Repair of Methylation Damage in DNA and RNA by Mammalian AlkB Homologues

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Human and Escherichia coli derivatives of AlkB enzymes remove methyl groups from 1-methyladenine and 3-methylcytosine in nucleic acids via an oxidative mechanism that releases the methyl group as formaldehyde. In this report, we demonstrate that the mouse homologues of the α-ketoglutarate Fed(II) oxygen-dependent enzymes mAbh2 and Abh3 have activities comparable to those of their human counterparts. The mAbh2 and mAbh3 release modified bases from both DNA and RNA. Comparison of the activities of the homogenous ABH2 and ABH3 enzymes demonstrate that these activities are shared by both sets of enzymes. An assay for the detection of α-ketoglutarate Fed(II) dioxygenase activity using an oligodeoxyribonucleotide with a unique modification shows activity for all four enzymes studied and a loss of activity for eight mutant proteins. Steady-state kinetics for removal of methyl groups from DNA substrates indicate that the reactions of the proteins are close to the diffusion limit. Moreover, mAbh2 or mAbh3 activity increases survival in a strain defective in alkb. The mRNAs of ABH2 and ABH3 are expressed most in testis for ABH2 and ABH3, whereas expression of the homologous mouse genes is different. The mAbh3 is strongly expressed in testis, whereas highest expression of mAbh2 is in heart. Other purified human AlkB homologue proteins ABH4, ABH6, and ABH7 do not manifest activity. The demonstration of mAbh2 and mAbh3 activities and their distributions provide data on these mammalian homologues of AlkB that can be used in animal studies.

Environmental and endogenous agents constantly subject DNA and RNA to damage. Alkylation agents cover a spectrum of chemicals that in their most simple form methylate DNA or RNA. The subsequent distribution of methylated products of the reaction of methylyating agents with nucleic acids depends on the nature of the agent. S$_2$1 alkylating agents react via a carbion ion intermediate whereas S$_2$2 agents interact directly with DNA (1). Under these circumstances different sites on nucleic acids are modified. In addition to the alkylating agent, the structure of the DNA also plays a role. Regions of single-stranded nucleic acids favor reaction at sites that are virtually untouched in double-stranded nucleic acids. For example, position 1 of Ade is not modified to any extent in double-stranded DNA (ds-DNA), whereas in single-stranded DNA (ss-DNA) it becomes a major target (1–4).

In 1983, an Escherichia coli mutant with sensitivity to S$_2$2 alkylating agents was isolated and named alkb (5). The cloning of the gene and the acknowledgment that it was linked to the ada operon responding to DNA alkylation damage produced enormous interest in this gene (6,7). Despite the knowledge that expression of alkb in human cells could increase resistance to alkylating agents (8, 9), AlkB had no known enzymatic function. The genetic work in E. coli was important, but without function, progress in the line of AlkB lagged behind that of other DNA repair studies (10–12). Even the isolation of a human homologue of AlkB, ABH1, failed to provide insight as to function of this group of proteins (13).

Using a host cell reactivation assay, alkb mutants were shown to efficiently process chemically methylated ds-DNA phage, but not chemically methylated ss-DNA phage (14, 15). That provided an important clue to the nature of AlkB. Crucial information in elucidating the enzymatic function of AlkB was provided by bioinformatics analysis that showed AlkB belonged to a large class of enzymes known as 2-oxo-glutarate- and iron-dependent dioxygenases (16).

Once the link between the AlkB and the 2OG family was established, two groups showed that AlkB was in fact a member of the family and that AlkB released methyl groups not only from 1-meAde and 3-meCyt, but also 3-meThy and 1-meGua (17–21). The identification of an activity for AlkB accelerated the field, and two homologues of human origin, ABH2 and ABH3, were isolated based on BLAST searches of databases. These proteins were both demonstrated to be active on methylated substrates (22, 23) (Fig. 1, a and b). Although ABH2 and ABH3 were active, ABH1, the first member in the ABH series, was not active in biochemical or complementation assays. Another bioinformatics search revealed a number of potential members of the series (24). This suggests that there are a number of homologues related to AlkB that cover most phyla.

One method to elucidate biological and biochemical function is to work in related organisms. Probably the most studied mammal aside from humans is mouse. The model presented by mouse has been exploited by targeted deletion of genes by homologous recombination. Therefore, we have isolated cDNAs coding for two mouse homologues of ABH2 and ABH3. We have expressed those sequences, produced proteins that were purified to homogeneity, demonstrated activity on both DNA and RNA substrates, determine the kinetics for the release of methyl groups from DNA, and show the tissue distribution in mouse and human of the RNA for both genes. We also have isolated and

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9 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) BI408770, BC018196, BC002820, BQ881117, BG71574G, BC004393, BC015183. On leave from CNRS UMR1772 Physicochimie et Pharmacologie des Macromolecules Biologiques. To whom correspondence should be addressed: Biology Division, Beckman Research Institute, City of Hope National Medical Center, 1450 E. Duarte Rd., Duarte, CA 91010. Tel.: 626-301-8220; Fax: 626-930-5366; E-mail: toconnor@coh.org.

