear extracts obtained from young mice (data not shown), but an AP site generated by preincubation of cccDNA containing a G:U mismatch with uracil-DNA glycosylase was repaired (data not shown). Long-patch BER activity of cccDNA containing a reduced AP site was 50% lower ($P < 0.05$) in MGC nuclear extracts isolated from middle-aged and old mice compared to MGC nuclear extracts prepared from young mice (Fig. 3).

BER protein composition in nuclear extracts. Relative proportions of BER proteins in nuclear extracts prepared from enriched populations of spermatogenic cells were determined by Western blot analyses (Fig. 4). The relative proportions of DNA ligase III and Xrcc-1 were 1.7-fold and 2.5-fold higher,

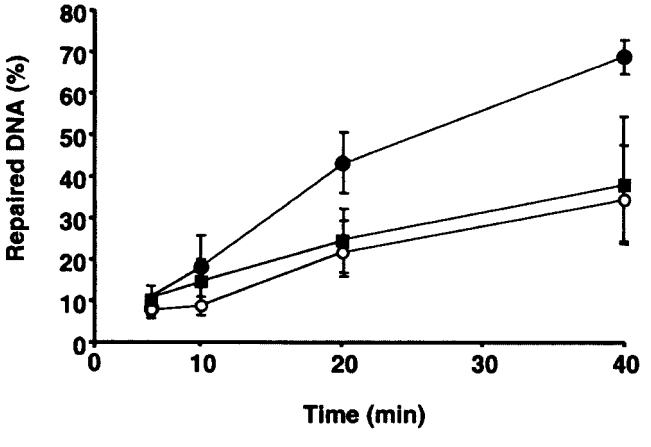

FIG. 3. Long-patch BER activities on reduced AP site cccDNA for MGC nuclear extracts prepared from 3-month-old, 16-month-old, and 28-month-old male B6D2F$_1$ mice. Results are presented as means ± SEM of assays for each of three independent nuclear extract preparations. Solid circles, 3-month-old mice; open circles, 16-month-old mice; solid squares, 28-month-old mice.

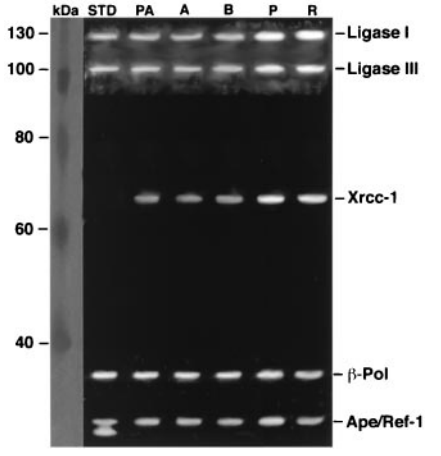

FIG. 4. Western blot analysis of BER proteins in nuclear extracts prepared from enriched populations of primitive type A spermatogonia (PA), type A spermatogonia (A), type B spermatogonia (B), pachytene spermatocytes (P), and round spermatids (R). Bands corresponding to DNA ligase I (130 kDa), DNA ligase III (93 kDa), Xrcc-1 (69 kDa), β-pol (39 kDa), and Ape/Ref-1 (37 kDa) proteins were visualized. Triplicate assays for each of three independent nuclear extract preparations were performed. Some variation between replicates was noted. A summary of all data is shown in Table 1. Molecular mass protein standards and purified DNA ligases I and III, β-pol, and Ape/Ref-1 (lane STD) are shown for comparison.

respectively, in pachytene spermatocytes and round spermatids than in spermatogonial cell types (Table 1).

