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Site-specific mutagenesis by O^6-alkylguanines located in the chromosomes of mammalian cells: Influence of the mammalian O^6-alkylguanine-DNA alkyltransferase

(alkylating agents/O^6-methylguanine/chemical carcinogenesis)

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ABSTRACT **A plasmid was constructed in which a single guanine residue was replaced with either O^6-methylguanine or O^6-ethylguanine, two of the DNA adducts formed by carcinogenic alkylating agents. The vectors were introduced in parallel into a pair of Chinese hamster ovary cells, in which one member of the pair was deficient in the repair enzyme O^6-alkylguanine-DNA alkyltransferase (*mex*$^-$) and the other was proficient in this activity (*mex*$^+$). The vectors integrated into and replicated within the respective host genomes. After intrachromosomal replication, the DNA sequence in the vicinity of the originally adducted site of each integrated vector was amplified from the host genome by using the polymerase chain reaction and was analyzed for mutations. High levels of mutation were observed from the O^6-methylguanine- and O^6-ethylguanine-containing vectors replicated in *mex*$^-$ cells ($\approx$19% for O^6-methylguanine and $\approx$11% for O^6-ethylguanine). DNA sequencing revealed the induced mutations to be almost exclusively G $\rightarrow$ A transitions. By contrast, little or no mutagenesis was detected when the adducted vectors were introduced into *mex*$^+$ cells, indicating the significant role of the O^6-alkylguanine-DNA alkyltransferase in the repair of O^6-methylguanine and O^6-ethylguanine in these mammalian cells.**

The treatment of cells with alkylating agents results in the formation of a diverse population of alkyl-DNA adducts, the processing of which is believed to lead to mutations and possibly cancer (1). Although many adducts form, a growing body of evidence has implicated the O^6-alkylguanine (O^6AlkGua) and O^4-alkylthymine adducts as the molecular precursors to the mutations that precede the appearance of the malignant phenotype (2). In both prokaryotic and eukaryotic systems, methylating and ethylating agents cause largely G·C$\rightarrow$A·T transitions (3–10), and this result has focused much attention on the O^6AlkGua adduct, which induces this mutation *in vitro* (11–14).

The most definitive way to establish that O^6AlkGua adducts are capable of causing the G $\rightarrow$ A transition *in vivo* is to situate the adduct at a known site in a genome, replicate the genome within cells and, finally, recover progeny and examine their DNA sequences in the vicinity of the originally adducted site (2). This approach of site-specific mutagenesis has revealed the quantitative and qualitative features of mutagenesis by O^6-methylguanine (O^6MeGua) in *Escherichia coli* (15–18). The mutations were exclusively G $\rightarrow$ A and their formation was inversely modulated by the competence of the cell to repair the adduct (15). Later studies revealed that this mutation was also the exclusive genetic event detected in the activated Ha-*ras 1* gene after induction of mammary tumors by treatment of rats with *N*-methyl-*N*-nitrosourea (19, 20), an alkylating agent that forms O^6MeGua in addition to other DNA adducts. The question arises as to whether O^6AlkGua adducts are indeed capable of inducing the oncogene-activating G $\rightarrow$ A mutation in mammalian cells. We describe here a system by which the mutagenesis of O^6MeGua and O^6-ethylguanine (O^6EtGua) was investigated in mammalian cells. The salient features of the system are (*i*) the integration of a vector containing O^6MeGua or O^6EtGua into the chromosomes of mammalian cells that either express or do not express mammalian O^6AlkGua-DNA alkyltransferase, (*ii*) the recovery of mutants directly from the replicated mammalian hosts by using the polymerase chain reaction (PCR) (21), and (*iii*) the characterization of the amount and type of mutation induced by the adduct *in vivo*.

MATERIALS AND METHODS

Preparation of Tetranucleotides Containing O^6MeGua or O^6EtGua. A tetradeoxynucleotide containing O^6MeGua, 5′-TpG$^{O^6\text{Me}}$pCpA-3′, was synthesized as described (22). A portion was converted to the O^6EtGua-containing tetramer, 5′-TpG$^{O^6\text{Et}}$pCpA-3′, by treatment of 1 A_{260} unit of pure, desalted, dry TG$^{O^6\text{Me}}$CA with 1 ml of a solution of ethyl alcohol saturated with sodium ethoxide for 1 week at 37°C. After drying in a centrifugal vacuum concentrator, the product was resuspended in 1 ml of water and adjusted to pH 6 with HCl. The yield of TG$^{O^6\text{Et}}$CA ranged from 30% to 60%. The tetranucleotide containing O^6EtGua was purified on a 25 × 0.46 cm Macherey Nagel C-18 (10 μm) HPLC column eluted at 1 ml/min. The gradient was 0.1 M ammonium acetate buffer (pH 7.5) to 50% acetonitrile in the same buffer over 60 min. The tetranucleotide TG$^{O^6\text{Me}}$CA eluted at $\approx$18 min, whereas TG$^{O^6\text{Et}}$CA was retained $\approx$3 min longer. Nucleoside composition analysis of TG$^{O^6\text{Et}}$CA was performed as described (22).