10 The abbreviations used are: ds, double-stranded; 2OG, α-ketoglutarate Fed(II)-dependent dioxygenase; ABH, human AlkB homologue; mAbh, mouse AlkB homologue; ODN, oligodeoxyribonucleotide; poly(A), polyribonucleotide A; poly(C), polyribonucleotide C; poly(dA), poly(deoxyribonucleotide A); poly(dC), poly(deoxyribonucleotide C); ss, single-stranded; NTA, nitrilotriacetic acid; [3H]MNU, N-[3H]methyl-N-nitrosourea; DMS, dimethylsulfate.
expressed cDNAs for ABH4–8 and discuss the results of experiments on the purported relationship between these proteins and the activities of ABH2 and ABH3.

MATERIALS AND METHODS

Strains—The F′-plasmid was introduced into E. coli strains HK81 (wild type) and HK82 (alkB) as described (15) to generate the strains HK81/F′ and HK82/F′, respectively (the HK81 and HK82 strains were kindly provided by Michael Volkert) (5, 7, 11). Briefly, overnight cultures of XL1-Blue (donor), HK81 (recipient), and HK82 (recipient) were diluted 20-fold and grown to approximately A600 0.5. Cells were mixed in ratio donor/recipient = 1:10 and incubated at 37 °C for 30 min. The cell suspension was streaked out on LB plates with 50 μg/ml streptomycin and 10 μg/ml tetracycline. HK82 (alkB DE3) was constructed using the ADE3 lysogenization kit (Novagen) according to the manufacturer’s instructions. The expression and purification of all the human or mouse AlkB homologues were conducted in this strain.

Purification of Human ABH2 and ABH3 and Mouse mAbh2 and mAbh3—Human ABH2 and ABH3 cDNAs were obtained by RT-PCR with total RNA of LNCaP prostate cancer cell line. Mouse Abh2 and Abh3 cDNAs were obtained from the IMAGE consortium, and IMAGE Clone IDs for expressed sequence tags (EST) clones are 4196765 (mouse ABH2) and 3993214 (mouse ABH3). The cDNAs were amplified by PCR using Pfu Turbo DNA polymerase, and subcloned into the EcoRI/XhoI sites (ABH2) or BamHI/SalI sites (ABH3, mAbh2, and mAbh3) of the expression vector pET28a(+) (Novagen). All sequences for mAbh2, mAbh3, Abh4–Abh8 have been submitted to GenBank™. His-tagged proteins were expressed in E. coli strain HK82 (alkB DE3) in LB medium. Bacterial pellets were lysed and sonicated in ice-cold lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole) with 1 mg/ml of lysozyme. The lysate was centrifuged at 10,000 × g for 25 min at 4 °C, and the supernatant was loaded onto a Ni-NTA flow column, which was washed first with lysis buffer containing 20 mM imidazole. The bound proteins were released from the column with elution buffer (50 mM NaH2PO4, 300 mM NaCl, and each of 50 mM, 100 mM, and 250 mM imidazole, respectively) (Qiagen). We further purified the fractions obtained from the Ni-NTA columns by ion exchange with CM Sepharose® Fast Flow and FPLC with Superose® 12 HR 10/30 column (Amersham Biosciences). Fractions were analyzed by SDS-PAGE, and the relevant fractions were pooled. Point mutations in human or mouse AlkB homologues were introduced by QuikChange® II XL site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The sequences of the mutant human ABH2 (D173A), ABH2 (H236A), human ABH3 (D193A), human ABH3 (H257A), mAbh2 (D151A), mAbh2 (H214A) mAbh3, and (D193A), mAbh3 (H257A) (H236A), human ABH3 (D193A), human ABH3 (H257A), mAbh2 (D151A), mAbh2 (H214A) mAbh3, and (D193A), mAbh3 (H257A) were confirmed by sequencing the entire coding sequence. The mutant proteins were expressed and purified in the same manner as the wild-type proteins.

