Identification and characterisation of the *Drosophila melanogaster* O\(^6\)-alkylguanine-DNA alkyltransferase cDNA

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ABSTRACT

The protein O\(^6\)-alkylguanine-DNA alkyltransferase (alkyltransferase) is involved in the repair of O\(^6\)-alkylguanine and O\(^4\)-alkylthymine in DNA and plays an important role in most organisms in attenuating the cytotoxic and mutagenic effects of certain classes of alkylating agents. A genomic clone encompassing the *Drosophila melanogaster* alkyltransferase gene (*DmAGT*) was identified on the basis of sequence homology with corresponding genes in *Saccharomyces cerevisiae* and man. The *DmAGT* gene is located at position 84A on the third chromosome. The nucleotide sequence of *DmAGT* cDNA revealed an open reading frame encoding 194 amino acids. The MNNG-hypersensitive phenotype of alkyltransferase-deficient bacteria was rescued by expression of the *DmAGT* cDNA. Furthermore, alkyltransferase activity was identified in crude extracts of *Escherichia coli* harbouring *DmAGT* cDNA and this activity was inhibited by preincubation of the extract with an oligonucleotide containing a single O\(^6\)-methylguanine lesion. Similar to *E.coli* Ogt and yeast alkyltransferase but in contrast to the human alkyltransferase, the *Drosophila* alkyltransferase is resistant to inactivation by O\(^6\)-benzylguanine. In an *E.coli* lac\(\alpha\)Z reversion assay, expression of *DmAGT* efficiently suppressed MNNG-induced G:C→A:T as well as A:T→G:C transition mutations in vivo. These results demonstrate the presence of an alkyltransferase specific for the repair of O\(^6\)-methylguanine and O\(^4\)-methylthymine in *Drosophila*.

INTRODUCTION

Alkylating agents such as \(N\)-methyl-\(N\)'-nitro-\(N\)-nitrosoguanidine (MNNG) and methylnitrosourea (MNU) introduce methyl adducts at various positions in DNA (1,2). Among the different methylated bases generated, O\(^6\)-methylguanine (O\(^6\)-MeG) and O\(^4\)-methylthy-
N-ethyl-N-nitrosourea (ENU) and ethylmethanesulphonate (EMS) induced primarily G:C→A:T and A:T→G:C transition mutations in germ cells (25,26). Surprisingly, G:C→A:T changes were almost absent after treatment with methylating agents such as MNU and MNNG, suggesting rapid repair of O6-MeG adducts from the DNA in *Drosophila* germ cells (27,28) and indicating that these cells may repair O6-MeG much more efficiently than O6-ethylguanine. Studies in other eukaryotes also indicate a preference of alkyltransferase for O6-MeG moieties in DNA (29).

In the case of alkylating agents acting more extensively at the ring nitrogen atoms in the DNA, a relatively high chromosome breakage effect was observed (30-32).

Extracts of *Drosophila* pupae, but not early embryos, have been reported to contain an alkyltransferase activity that acts on O6-MeG and possibly also N7-methylguanine and N3-methyladenine in DNA and two non-inducible alkyltransferase activities of 30 and 19 kDa that transferred methyl groups from methylated DNA *in vitro* were later identified (33,34). In the present study we report the identification of the *Drosophila* O6-alkylguanine-DNA alkyltransferase gene, *DmA GT*. Sequence analysis revealed a significant homology with alkyltransferase genes of *E.coli*, yeast and mammals. Expression of *DmA GT* in alkyltransferase-deficient bacteria suppressed MNNG-induced G:C→A:T and A:T→G:C transition mutations.

**MATERIALS AND METHODS**

**Bacterial strains and media**

The *E.coli* strains AB1157, GWR111 (AB1157, Δada-25::CamΔΔgt::kan), FC218 (ogg-1::kan Δada-25::CamΔΔgt) and FC326 (ogg-1::kan Δada-25::CamΔΔgt) were kindly provided by Dr Leona Samson. Strains FC218 and FC326 have, respectively, an A:T→G:C and a G:C→A:T transition mutation in their lacZ genes which make them unable to use lactose as carbon source (35).