The relative proportions of BER proteins in MGC nuclear extracts prepared from young, middle-aged, and old mice were also determined by Western blot analyses (Fig. 5). Quantification of chemiluminescence from Western blots revealed no significant changes in the abundances of DNA ligase I, DNA ligase III, Xrcc-1, and β-pol among the age groups (Table 2). In contrast, MGC nuclear extracts prepared from 16-month-old mice exhibited a 38% reduction in Ape/Ref-1 compared to nuclear extracts from 3-month-old mice (Table 2). Abundance of Ape/Ref-1 in MGC nuclear extracts prepared from 28-month-old mice was reduced by 62% compared to nuclear extracts from 3-month-old mice ($P < 0.05$; Table 2).

Identification of limiting BER enzyme activities in MGC nuclear extracts. Due to the concomitant reduction in UDG-BER activity and decreased Ape/Ref-1 levels in MGCs obtained from old mice, the ability of purified Ape/Ref-1 to restore UDG-BER activity in nuclear extracts from old mice was tested. Young heterozygous *Apex* knockout mice (35) were used as controls for reduced Ape/Ref-1 activity. Addition of purified APE/REF-1 to MGC nuclear extracts prepared from 3-month-old mice had no effect on UDG-BER activity (Fig. 6). MGC nuclear extracts prepared from heterozygous *Apex* knockout mice exhibited 50% of the UDG-BER activity of nuclear extracts from MGCs obtained from young wild-type mice (Fig. 6). Purified APE/REF-1 (5 ng) added to MGC nuclear extracts prepared from *Apex* heterozygous knockout mice was sufficient to restore the UDG-BER activity to that of young wild-type animals. Addition of 5 ng of APE/REF-1 resulted in a significant increase in UDG-BER activity for nuclear extracts from MGCs obtained from 28-month-old mice

TABLE 1. Abundance of BER proteins in nuclear extracts prepared from enriched spermatogenic cell types

Protein	IDV/10^3 cells ± SEM (% of total)[a]				
	Primitive type A spermatogonia	Type A spermatogonia	Type B spermatogonia	Pachytene spermatocytes	Round spermatids
DNA ligase I	21.0 ± 3.1 (26.6)	20.7 ± 2.1 (26.0)	20.8 ± 0.7 (24.9)	22.6 ± 1.8 (20.4)	22.1 ± 0.7 (20.2)
DNA ligase III	15.3 ± 2.6 (19.3)	15.2 ± 1.9 (19.1)	16.3 ± 1.1 (19.6)	**26.5 ± 0.4 (24.3)**	**26.3 ± 1.0 (24.1)**
Xrcc-1	9.3 ± 0.2 (11.7)	9.0 ± 0.8 (11.2)	8.6 ± 0.2 (10.3)	**22.1 ± 1.8 (20.3)**	**22.8 ± 0.2 (20.9)**
β-Pol	19.0 ± 2.5 (18.4)	19.0 ± 0.6 (20.0)	18.9 ± 1.8 (22.5)	19.0 ± 0.7 (17.5)	19.1 ± 0.3 (17.3)
Ape/Ref-1	14.6 ± 2.7 (24.0)	15.9 ± 1.3 (23.7)	18.8 ± 0.6 (22.7)	19.0 ± 2.8 (17.5)	18.9 ± 1.3 (17.5)

[a] Values are expressed as integrated density value (IDV) per 10^3 cells ± SEM, with the percentage of total chemiluminescence for a tissue, calculated by (protein IDV/total IDV for 10^3 cells) × 100. Values in boldface type are significantly different from values for proteins in other cell types.

(Fig. 6), and supplementation with 20 ng of purified APE/REF-1 resulted in UDG-BER activity similar to that of MGCs obtained from young mice.

The addition of 1.25 ng of purified uracil-DNA glycosylase significantly increased UDG-BER activity in nuclear extracts prepared from MGCs obtained from 3-month-old mice (Fig. 7a). Furthermore, UDG-BER activity was increased approximately 2-fold after addition of 20 ng of purified uracil-DNA glycosylase to MGC nuclear extracts prepared from 3-month-old mice. In comparison, addition of purified uracil-DNA glycosylase to MGC nuclear extracts prepared from 28-month-old mice did not alter UDG-BER activity. Purified DNA ligase IIIβ also significantly increased UDG-BER activity in nuclear extracts prepared from 3-month-old mice. However, no significant change in UDG-BER activity was observed after addition of purified DNA ligase IIIβ to MGC nuclear extracts prepared from 28-month-old mice (Fig. 7b).