Preparation of Methylated DNA and RNA—Poly(A), poly(C), poly(dA), and poly(dC) were obtained from Amersham Biosciences. The homopolymers, single-stranded M13mp18, and single-stranded DNA oligo 5′-AAAGCAGCAATTCCGAAAAGCGG−3′, 5′-AAAGCAGCAGGGTTCAAAAGCGG−3′. The ss-DNA oligos (at least in 3-fold molar excess compared with the ABH2 or 3) were labeled with [γ−32P]ATP at the 5′-end, and incubated with 50 pmol of human or mouse ABH2 and ABH3 and for 30 min at 37 °C in the same buffer as for the complementation assay. After phenol/chloroform extraction and ethanol precipitation, ss-DNA oligos were annealed with a 3-fold molar excess of non-methylated complementary strand for 20 min at 37 °C in 10 mM Tris buffer (pH 8.0), and then subjected to EcoRI or HpaII treatment for 30 min at 37 °C, followed by phenol/chloroform extraction and ethanol precipitation. The restriction enzyme cleavage was verified on 20%(w/v) polyacrylamide gels containing 7% urea. The gels were dried and exposed overnight to storage phosphor screens and were scanned at 200 micron resolution using a Typhoon 9410 Variable using ImageQuant 5.2 software (Molecular Dynamics) Mode Imager (Amersham Biosciences).

Steady-state Kinetics of ABH2 and ABH3—Determination of the kcat and Km values of the ABH2 and ABH3 was made by varying the substrate concentration (10, 20, 50, 100, 200, and 400 nM). The substrates used were the same ODNs as those in the previous section. Both ss- and ds-DNA substrates were used. The total protein concentration in each case was 5 μM. Reactions were performed for 60 min or adjusted to ensure that less than 20% of the substrate was expended. All reactions were performed in triplicate and analyzed using Hanes plots according to Equation 1.

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\frac{[ODN_{Total}]}{V} = \frac{[ODN_{Total}]}{V_{max}} + \frac{K_m}{V_{max}} \tag{Eq. 1}
\]

where ODN_{total} is the total ODN substrate concentration, V_{max} is the enzyme maximum reaction velocity, v is the initial reaction velocity, and K_m is the Michaelis constant. Reactions of ds-DNA substrates with
ABH2 were performed in 150 mM NaCl in the reaction buffer based on results with respect to dependence on salt concentration.

NMR Studies—The wild-type and mutant ABH2 and ABH3 proteins were characterized by one-dimensional NMR experiments to ensure that the amino acid substitutions do not disrupt the overall structural integrity of the protein. The samples for NMR measurements were at 1 mg/ml concentration, in 10 mM sodium phosphate buffer in 95% H2O, 5% D2O at pH 6.0. One-dimensional 1H experiments were performed on a Bruker Avance 500 MHz spectrometer equipped with four channels, pulse shaping, and pulsed field gradient capabilities. The spectra were collected with 8192 complex points and 8000 Hz spectral width.

Northern Blot Analysis—Human or mouse multiple-tissue poly(A)+ Northern blots were obtained from Clontech. The membranes were hybridized with a probe containing the complete cDNA of human or mouse AlkB homologues. A probe specific for β-actin was used as a control. Membranes were exposed overnight to storage phosphor screens and were scanned at 200 micron resolution using a Typhoon 9410 Variable Mode Imager (Amersham Biosciences). Signal quantification was performed using ImageQuant 5.2 software (Molecular Dynamics).

RESULTS

Cloning and Sequence Alignment of mABH2 and mABH3—Mouse homologues of human ABH2 and 3, mAbh2 and mAbh3, were cloned by homology to the corresponding human proteins. The alignment of the proteins using ClustalW is shown in Fig. 1c. The identity between the mouse and human proteins suggests that the function should be conserved in these derivatives. The identity between mAbh2 and ABH2 is over 75.1%, but that is due mainly to differences in the N-terminal end of the proteins. In contrast, the identity for mAbh3 and ABH3 is 85.7% with the majority of bases that differ spread throughout the two sequences. There are a number of sequences that are conserved for all five sequences in Fig. 1c. These sequences indicated in yellow could have structural or catalytic roles. Among these, there is a cluster that has H separated by an amino acid and DDE (for ABH2, D173, Fig. 1c). This region could have a role in catalysis. The AlkB protein is very close in its sequence alignment in this region with the only difference being a lysine substituted in the sequence to separate the two consecutive aspartic acids. These alignments are close, but functional assays on the proteins must be performed to indicate that the proteins have the same activities. Interestingly, there is only 27% identity between mAbh2 and mAbh3.
The identity drops to 19.1 or 12.6% when the AlkB is compared independently to mAbh2 or mAbh3. Even though the identity in bioinformatics alignments involving mAbh2 and mAbh3 with AlkB is low, both proteins still are active against similar substrates (see below).

**Expression and Purification of mAbh2 and mAbh3**—To study the biochemical properties of mAbh2 and mAbh3, we expressed the mAbh2 and mAbh3 proteins using a T7 RNA polymerase promoter-based system in *E. coli* HK82(DE3) (*alkB*/H11002) with His6 tags to use Ni-NTA chromatography in the initial purification step. Further purification of the proteins was performed using CM-Sepharose and gel filtration chromatography to obtain homogeneous proteins (Fig. 2a). To compare the proteins to their human counterparts, the ABH2 and ABH3 were isolated using similar methods. To ensure that the major peaks from the final columns corresponded to the activity of the peaks observed for the proteins, we monitored the activity against a number of 3H-labeled substrates (Fig. 2b). Both mAbh2 and mAbh3 (not shown) purification demonstrated that the major peaks obtained in the final purification were the major activities obtained. Expression of the ABH2, ABH3, mAbh2, and mAbh3 produced more than 10 mg/liter of purified protein.