After MNNG treatment, strains FC218 and FC326 were grown on minimal medium plates containing 0.025% thiamine, 40 μg/ml methionine and 0.025% glucose or 0.025% lactose. For the preparation of bacterial extracts *E.coli* strains BS21 (thyA, his, sula, ada+) (36) and UC978 (Δada::kan AΔgt::Tet) were used (37). Plasmid-transformed strains were grown in LC medium or on LC agar plates, containing 50 μg/ml ampicillin and the other appropriate antibiotics, at 37°C.

**Cloning of the *DmA GT* cDNA**

Based on the nucleotide sequence of the region of clone DS00004 (38), which encodes a putative protein with homology to the human and *S.cerevisiae* alkyltransferases, primers DMT1 (5’-GAACATCTCCATCACTGCTCC, sense) and DMT2 (5’-CATCGCCAGAAGCAGTGGTAC, antisense) were designed. Total plasmid DNA, derived from a *Drosophila* embryonic cDNA library in *λ* ZAPII (a kind gift of Dr Akira Yasui) was used to template the complete coding region was cloned in one step downstream of the lacZ promoter of pUC18, resulting in plasmid pDMT2. Other primers, used for cloning and sequencing were DMT3 (5’-CTCTGACCGCCGTTGGAACG; antisense), DMT4 (5’-GTAAGTGGTCTCCCTCCCTTCC, antisense) and DMT5 (5’-GAATTTGTTGTCGCCAAACG, sense).

**MNNG-induced survival assay**

A stock solution of 1 mg/ml MNNG in 100 mM NaAc (pH 5.0) was frozen in aliquots at −20°C. Thawed aliquots were used only once. Overnight cultures of plasmid-transformed GWR111 or AB1157 were diluted 1:25 in LC broth containing ampicillin (LC/amp) and grown at 37°C to OD600 = 0.5 (±108 cells/ml). Cells were washed and resuspended in M9 salts. Samples were treated with different concentrations of MNNG for 10 min at 37°C. The cells were washed and resuspended again in M9 salts. Appropriate dilutions were plated on LC/amp plates, incubated overnight at 37°C and the next day the colonies were counted and survival calculated.

**Alkyltransferase assay**

Overnight cultures of BS21 (enhanced ada expression) or UC978, transformed with either pDMT2 (*DmA GT* cDNA in pUC18) or pHAT (*HsAGT* cDNA in pRBS), were sonicated in buffer I (50 mM Tris–HCl, pH 8.3, 3 mM DTT, 2 mM EDTA). Protein extracts were assayed for alkyltransferase activity by measuring the transfer of radioactivity from [3H]MNNG-treated calf thymus DNA to acid-resistant protein as described previously (39). The incubation temperature of the assay was 27°C unless indicated otherwise. To investigate methylphosphotriester repair, the same substrate was pretreated with excess of a truncated version of Ada (called Sx), containing only the O6-MeG repair function, and the DNA recovered (39,40). This substrate contains little or no O6-MeG due to repair of this lesion by the Sx protein. The sequence of oligo 320 used in the competition assay was 5’-GGCGCCCP- Meadowgrass-5’T and the control oligo 348 contained guanine in place of O6-MeG at position 7. The oligos were annealed to their complementary oligo 313 before preincubation.

**MNNG-induced mutagenesis assay**

Plasmid pWX1023 (human alkyltransferase cDNA cloned in pUC19) was kindly provided by Dr Leona Samson (41). The MNNG-induced mutagenesis assay was performed as described (41). In brief, FC218 or FC326 cells, transformed with either pDMT2, pWX1023 or pUC18, were grown in LC medium to OD600 = 0.7 (±5×108 cells/ml). The cells were washed with M9 salts, treated with MNNG for 15 min at 37°C and washed again with M9 salts. Finally, appropriate dilutions were plated on minimal medium plates containing glucose or lactose as carbon source. The number of colonies on the glucose plates was used to calculate the number of surviving cells and the number of colonies on the lactose plates was used to calculate the number of LacZα revertants/105 surviving cells for FC218 and revertants/105 surviving cells for FC326.