Addition of 10 or 20 ng of purified β-pol modestly but significantly increased UDG-BER activity in nuclear extracts prepared from MGCs obtained from 3-month-old (1.35-fold) and 28-month-old (1.5-fold) mice (Fig. 7c). No significant changes in UDG-BER activity were detected with the addition of DNA ligase I to nuclear extracts from young or old mice (Fig. 7d).

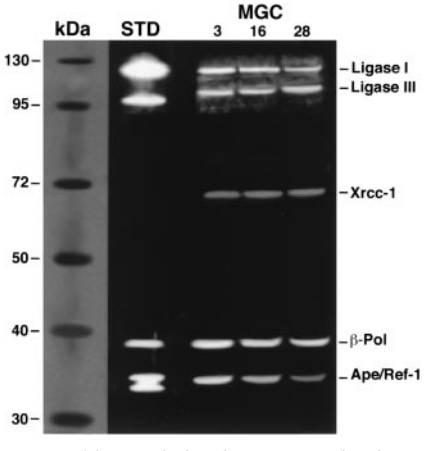

FIG. 5. Western blot analysis of BER proteins in MGC nuclear extracts prepared from 3-month-old, 16-month-old, and 28-month-old mice. Bands corresponding to DNA ligase I (130 kDa), DNA ligase III (93 kDa), Xrcc-1 (69 kDa), β-pol (39 kDa), and Ape/Ref-1 (37 kDa) proteins were visualized. Triplicate assays were performed for each of three independent nuclear extract preparations. Some variation in signal intensities was observed among Western blots. A summary of all data is shown in Table 2. Molecular mass protein standards and purified DNA ligases I and III, β-pol, and APE/REF-1 (lane STD) are shown for comparison.

DISCUSSION

Based on previous observations identifying variable DNA repair gene expression among defined populations of murine spermatogenic cell types (1, 11, 25, 36, 59, 61, 64), differences in spontaneous mutation frequencies among spermatogenic cell types (60), and differences in induced mutagenesis among murine spermatogenic cell types (reviewed in reference 53) nuclear extracts prepared from defined populations of spermatogenic cell types were examined for short-patch UDG-BER activity to determine if there was cell type variation in repair activity.

The modest increase in activity observed for nuclear extracts prepared from pachytene spermatocytes and round spermatids compared to earlier spermatogenic cell types is generally consistent with elevated DNA repair gene expression and inversely correlated with decreased spontaneous mutant frequencies (1, 11, 25, 36, 59, 61, 64, 66). These results suggest that high UDG-BER activity measured in spermatogenic cell types may contribute to the overall reduced mutation frequency observed in male germ cells. Consistent with Olsen et al. (48), we detected no variation between the relative abundances of Ape/Ref-1, DNA ligase I, and β-pol in premeiotic, meiotic, and postmeiotic male germ cells from young mice. However, increases in Xrcc-1 and DNA ligase III were detected in meiotic and postmeiotic mouse male germ cells, although these increases in protein are modest compared to the 10-fold increase reported for *Xrcc-1* (59) and *LigIII* (11) transcript levels. Transcription ceases in condensing spermatids, and several genes required in later stages of spermatogenesis are expressed and accumulated in meiotic and postmeiotic spermatogenic cell

TABLE 2. Abundance of BER proteins in nuclear extracts prepared from MGCs obtained from different-aged mice