**mAbh2 and mAbh3 Reactions Complement Sensitivity of *E. coli* alkB− Strain HK82 to MMS-treated M13mp18**—To test the activities of the purified proteins and their capacity to complement an alkB-deficient *E. coli* strain, ss-M13mp18 DNA was treated with MMS, the methylated ss-DNA treated with one of the AlkB homologue proteins, and transformed into an alkB− strain (HK82) or as a control, a wild-type strain (HK81) (Fig. 3). If the methylated ss-DNA is not reacted with one of the AlkB homologue proteins, the transformation efficiency in the HK82 alkB− strain as determined by plaque formation is almost two orders of magnitude less than when the modified ss-DNA is treated with an AlkB homologue prior to transformation. In this assay, transformation efficiency is determined by the number of plaques formed compared with unmodified ss-M13mp18 DNA. The modified ss-DNA was trans-
formed into the wild-type strain, HK81, without reaction with any of the AlkB homologues and demonstrated to have similar transformation efficiency to that of the modified ss-DNA treated with AlkB homologues, but transformed into the HK82 alkB-deficient strain. Any of the AlkB homologues (ABH2, ABH3, mAbh2, or mAbh3) used for treatment of modified ss-DNA yielded transformation efficiencies comparable to that for transformation into the wild-type HK81 strain. Therefore, the mAbh2 and mAbh3 have similar activities to ABH2 and ABH3 that restore the transformation efficiency of methylated ss-DNA.

mAbh2 and mAbh3 Remove Methyl Groups from 1-meA and 3-meC—The AlkB homologues repair the alkylated base via an oxidative mechanism that releases formaldehyde and does not involve scission of the phosphodiester backbone (17, 22, 25–27). To study the release of methyl groups by the 2OG activity of AlkB homologues, we used an assay based on ODNs with unique modifications of 1-meA or 3-meC in restriction enzyme cleavage sites. The modified ODN isfirst treated with the AlkB homologue. If ss, the ODN must be annealed to the complementary ODN prior to restriction enzyme cleavage. The ds-ODN is then reacted with a restriction enzyme and the ODN is cleaved if the methyl group on 1-meA or 3-meC has been removed. Following restriction endonuclease cleavage, the products are separated using denaturing PAGE. Fig. 4 shows results from the use of this assay for ss-DNA substrates. If there is no modified ODN, all DNA is cleaved by EcoRI. However if a 1-meA is present in the first A in the EcoRI restriction site, the DNA is not cleaved by the restriction enzyme. Partial removal of the 1-meA by the ABH2, ABH3, mAbh2, or mAbh3 restores the restriction site and permits cleavage by EcoRI. To test the universality of this assay, we also built a 1-meA ODN with a HindIII site and placed the 1-meA at the first A of the recognition sequence. In this case, no inhibition of the cleavage is observed (data not shown). Therefore, this is a useful assay, but not every restriction site can be employed.

An assay to monitor 3-meC activity based on the same principles was also developed. In this case, 3-meC was inserted into an ODN at the first position of a HpaII site. The same procedure was followed to demonstrate activity for the 1-meA assay, except that HpaII restriction endonuclease cleavage was used. Fig. 5 unambiguously demonstrates that there is only cleavage of the ODN when 3-meC has been removed from the HpaII site. We also tested KpnI and HhaI restriction sites in a similar assay, but found that KpnI and HhaI cleavage, although inhibited, did not yield clear results as did use of the HpaII site (data not shown). The use of this assay method should greatly simplify the determination of kinetics and the study of AlkB homologues.

The structural integrity of mutant AlkB proteins was evaluated by one-dimensional NMR spectra. The one-dimensional NMR spectra of the wild-type and mutant proteins were nearly identical, particularly in the region of the spectra corresponding to signals of methyl and methylene groups, which include those from amino acid residues in the hydrophobic core of the protein, such as Ile, Leu, and Val. The one-dimensional spectra indicate that the hydrophobic core of the protein is intact in the mutants, and therefore, the amino acid substitutions do not disrupt the overall three-dimensional structure of the protein (data not shown).