Preparation of Vector Genomes Containing a Single O^6MeGua or O^6EtGua Adduct. The construction of a vector, pKE15, containing a single O^6MeGua or O^6EtGua adduct, or the unmodified base, guanine, was accomplished by ligating the adducted or unmodified tetranucleotides into a 4-base gap in one of the *Pst* I sites of the plasmid (23). The ligation reaction produced approximately equal amounts of covalently closed circular and nicked circular DNA; this mixture is designated A. Covalently closed circular DNA was separated from nicked circular DNA by electrophoresis through a 1% low melting point agarose gel. Covalently closed circular DNA was excised from the gel and extracted as described (23); this material is designated B.

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Abbreviations: O^6AlkGua, O^6-alkylguanine; O^6MeGua, O^6-methylguanine; O^6EtGua, O^6-ethylguanine; PCR, polymerase chain reaction; G418^r, G418 resistant; W, Watson strand of the vector; C, Crick strand of the vector.

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Cell Lines and Transfections. The Chinese hamster ovary (CHO) cell lines CHO-D422 wild type (*mex*$^-$) and an O^6AlkGua repair-proficient (*mex*$^+$) variant (24) were obtained from E. Bresnick (Dartmouth College). pKE15-O^6MeGua, pKE15-O^6EtGua, pKE15-Gua, or pKE15 (300–500 ng) was transfected into CHO cells as a calcium phosphate coprecipitate, and G418-resistant (G418^r) clones were selected as described (23). The G418^r clones in a given transfection were pooled and expanded to $\approx 4 \times 10^7$ cells, from which genomic DNA was isolated (25).

Recovery of Plasmid Sequences from CHO Cells. A fragment of the vector containing the *Pst* I site at which the adducts were originally located was amplified from the genomic DNA by using the PCR. Two 28-mer primers were synthesized on an Applied Biosystems DNA synthesizer (model 381A) and purified on a 20% polyacrylamide/8 M urea electrophoresis gel. Each primer was bounded at the 5′ end by either an *Xba* I or a *Hin*dIII site to facilitate the cloning of the amplified DNA. PCR amplifications were performed on 3 μg of genomic DNA or 10 pg of plasmid by using *Thermus aquaticus* DNA polymerase (*Taq* polymerase) (Perkin–Elmer/Cetus) (26). Thirty cycles on a DNA thermal cycler (Perkin–Elmer/Cetus) were used with the denaturation/primer annealing/primer extension cycles as follows: denaturation (94°C, 1 min), annealing (60°C, 2 min), and extension (72°C, 3 min).

Determination of Mutation Frequency. The amplified DNA from the PCR was resolved from the template genomic DNA by electrophoresis in an 8% polyacrylamide gel followed by extraction from the gel (25). A 5′ overhang was generated at the primer-encoded *Hin*dIII site at one end of the fragment by digestion with *Hin*dIII and was labeled by using the *E. coli* DNA polymerase I Klenow fragment (Boehringer Mannheim) and [α-^{32}P]dATP as described (25). The ^{32}P-end-labeled PCR products were mixed with 0.5 μg of unlabeled carrier pKE15 DNA and digested with *Pst* I. One-fifth of the reaction mixture was electrophoresed in a 1% agarose gel in the presence of 0.5 μg of ethidium bromide per ml to check for complete digestion of the carrier. After serial extractions with phenol and with chloroform/isoamyl alcohol (24:1) and ethanol precipitation, a portion of the remaining DNA ($\approx$2000 cpm) was electrophoresed in an 8% polyacrylamide gel, which was subsequently dried. The number of cpm in the uncut and *Pst* I-digested bands was determined by using a Betascope 603 blot analyzer (Betagen, Waltham, MA). The mutation frequency was calculated as the % cpm remaining in the uncut band relative to the total number of cpm in the uncut plus *Pst* I-digested bands.

DNA Sequencing. Ten micrograms of genomic DNA was digested with *Pst* I to enrich for mutant DNA and was then used as the template for the PCR. The amplified DNA was digested with *Pst* I and *Xmn* I, after which DNA resistant to these enzymes was isolated from an 8% polyacrylamide gel (25). The primer-encoded *Xba* I and *Hin*dIII restriction sites bounding the amplified fragment were used to clone the mutant DNA into M13mp18. Single-stranded DNA from individual clear plaques was sequenced by using Sequenase (United States Biochemical) and the chain-termination method (27).