Protein	Mean IDV/10^3 cells ± SEM (% of total)[a]		
	3-month-old mice	16-month-old mice	28-month-old mice
DNA ligase I	12.0 ± 1.3 (24.2)	12.2 ± 0.3 (26.5)	11.9 ± 0.4 (26.5)
DNA ligase III	11.7 ± 0.8 (23.6)	11.4 ± 0.3 (24.8)	12.8 ± 0.4 (28.5)
Xrcc-1	5.0 ± 0.6 (10.1)	5.3 ± 0.3 (11.4)	5.1 ± 0.1 (11.3)
β-Pol	13.3 ± 0.6 (26.8)	12.3 ± 0.4 (27.4)	12.4 ± 0.4 (27.7)
Ape/Ref-1	7.6 ± 0.9 (15.3)	4.6 ± 0.3 (9.9)	**2.7 ± 0.9** (6.0)

[a] See Table 1, footnote *a*.

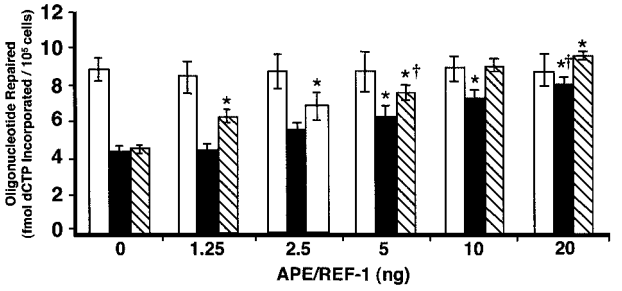

FIG. 6. UDG-BER activities for MGC nuclear extracts prepared from 3-month-old B6D2F$_1$ (open), 28-month-old B6D2F$_1$ (solid), and 3-month-old *Apex* heterozygous knockout mice (striped). Results are presented as means ± SEM of three replicate assays for each of three independent nuclear extract preparations. ∗, significantly ($P < 0.05$) different from addition of 0 ng of protein within a specific group. †, amount of purified APE/REF-1 required to restore activity to that of 3-month-old mice with 0 ng of protein added.

types (23, 29, 30, 37, 38, 43, 58). Such a phenomenon may explain the discrepancy between transcript and protein levels observed for these BER genes.

Pachytene spermatocyte and round spermatid nuclear extracts were prepared from 30-day-old and 60-day-old animals to determine whether there were differences in activity between cells in the first wave of spermatogenesis (30-day-old) and later (60-day-old). No significant differences were detected. These results were reinforced by the comparisons among MGC nuclear extracts prepared from 6-day-old (composed principally of primitive type A spermatogonia), 8-day-old (types A and B spermatogonia), and 3-month-old mice (all stages of spermatogenesis and after the first wave of spermatogenesis). Accordingly, no significant difference was detected among nuclear extracts prepared from different-aged mice reflecting the first wave of spermatogenesis (6-day-old and 8-day-old) compared to animals that have progressed beyond the first wave of spermatogenesis (3-month-old).

A paternal age effect has long been recognized in humans due to the corresponding impact on paternally derived de novo mutations associated with certain autosomal dominant disorders. A similar age-related increase in spontaneous mutant frequencies for murine spermatogenic cells has been observed using *lacI* transgenic mice (60). Because DNA repair pathways contribute to maintaining genetic integrity and because BER in particular is largely responsible for ameliorating spontaneous base damage (22, 33), short-patch and long-patch UDG-BER assays were employed to assess repair activity in relation to age. A decrease in short-patch UDG-BER activity measured in MGC nuclear extracts from 16-month-old mice compared to young mice and an additional reduction in nuclear extracts from 28-month-old mice were observed.