ABH2 and mAbh2 Remove Methyl Groups from Single-stranded RNA and DNA Substrates—The AlkB homologues were tested against a number of substrates for activities removing methylation damage. The substrates were ODNs, polydeoxynucleotides, or polynucleotides that were modified with [3H]MNU. The rate of release of radioactivity is similar for mAbh2 and ABH2 for ODNs, either single- or double-stranded that are modified with [3H]MNU (Fig. 6, a and c). By
the reaction of the four AlkB homologues all released formaldehyde release of formaldehyde using the Nash assay. This demonstrated that the reaction of the four AlkB homologues all released formaldehyde (data not shown). The ensemble of the experiments on the DNA substrates shows that the mAbh2 and mAbh3 release methyl groups from DNA using a mechanism similar to that of AlkB or the ABH2 or ABH3.

**ABH2 and ABH3 Have Different Salt-dependent 2OG Activities for Removal of 1-meA or 3-meC—** Some data on the ionic strength dependence of ABH2 and ABH3 are available using MgCl₂, but those data use polydeoxyribonucleotide substrates with ³H release (20). We performed a titration for the ss and ds substrates with either 1-meA or 3-meC substrates using ABH2 and ABH3 and the ODN assay described in Figs. 4 and 5. Fig. 7a shows that with ABH2, the ionic strength dependence is different for the ss and ds substrates. At salt concentrations below 100 mM NaCl, the ABH2 removes 1-meA or 3-meC more efficiently from ss substrates. However, at NaCl concentrations above 100 mM, the ds 1-meA or 3-meC begin to be removed more efficiently by ABH2. The efficiency of ABH2 is maintained until at least 500 mM NaCl (Fig. 7a). This is in contrast to the ABH3 where both the ss and ds 1-meA or 3-meC substrates maintain almost identical dependence with respect to NaCl concentration (Fig. 7b).

**Mouse AlkB Homologues**

**FIGURE 5. Repair of 3-meC from a site specifically modified ODN.** Experiments were performed using a ss-ODN substrate (150 pmol) with a unique modified 3-meC or an unmodified ODN and one of the AlkB homologues or mutants using 50 pmol of protein in a reaction volume of 100 μl. Following the AlkB homologue reaction, a complementary ODN was annealed and reacted for 30 min with EcoRI. The lower band represents the product, and the upper band starting ODN. Lanes from left to right: Lane 1, Cyt containing ODN; lanes 2–6 indicate that EcoRI cleavage is not inhibited by reaction of the unmodified ODN with either the mouse or human AlkB homologues; lane 7, 3-meC-containing ODN; lane 8, 3-meC ODN is not cleaved by EcoRI; lane 9, reaction of 50 pmol of ABH2 followed by EcoRI; lane 10, reaction of 50 pmol of ABH2 D173A mutant followed by EcoRI does not show cleavage; lane 11, reaction of 50 pmol of ABH2 H236A followed by EcoRI cleavage; lane 12, reaction of 50 pmol of ABH3 followed by EcoRI cleavage; lane 13, reaction of 50 pmol of ABH3 D193A mutant followed by EcoRI; lane 14, reaction of 50 pmol of ABH3 H257A mutant followed by EcoRI; lane 15, reaction of 50 pmol of mAbh2 followed by EcoRI; lane 16 reaction of 50 pmol of mAbh2 D151A mutant followed by EcoRI; lane 17, reaction of 50 pmol of mAbh2 H214A mutant followed by EcoRI; lane 18, reaction of 50 pmol of mAbh3 followed by EcoRI; lane 19, reaction of 50 pmol of mAbh3 D193A mutant followed by EcoRI; lane 20, reaction of 50 pmol of mAbh3 H257A mutant followed by EcoRI.

Contrast, mAbh3 and ABH3 release radioactivity from ss-ODNs much faster than from ds-ODNs (Fig. 6, b and d).

The ss-polydeoxyribonucleotides, ss-polyribonucleotides, and M13mp18 were also examined for the release of radioactivity. For mAbh2 and ABH2, there are again similarities. Release of the radioactivity from [³H]CH₃-poly(dA) or [³H]CH₃-poly(dC) is at least 10–50-fold more rapid than release of radioactivity from [³H]CH₃-poly(A) or [³H]CH₃-poly(C) (Fig. 6, e and g). We also examined the release of radioactivity from [³H]CH₃-M13mp18 and found that less than 10% of the total radioactivity in the assay was released by mAbh2 or ABH2 treatment. However, release of radioactivity from the [³H]CH₃-M13mp18 substrate by mAbh2 was significantly lower than for ABH2. Thus, the mAbh2 removes 1-meA or 3-meC from both RNA and DNA, but there is a distinct bias for the removal from DNA.

For mAbh3 and ABH3, release of methyl groups from polydeoxyribonucleotide and polyribonucleotide substrates had similar efficiencies, showing that mAbh3 and ABH3 recognize RNA and DNA (Fig. 6, f and h).