**Nucleotide accession number**

The nucleotide sequence of the *DmA GT* cDNA is listed in the EMBL/Genbank database under accession no. AF063906.
RESULTS

Cloning of the DmAGT cDNA

Sequence analysis of alkyltransferases from E.coli, yeast and mammals indicated significant conservation at the amino acid level. This conservation is most apparent in the C-terminal part of these proteins, which contain the cysteine acceptor site. The human (15–17) and S.cerevisiae (9) protein sequences were used as a query to search the Drosophila sequence database with the TBLASTN algorithm (42), resulting in the identification of P1 clone DS00004 (38). Within the 30 kb sequence of this clone an open reading frame (ORF) was identified, coding for a putative protein with significant homology to the yeast and human alkyltransferases. Based on the sequence of this ORF, primers DMT1 and DMT2 were designed. With these primers a 134 bp PCR product was obtained using total plasmid DNA from a Drosophila embryonic cDNA library as template. The 3′-end of the putative Drosophila alkyltransferase gene, DmAGT, was amplified with a vector-specific primer (M13) and DMT1, followed by a nested PCR with a second vector-specific primer (T7) and DMT5. The same technique was used to amplify the 5′-end of the cDNA using the vector-specific primers Reverse M13 and T3 and the cDNA-specific primers DMT3 and DMT4. In addition, 5′-RACE experiments using total ovarian RNA as template were performed, showing a 5′-untranslated region of at least 78 nt. Based upon these analyses, a composite cDNA of 695 bp was derived. Within this sequence a 582 bp ORF between nt 78 and 660 could be recognised, encoding a putative protein of 194 amino acids. The alignment of the DmAGT protein and alkyltransferases from man, S.cerevisiae and E.coli (Ogt) is shown in Figure 1. The overall identity of DmAGT with these three proteins varies between 23% (human) and 30% (yeast). The identity in the C-terminal part is 44% with yeast and E.coli Ogt alkyltransferases and 49% with the human protein. With the exception of the rabbit alkyltransferase, mammalian alkyltransferases are characterised by approximately 30 amino acids following the conserved region whereas Ogt and the alkyltransferases from yeast and Drosophila lack this tail. Clone DS00004 has been localised by in situ hybridisation to the region 84A1–84A2, on the third chromosome. This is a well-characterised part of the Drosophila genome, including the antennapedia complex and the Edg84A gene, encoding a cuticle protein (43). The DmAGT gene is localized proximal of the Edg84A gene. Comparison of the DmAGT cDNA sequence to the genomic sequence of clone DS00004 did not reveal any introns. To determine whether the Drosophila alkyltransferase is encoded by a single copy gene located in region 84A or additional DmAGT genes are present, low stringency Southern blot hybridisations were performed. The results were consistent with the presence of a single DmAGT gene per haploid genome (data not shown). Northern blot experiments indicated that the DmAGT gene is expressed at a very low level: hybridisation with a 0.7 kb RNA probe could only be detected in early embryos and in ovaries (Kooistra et al., preliminary results).

DmAGT complements the MNNG-sensitive phenotype of an ada− ogt− E.coli strain

To confirm that the isolated DmAGT cDNA encodes a protein with alkyltransferase activity, the coding region was expressed in alkyltransferase-deficient E.coli cells. Plasmid pDMT2, which contains the DmAGT coding region cloned downstream of the lacZ promoter of pUC18, was introduced into E.coli strain GWR111 (ada− ogt−). Bacteria were treated with different doses of MNNG and plated on LC/amp plates. The next day individual colonies were counted for survival. GWR111 cells and wild-type AB1157 cells both transformed with pUC18 were used as controls. Figure 2 shows the relative survival for the indicated doses of MNNG. Almost no cell killing took place for the wild-type AB1157/pUC18 strain at 20 µg/ml MNNG, whereas for the GWR111/pUC18 strain at this dose <1% of the bacteria survived. Plasmid pDMT2 suppressed the MNNG-hypersensitive phenotype of GWR111 to almost wild-type level, demonstrating that the DmAGT cDNA encodes a protein with alkyltransferase activity. Similar results were obtained using a plasmid expressing the human AGT (15).