Despite the decline in UDG-BER activity in MGCs from old animals, the activity is greater than that found in thymocytes and Sertoli cells obtained from 6-day-old animals that display relatively high UDG-BER activity for somatic cell types. Analogous to short-patch BER, a decline in long-patch BER was detected in nuclear extracts prepared from 16- and 28-month-

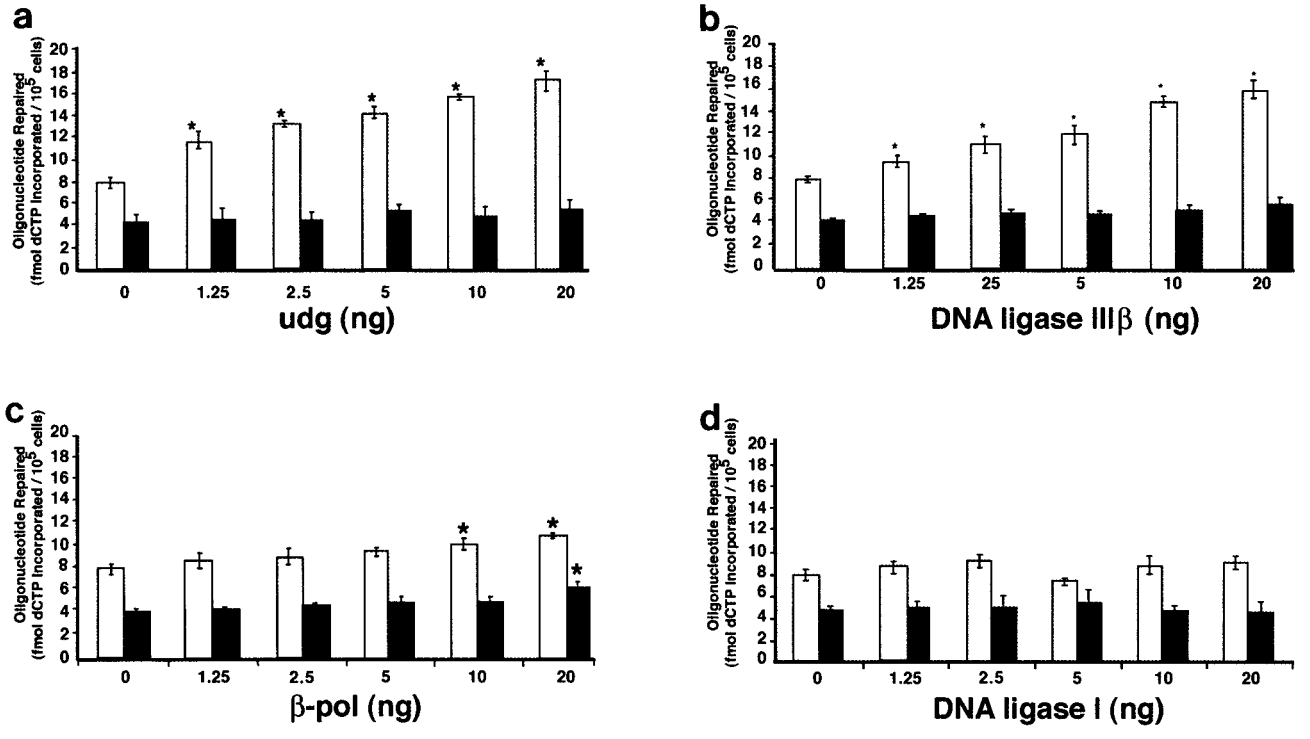

FIG. 7. UDG-BER activities for MGC nuclear extracts prepared from 3-month-old (open) and 28-month-old (solid) male B6D2F$_1$ mice. Results are presented as means ± SEM of three replicate assays for each of three independent nuclear extract preparations. Results are for UDG-BER assays in which purified uracil-DNA glycosylase (a), DNA ligase III (b), β-pol (c), and DNA ligase I (d) were added to MGC nuclear extracts. ∗, significantly ($P < 0.05$) different from addition of 0 ng of protein within a specific age group.

old mice with the reduced AP site-containing substrate. Together, these data indicate that an overall decline in BER activity may contribute to the increased mutant frequency observed in spermatogenic cells from old mice.