It is also possible that the mAbh2, mAbh3, ABH2, or ABH3 could harbor activities associated with exo or endonucleases that would release radioactivity in the assays. To show that the activities are not associated with exo or endonucleases, we end-labeled either an ODN or a polyribonucleotide and treated these substrates with the enzymes. Only minor amounts of radioactivity were released by the purified AlkB homologues (see supplementary data) from the 3'-end of the ODN. A control using exonuclease I, a 3' → 5' exonuclease, shows that the pattern expected for the degradation for that type of exonuclease. No degradation of the ODN by the AlkB homologues is observed, consistent with the absence of exo or endonuclease activity. The fact that the intensities of the full-length ODNs that are 5'-end-labeled do not change, argues that there is no 5' → 3' exonuclease.
Active Site Residues of mAbh2, Abh3, ABH2, and ABH3—Using the alignment of the AlkB homologues, we introduced non-conservative site-specific modifications into the recombinant mouse and human proteins at the first aspartic acid in the DDE sequence shown in Fig. 1c (for ABH2, D173). The structure of the ABH2, and its mutants were verified using 1H-NMR. We then tested the activity of the mutant AlkB homologues for removal of 1-meA and 3-meC. These results shown in Figs. 4 and 5 demonstrate that modification of a single residue from Asp → Ala in the conserved regions in Fig. 1c abolishes the 2OG activity for all four proteins. Moreover, the reductions in activity from the tritium-labeled substrates show that for all the mutants, over a 10-fold decrease in activity is observed (TABLE ONE). Mutation of a series of conserved His residues to Ala (Fig. 1c, for ABH2, H236) reduced but did not eliminate 2OG activity of the His → Ala mutant proteins (Figs. 4 and 5), indicating that the conserved His residues are not as important for activity as the mutated Asp residues. These mutations should help in defining amino acids important in the active site of the enzymes.

Steady-state Kinetics of ABH2 and ABH3—Polydeoxyribonucleotide and polyribonucleotide substrates for the AlkB homologues are useful, but one difficulty is the fact that these substrates have a number of modifications and therefore are not as well defined substrates as ODNs with bases modified at single sites. Therefore, we used the 1-meA and 3-meC ODNs with the assay presented in Figs. 4 and 5 to determine the steady-state kinetics of ABH2 and ABH3. This provides quantitative data on efficiency of both proteins for both ss- and ds-DNA. The steady-state kinetics results are summarized in TABLE TWO for both ABH2 and ABH3 with the 1-meA and 3-meC substrates in both ss- and ds-DNA configurations. The pseudo second-order rate constants comparing \( k_{cat}/K_m \) yield a measure of the efficiency of excision for each substrate. These data can be more easily compared if they are converted to relative \( k_{cat}/K_m \) values (TABLE TWO and supplementary data). The ds 1-meA and ds 3-meC substrates are more efficiently processed by the ABH2 than any of the other substrate-enzyme pairs studied. By contrast, the ds 1-meA and ds 3-meC substrates are least efficiently processed by ABH3 compared with any of the
enzyme-substrate pairs. Even for the ss 1-meA and ss-3-meC, the $k_{\text{cat}}/K_m$ value for ABH3 is almost a factor of three lower than the most favored substrates for ABH2.

**Binding of ABH2 and ABH3 to 1-meA and 3-meC Substrates**—A number of DNA repair enzymes have been examined for their capacity to interact with DNA substrates using electrophoretic mobility shift assays (EMSA). One of the most notable are DNA glycosylases (28–32). To examine the ability of the ABH2, ABH3, mAbh2, and mAbh3 enzymes to be used in gel shift assays, a number of experiments were conducted. None of the proteins manifested significant mobility shifts using either ss- or ds-DNA substrates at concentrations of even 5:1 protein:DNA substrate (data not shown). However, minor amounts of the DNA substrate (10% or less) manifested a slower mobility.

**Tissue-dependent Expression of the ABH2 and ABH3 in Humans**—We compared the distribution of the mRNA in human tissues using Northern blots (Fig. 8). Highest expression levels of ABH2 messenger occur in testis, skeletal muscle, liver, prostate, ovary, and heart. The RNA is detectable in lower amounts in peripheral blood leukocytes, colon, thymus, brain, pancreas, spleen, and kidney. By Northern blotting, no signal was detected in lung, placenta, or brain tissue, suggesting that the levels of mRNA for ABH2 in those tissues are low. We also compared the ABH2 using another available human tissue Northern blot. We found that the levels of the DNA substrate (10% or less) manifested a slower mobility.

**Tissue-dependent Expression of the ABH2 and ABH3 in Humans**—We compared the distribution of the RNA in human tissues using Northern blots (Fig. 8). Highest expression levels of ABH2 messenger occur in testis, skeletal muscle, liver, prostate, ovary, and heart. The RNA is detectable in lower amounts in peripheral blood leukocytes, colon, thymus, spleen, pancreas, and kidney. By Northern blotting, no signal was detected in lung, placenta, or brain tissue, suggesting that the levels of mRNA for ABH2 in those tissues are low. We also compared the ABH2 using another available human tissue Northern blot. We found that the levels of the DNA substrate (10% or less) manifested a slower mobility.