Activity of DmAGT protein in vitro

Crude extracts of E.coli UC978 (ada− ogt−) cells containing pDMT2 were used to assay alkyltransferase activity in vitro. Increasing amounts of protein extracts were tested at different temperatures and the specific activities were calculated (Table 1). UC978 cells transformed with plasmid pHAT, containing the human methyltransferase cDNA (37), were assayed in parallel. The temperature optimum for DmAGT extract appeared to be ∼27°C. The specific activity of ∼1500 fmol/mg protein is ∼10-fold lower than observed for the human alkyltransferase protein at 27°C, even though its optimum temperature is 37°C.

Table 1. Specific activity (fmol/mg protein) at various temperatures of extracts from bacteria expressing human and Drosophila alkyltransferases

<table>
<thead>
<tr>
<th>Incubation temperature (°C)</th>
<th>22</th>
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<td>Drosophila melanogaster</td>
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<td>Human</td>
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Figure 2. DmAGT rescues the MNNG-hypersensitive phenotype of ada–, ogt– E.coli cells. Bacterial cultures were treated with the indicated doses of MNNG for 10 min at 37°C and grown overnight on LC/Amp plates. ▲, AB1157/pUC18; ■, GWR111(ada–, ogt–)/pUC18; ●, GWR111(ada–, ogt–)/pDMT2.

Figure 3. Time course of in vitro [3H]methyl group transfer under protein limiting conditions. Escherichia coli protein extracts were assayed for alkyltransferase activity as described in Materials and Methods. ▲, UC978(ada–, ogt–)/pDMT2; ●, UC978(ada–, ogt–)/pHA T.

The kinetics of the alkyltransferase reaction were determined in a time course experiment under protein limiting conditions. Crude extracts of UC978/pHA T were diluted 1:10⁴ and assayed for alkyltransferase activity with increasing incubation times. Extracts of UC978/pDMT2 were diluted only 1:10. The results of this time course experiment are shown in Figure 3. Rapid transfer of methyl groups was observed for the human and the Drosophila alkyltransferases. After 10–15 min the activity of both proteins starts to level off, but a plateau is still not reached after 2 h, indicating that after this period the alkyltransferase protein in the extracts is still accepting methyl groups from the DNA substrate.

To confirm that the DmAGT protein did not act on methylphosphotriester lesions in the substrate DNA, BS21 (Ada), UC978/pDMT2 and UC978/pHA T protein extracts were assayed using a modified substrate DNA containing methylphosphotriesters but little or no O⁶-MeG. In contrast to the BS21 extract, the extracts of UC978 expressing DmAGT or HsAGT showed very little activity (Fig. 4A). In addition, the same extracts were preincubated for 1 h with a 15mer oligonucleotide containing a single O⁶-MeG lesion at position 7 and subsequently assayed for residual alkyltransferase activity, using the normal substrate. The activity of the human alkyltransferase was almost completely inhibited by this preincubation and the DmAGT activity was inhibited by ~80%, as shown in Figure 4B. However, the Ada protein still showed considerable activity after preincubation with the O⁶-MeG-containing oligonucleotide, because the oligonucleotide did not inactivate the phosphotriester alkyltransferase activity. The residual activity seen in the case of the DmAGT protein could be explained by less efficient repair of O⁶-MeG present in oligonucleotides in comparison to genomic DNA. Preincubation with the same oligonucleotide without the O⁶-MeG lesion had no effect on the activity of either alkyltransferase. Together these experiments indicate that DmAGT is not active on methylphosphotriesters in methylated DNA.

DmAGT is resistant to O⁶-benzylguanine

The E.coli Ada and yeast alkyltransferase proteins are resistant to inhibition by O⁶-benzylguanine (O⁶-BG) whereas mammalian alkyltransferases lose their activity when preincubated with this
alkylated base (44,45). To test the sensitivity of DmAGT to O6-BeG, protein extracts were preincubated for 1 h at 27°C with different concentrations of O6-BeG. Figure 5 shows that concentrations up to 5 μM O6-BeG have no effect on the DmAGT activity whereas with the human protein a concentration of 0.08 μM decreases alkyltransferase activity by 50% and the activity is almost completely lost at 0.5 μM O6-BeG.