The limiting enzyme activity in the short-patch BER pathway has not been conclusively identified, although it has been reported that in some cell culture lines Ape/Ref-1 is limiting (27, 50). However, the lyase activity of β-pol appears to be rate limiting using a reconstituted BER pathway (2, 57). The apparent discrepancies may be explained by the different assay systems used; the limiting activity may vary among cell types and tissues and may vary for different DNA lesions.

In this study we show that uracil-DNA glycosylase appears to be a major limiting activity for short-patch BER in MGC nuclear extracts prepared from young mice. The cccDNA substrate containing a G:U mismatch was not repaired at a detectable level by MGC nuclear extracts prepared from young male mice. Due to the smaller amount of cccDNA used for the long-patch assay compared to the short-patch assay, it is possible that the same relative proportion of G:U-containing substrate was repaired via the long-patch pathway, but the actual amount of product was too small to be detected. However, cccDNA substrate containing a natural AP site was repaired efficiently. This suggests that uracil-DNA glycosylase is also one of the rate-limiting factors in long-patch BER.

Because there are many DNA glycosylases (22), it is reasonable to speculate that the BER proteins functioning downstream of these enzymes are present at greater activity levels than any one DNA glycosylase, so that the BER pathway can be completed. In this scenario, the initiating DNA glycosylase may be expected to be rate limiting for any specific base lesion.

Therefore, it was somewhat surprising that DNA ligase IIIβ was nearly as effective as uracil-DNA glycosylase in elevating the overall UDG-BER activity of MGC nuclear extracts prepared from young mice. Two forms of DNA ligase III are present in the testis (36): DNA ligase IIIα (103 kDa), which is ubiquitously expressed, and DNA ligase IIIβ (96 kDa), which is expressed in meiotic and postmeiotic cell types. However, peak expression of DNA ligases IIIα and IIIβ occurs in pachytene spermatocytes (11, 36).

The relative contributions of DNA ligase IIIα and IIIβ in BER have not been clearly delineated. Although our results suggest that DNA ligase IIIβ can participate in short-patch BER, the data do not exclude a contribution from DNA ligase IIIα. Notably, DNA ligase IIIα has been shown to interact with Xrcc-1 to facilitate ligation of DNA single-strand breaks during BER (8, 9, 10, 36, 62). The addition of APE/REF-1, β-pol, and DNA ligase I to MGC nuclear extracts obtained from young animals had little to no effect on UDG-BER activity, suggesting that uracil-DNA glycosylase and DNA ligase III are the major limiting activities in these nuclear extracts.

In contrast, the major limiting activity in MGC nuclear extracts prepared from old mice appears to be Ape/Ref-1. Decreased signal for Ape/Ref-1 in Western blots was observed for MGC nuclear extracts obtained from 28-month-old mice compared to young mice, and addition of APE/REF-1 to extracts from old mice restored short-patch BER activity to the levels observed for extracts obtained from young mice. Recently, cell cultures derived from *Apex* heterozygous knockout mice were shown to be more susceptible to oxidative stress than wild-type cells (42). Together, these data reveal biological consequences of reduced Ape/Ref-1 levels. In agreement with earlier reports, no changes were detected in the relative abundance of Xrcc-1 with increased age (61).

In conclusion, there is only modest variation in short-patch UDG-BER activity between spermatogenic cell types. However, there are differences between BER activity in spermatogenic cells from young and old mice. First, short-patch and long-patch BER activities are substantially lower in germ cell nuclear extracts prepared from old mice compared to extracts prepared from young mice. Second, uracil-DNA glycosylase and DNA ligase III appear to be the principal limiting activities in short-patch UDG-BER for MGC nuclear extracts obtained from young mice, while Ape/Ref-1 is the major limiting activity in MGC nuclear extracts obtained from old mice.

Considered in conjunction with the recently reported low level of nucleotide excision repair in rat spermatogenic cell types (28), our data suggest that BER is an important pathway for maintaining genome integrity in male germ cells and that a reduction in BER with age may contribute to the increased mutant frequency observed for male germ cells obtained from old mice.

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