**Tissue-dependent Expression of mAbh2 and mAbh3 in Mouse**—To examine the expression of the mAbh2 and mAbh3 in mouse tissues, we performed Northern blots for various mouse tissues. The Northern blots shown in Fig. 9 demonstrate that mAbh2 is expressed largely in heart, and to a much lesser extent in testis, where there may be some splicing variation. No expression was detected in skeletal muscle, lung or spleen. For mAbh3, the highest expression levels of the mRNA are found in testis, kidney, liver, and heart. No mAbh3 expression is observed in lung, spleen, and brain. These data suggest that there is little or no protection from 1-meA or 3-meC damage in some tissues by the AlkB homologues studied in this report.

**Other Human AlkB Homologues**—A number of AlkB homologues have been postulated based on bioinformatics (24). To examine those proteins, we cloned and expressed human ABH4–8. We found that only ABH4, ABH6, and ABH7 produced soluble protein in E. coli that we were able to purify to homogeneity (Fig. 10). These proteins were tested for activity using the ODN substrates as either single or double strands, but no activity was observed. We also used the assay with the M13 ss-DNA modified with DMS transfected into alkB– E. coli and found no capacity to increase the transformation efficiency for any of the

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**TABLE ONE**

| Percentage activity of Asp → Ala mutants compared to wild type AlkB homologues |
|-----------------------------------|-------------------|-------------------|-------------------|-------------------|
|                                    | pmol              | Poly(C)           | Poly(A)           | Poly(dC)          | Poly(dA)          |
| ABH2 D173A                         | 50               | 0.0              | 1.5              | 0.8              | 2.3              |
|                                   | 100              | 5.9              | 0.8              | 0.6              | 3.4              |
| ABH3 D193A                         | 50               | 3.5              | 4.7              | 1.0              | 0.7              |
|                                   | 100              | 4.1              | 5.9              | 4.0              | 0.0              |
| mAbh2 D151A                        | 50               | 0.0              | 10.3             | 3.8              | 2.5              |
|                                   | 100              | 4.9              | 7.2              | 3.9              | 2.1              |
| mAbh3 D193A                        | 50               | 6.6              | 0.2              | 0.0              | 0.0              |
|                                   | 100              | 3.1              | 0.4              | 0.0              | 0.1              |

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**FIGURE 7.** Ionic strength dependence of ABH2 and ABH3. % maximal activity of each substrate with ABH2 or ABH3 as a function of NaCl concentration. Standard assays using 50 pmol of substrate and 150 pmol of protein incubated for 30 min were used to evaluate activities. a, activity of ABH2 with respect to NaCl concentration. b, activity of ABH3 with respect to NaCl concentration.
the three proteins isolated (assay in Fig. 3). This could mean that the proteins were inactive; that other conditions or cofactors are necessary to observe activity. Alternatively and more likely, it could also mean that these proteins have other activities that remain to be ascertained.

**DISCUSSION**

In this report we have demonstrated the activities of mouse homologues (mAbh2 and mAbh3) of human ABH2 and ABH3. We have introduced a simple assay that should simplify the determination of 2OG activity using ODNs. Data on the tissue-dependent distribution of AlkB homologues is also presented for humans and mice. Moreover, we have demonstrated that both ABH2 and ABH3 derivatives have 2OG activity at physiologically salt concentrations. We have also shown that similar proteins (ABH4, ABH6, and ABH7) postulated to have 2OG activity do not manifest activities against any of the substrates examined in this report.

The alignments of the ABH2, ABH3, mAbh2, and mAbh3 show several conserved regions, including a region at the C-terminal end of the region associated with RVNLTFR. A BLAST search over the human data base returns a number of proteins, among them cytochrome oxidases. There are at least two other highly conserved sequences in the mammalian AlkB homologues. Those include HXDDEXLX4ASXSFGAXRXFX2RXK and LXHGXLLXMX3TX3WXHX2P. The importance of all these sequences in the 2OG activity of AlkB homologues remains to be shown, but we have identified at least two residues that when altered significantly alter 2OG repair activity. AlkB has been shown to have a Trp at position 178 (Fig. 1) and a C-terminal Trp234 that release the ethyl groups as acetaldehyde (19, 23). These data suggest that the upper limit for removal of damage by the AlkB homologues has not yet been observed. The addition of a simple ODN-based assay for removal of 1-meA or 3-meC makes it possible to more easily study the kinetics of removal of DNA damage and will provide some important comparisons between substrates. The use of these defined substrates will also make it possible to more easily study the kinetics of removal of DNA damage and will provide some important comparisons between substrates. The use of these defined substrates will also make it possible to more easily study the kinetics of removal of DNA damage and will provide some important comparisons between substrates.