**DmAGT repairs O6-MeG and O4-MeT in *in vivo***

It has been shown that alkyltransferases from *E.coli*, yeast and mammals repair O6-MeG and O4-MeT in *in vitro* (9,19,20,46–48). In previous experiments we have shown that the *Drosophila* alkyltransferase also repairs O6-MeG adducts in *in vitro*. In order to test whether DmAGT can also repair O6-MeG and O4-MeT in *in vivo*, pDMT2 was introduced into the alkyltransferase-deficient *E.coli* strain FC218 and FC326. These strains have mutations in their lacZ genes that can be reverted to LacZ+ via a G:C transition and an A:T→G:C transition, respectively. The LacZ reversions were measured as colonies that were able to grow on minimal medium plates containing lactose. Treatment of FC218/pUC18 and FC326/pUC18 with MNNG resulted in a dose-dependent increase in the frequency of G:C→A:T and A:T→G:C transitions, respectively (Fig. 6). The induction of transition mutations is extensively suppressed by expression of DmAGT, indicating that O6-MeG and O4-MeT adducts, respectively, are repaired by the *Drosophila* protein in *in vivo* (Fig. 6A and C). Expression of the human alkyltransferase conferred full protection against MNNG-induced G:C→A:T transitions (Fig. 6B). In the FC326 strain, a significant protection against A:T→G:C transitions by the human protein was observed (Fig. 6D). Similar results have been reported for mouse alkyltransferase (49) but protection against A:T→G:C mutations by the human alkyltransferase was not seen by Samson et al. (41). The fact that A:T→G:C mutations are induced at a relatively low level may be an explanation for this discrepancy.

**DISCUSSION**

Alkylating agents introduce toxic, mutagenic and carcinogenic lesions into DNA. To counter the biological effects of O6-MeG and O4-MeT, most organisms employ the DNA repair protein O6-alkylguanine-DNA alkyltransferase (AGT) which transfers the methyl groups from the lesions to a cysteine residue in the protein. There is a significant degree of homology between alkyltransferases from bacteria, yeast and mammals: in particular, the C-terminal regions of the proteins contain several stretches of highly conserved amino acids. On the basis of such conserved sequences we identified the alkyltransferase gene from *D.melanogaster* by database screening using the human and yeast alkyltransferase sequences as queries. To confirm that the isolated cDNA encoded an active alkyltransferase, the DmAGT cDNA was introduced in *ada*′ *ogt*′ *E.coli* strains. Expression in these cells resulted in almost complete rescue of these bacteria from the killing effects of MNNG. More direct evidence that DmAGT repairs O6-MeG was the *in vitro* transfer of methyl groups from methylated DNA to protein by extracts of *ada*′ *ogt*′ *E.coli* cells expressing DmAGT. A rapid transfer was observed in the first minutes with a slower, continuing transfer for up to 2 h. This transfer was inhibited by preincubation of the extracts with an oligonucleotide that contained a single O6-MeG residue. Similar to other eukaryotic AGTs, DmAGT appears not to repair methylphosphotriesters (Fig. 4A and B).

A LacZ reversion assay was used to study the *in vivo* repair of O6-MeG and O4-MeT in DNA. Expression of DmAGT efficiently suppressed the formation of methylation-induced G:C→A:T and A:T→G:C transition mutations, indicating efficient *in vivo* repair of O6-MeG and O4-MeT by the *Drosophila* alkyltransferase. Similar results based on *in vivo* studies have been reported for prokaryotic and eukaryotic alkyltransferases (41,49). *In vitro* studies using purified protein also demonstrated the removal of O6-MeG and O4-MeT lesions from DNA (46–48).