The assay used in this report is similar to assays used by other investigators for enzymatic cleavage. This was first employed to monitor repair of DNA mismatches (40, 41). Similar methods have been used to examine repair of O6-methylguanine (42–45). It should be noted that the specificity of the assay used in this study depends on the restriction endonuclease used. Not all restriction sites yield a clear assay, thus making it necessary to determine empirically which sites are useful.
efficient at removing 1-meA and 3-meC from ds-DNA compared with single-stranded DNA. However, for ABH3, 1-meA and 3-meC are much more efficiently removed from ss-DNA than from ds-DNA. Overall, in DNA, ABH2 more efficiently removes 1-meA and 3-meC than ABH3. The efficiencies of the reactions using the different substrates as noted by the pseudo second-order rate constants in TABLE TWO are close to the diffusion limit and are above $10^7 \text{ M}^{-1} \text{s}^{-1}$ for almost all the ABH2 and ABH3 substrates. Compared with MPG and
proteins purified in the same manner as those for ABH2, ABH3, mAbh2, and mAbh3. Use of mobility shifts with the native proteins. Therefore, the interaction of the AlkB homologues with their substrates is too slow to be observed by mobility shifts with the native proteins. This is quite different from many DNA glycosylases that remove modified bases from DNA and RNA. It is interesting to note that the highest mRNAs appear to be in testis and ovary. This could indicate a role of these enzymes in protecting DNA or RNA during meiosis.

There has been speculation concerning other AlkB derivatives identified by informatics screening of human cDNA sequences. We have shown that at least ABH4, ABH6, and ABH7 do not have activity that repair 1-meA or 3-meC. Although other proteins or cofactors could be involved in conferring activities, it is more probable that ABH4, ABH6, and ABH7 are similar to ABH1, in that these proteins do not yet have a defined function in the repair of DNA. It is also possible that improper folding of the proteins does not confer activity. The other cDNAs for ABH5 and ABH8 when expressed in E. coli do not yield soluble proteins following purification and therefore, it is difficult to assess if these proteins lack proper folding to have activity or if the proteins properly folded to not have 2OG activity. Although reaction conditions or protein folding may play a role, it is unlikely that ABH4, ABH6, and ABH7 would have no activity with any of the substrates. Therefore, it would prove useful to examine if any of the mammalian AlkB homologues increase survival with respect to DMS or MMS treatment to determine if these expressed proteins have any role in protection of mammalian cells against alkylation damage. These data are consistent with ABH2 and ABH3 homologues with 2OG activity and ABH4, ABH6, and ABH7 lacking such activity. This is the first evidence that a number of these proteins may not be active against 1-meA and 3-meC substrates. Therefore, if these other proposed AlkB homologues do not have 2OG activity, it would prove easier to probe for the biological role of ABH2 and ABH3 than if all 8 AlkB homologues were active. This could be accomplished using either mouse models (mAbh2- and mAbh3-targeted deletions) or siRNA knockdown of the mRNAs.

In the future, the identification of other active site residues and an understanding of the structure of AlkB homologues will help to determine how these enzymes function. Other work to evaluate the mutagenesis and repair in vivo of 1-meA and 3-meC in DNA will help to yield information on the effects of these adducts in mammalian cells. The identification of residues critical for the mAbh2 and mAbh3 yield the possibility for the construction of targeted deletion by elimination of the 2OG activity of a particular exon. Moreover, the role of these enzymes in the repair of RNA in mammalian cells is an extremely important area that will require much study in the future to comprehend the limits of RNA repair.

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Mouse AlkB Homologues

FIGURE 9. Tissue-dependent distribution of mAbh2 and mAbh3 in mice. a, mouse RNA tissue blots were obtained from BD Clontech. b, tissue distribution of β-actin. Note that in skeletal muscle and heart the actin transcript is different that in the other tissues shown.

FIGURE 10. Purification of ABH4, ABH6, and ABH7. The cDNAs were expressed and the proteins purified in the same manner as those for ABH2, ABH3, mAbh2, and mAbh3.

AlkA, representative members of DNA glycosylases that remove other alkylated bases, the pseudo second-order rate constants are often on the order of 10^4-10^5 M^-1 s^-1 (32). In the future, RNA substrates with unique modified bases will provide the direct comparison of the efficiency of removal of modified bases from DNA and RNA.

We also attempted to examine the electrophoretic mobility shift using these enzymes. The fact that the reactions observed are close the diffusion-controlled limit suggests that the interaction with the substrate is rapid. This is quite different from many DNA glycosylases that often remain bound to the product even hours after the reaction. Therefore, the interaction of the AlkB homologues with their substrates is too fast to be observed by mobility shifts with the native proteins. Use of mutant proteins with much slower exchange rates may make it possible to study the interaction of the AlkB homologues with DNA substrates using mobility shift and other biophysical techniques such as NMR.