In crude extracts of *E.coli*, the specific activity of *Drosophila* alkyltransferase is extremely low in comparison with the human alkyltransferase. Whilst this difference may be a consequence of the reduced expression level of the DmAGT construct in comparison with the human alkyltransferase cDNA plasmid, it might also be due to reduced stability of the *Drosophila* alkyltransferase, as has been observed for the yeast protein (9). In undiluted UC978/pDMT2 protein extracts, no alkyltransferase activity could be measured after 24 h incubation on ice, whereas 1:100 diluted UC978/pHAT extracts only showed minimal loss of activity after this period (data not shown). Furthermore, in contrast to the human alkyltransferase there was a 50% loss of DmAGT activity following a 1 h preincubation at 27°C (data not shown). It should be noted that this had no effect on the results of the inhibition experiment because all control levels were also measured after a 1 h preincubation. These observations suggest a greatly reduced stability of the *Drosophila* alkyltransferase in comparison with the human protein (Table 1).

Extracts prepared from different stages of *Drosophila* development and from adult flies have been tested for the transfer of radioactivity from [3H]methylated substrate DNA to protein. Surprisingly, transferase activity could only be detected in pupal extracts and not in protein extracts prepared from early embryos (34). Since northern blot hybridisations indicated the presence of DmAGT transcripts in early embryos the possible absence of alkyltransferase activity does not seem very likely. Furthermore, the
Figure 6. Biological evidence that DmAGT and human alkyltransferase repair O^6-MeG and O^4-MeT adducts. LacZ reversions in E.coli strain FC218 or FC326 were induced by the indicated doses of MNNG. Cells were plated on minimal medium plates containing either glucose, to estimate the number of surviving cells, or lactose, to estimate the number of revertants. Note that the MNNG concentration for measuring A:T→G:C transitions is 10-fold higher than for G:C→A:T transitions while the revertant induction is ∼100-fold lower. (A) Δ, FC218/pUC18; s, FC218/pDMT2. (B) Δ, FC218/pUC18; l, FC218/pWX1023. (C) Δ, FC326/pUC18; s, FC218/pDMT2. (D) Δ, FC326/pUC18; l, FC326/pWX1023.

The data presented in Figure 4 can also refute the suggested repair of N7-methylguanine and N3-methyladenine by DmAGT (33,34).

There is considerable current interest in the inhibition of alkyltransferase activity in mammalian cells, in order to improve the therapeutic use of certain alkylating agents in cancer treatment. One of the most potent inhibitors so far described is O^6-BeG and all wild-type mammalian alkyltransferases are very sensitive to inhibition by this compound: the E.coli Ogt protein is sensitive, although much higher doses are necessary for inhibition (45). The DmAGT protein is, like E.coli Ada and the yeast methyltransferase proteins, resistant to inactivation by O^6-BeG. Studies with site-directed mutant alkyltransferase proteins have revealed several amino acid residues that could play a role in the reaction with O^6-BeG. Residue G160 of the human alkyltransferase (corresponding to W161 of E.coli Ada and to W177 of DmAGT) when mutated to W increases O^6-BeG sensitivity by 3- to 5-fold whereas G160R mutation decreases the sensitivity by ∼20-fold (50,51). Furthermore, P138K, P140A and G156A mutations all resulted in increased resistance to O^6-BeG (52). The amino acids at these positions are not conserved in alkyltransferases from bacteria, yeast and Drosophila. The only exception is P132 from E.coli Ogt (corresponding to P138 of the human alkyltransferase) which is conserved in all O^6-BeG-sensitive proteins, suggesting a key role for this proline in the O^6-BeG-mediated inactivation of alkyltransferase.

Animal models have been generated to further elucidate the biological function of the alkyltransferase protein (53–56). Transgenic mice overexpressing the E.coli ada or the human alkyltransferase gene show a reduced induction of thymic lymphomas by MNU (57). Furthermore, alkyltransferase null mutant mouse strains are very sensitive to killing by MNU and show an increase in MNU-induced thymic lymphomas and lung adenomas compared to alkyltransferase-proficient mice (58,59). In Drosophila, several third chromosome mutagen-sensitive (mus) mutants have been identified but none of these mus mutations have been genetically mapped to region 84A, where DmA GT is localised (60). Therefore, classical fly techniques are currently being used to isolate an alkyltransferase-deficient
Drosophila strain. Such a strain could be very useful to study the mutagenic properties of specific chemotherapeutic drugs in a multicellular organism.

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