RESULTS

Strategy for Analyzing O^6MeGua- and O^6EtGua-Induced Mutations in CHO Cells. We recently reported the construction and characterization of a vector containing a single O^6MeGua adduct at a defined site for use as a probe for mutagenesis in mammalian cells (23). The vector (pKE15; Fig. 1) contains bacterial and simian virus 40 origins of replication and the simian virus 40 early promoter linked to the neomycin-resistance gene from transposon Tn*5*. This gene is part of a transcriptional cassette that confers G418^r to mammalian cells harboring the vector. An O^6MeGua or O^6EtGua adduct was incorporated into the vector at a *Pst* I

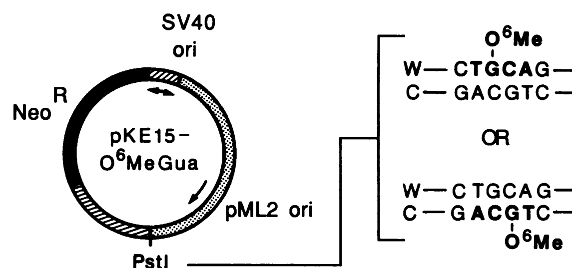

FIG. 1. Structure of the vector, pKE15, containing an O^6MeGua adduct in either the W or the C strand of a *Pst* I site. Neo^R, neomycin-resistance gene; SV40, simian virus 40.

recognition site. The techniques used situated the adducts with equal likelihood in either of the complementary strands of the vector [strands arbitrarily designated as Watson (W) or Crick (C); Fig. 1]. The placement of the adducts in a *Pst* I site facilitated the isolation of mutants, which were selected by their resistance to digestion by this enzyme.

The vectors pKE15-O^6MeGua or pKE15-O^6EtGua were introduced by transfection into CHO cells. These cells were chosen because the parental line is deficient in the O^6AlkGua-DNA alkyltransferase (28) (i.e., they are *mex*$^-$). A *mex*$^+$ variant containing the alkyltransferase repair activity was derived by transfecting DNA from human liver into the *mex*$^-$ cell line (24). It was anticipated that the O^6AlkGua adducts might be more mutagenic in the alkyltransferase-deficient host than in the cells expressing the alkyltransferase activity in view of the higher sensitivity of the former to alkylating agent-induced mutagenesis (29). Two guanine-containing control genomes were also transfected into CHO cells. One, pKE15-Gua, was produced in the same manner as pKE15-O^6MeGua and pKE15-O^6EtGua, but an unmodified tetranucleotide replaced those containing the adducts. This genome was a control for possible artifactual mutations arising from the techniques used to produce the site-specifically modified vectors. The second control was for spontaneous mutations arising in the CHO cells and was simply unmodified pKE15 isolated from *E. coli*. Once inside the cells, the vectors integrated into the host genome due to the lack of an origin for episomal replication in CHO cells. Clones of cells harboring the vector were selected with G418. Presumably, mutations arising from the adducts were fixed during the replication of the host chromosome during the time required for G418^r clones to become visible ($\approx$15 cell generations).

Analysis of the type and amount of adduct-induced mutation required that the integrated sequences of the original adducted vector, presumably a mixture of mutant and wild-type DNA, be rescued from the chromosomes of the host. This was accomplished by the use of the PCR to amplify selectively the region of the integrated vector containing the *Pst* I site at which the adducts had been situated. PCR primers were chosen that enabled the amplification of a 292-base-pair (bp) fragment of pKE15. The amplified DNA was end-labeled with ^{32}P, digested with *Pst* I, and then analyzed by polyacrylamide gel electrophoresis and autoradiography (Fig. 2). *Pst* I cleavage of nonmutant DNA resulted in two fragments of 201 and 91 bp,‡ whereas mutant DNA was

‡The method for labeling the amplified DNA was expected to label only one end of the fragment (see *Materials and Methods*). Examination of Fig. 2 reveals that both ends of the amplified fragment of DNA became labeled, as shown by the presence of both the 201- and 91-bp *Pst* I-digested bands on the autoradiogram. Possible explanations for this are (*i*) the 3′ → 5′ exonuclease activity of the Klenow fragment of DNA polymerase I may have generated a recessed 3′ terminus that was labeled by the 5′ → 3′ polymerase activity and [α-^{32}P]dATP, or (*ii*) the ends of the amplified DNA from the PCR were not completely blunt.

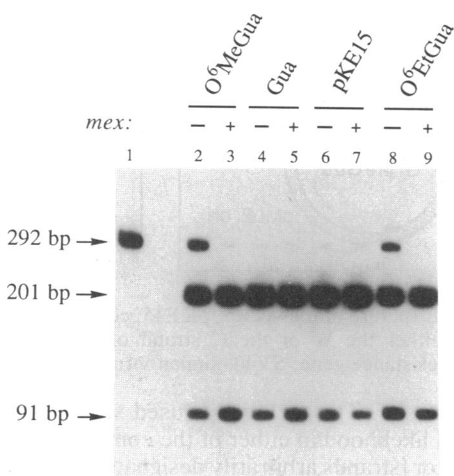

FIG. 2. Amplified DNA resulting from the genomic DNA of *mex*$^-$ or *mex*$^+$ CHO cells transfected with adduct-containing and control vectors. The PCR products from the genomic DNA derived from *mex*$^-$ or *mex*$^+$ CHO cells transfected with pKE15-O^6MeGua, pKE15-O^6EtGua, pKE15-Gua, or pKE15 were purified, end-labeled with ^{32}P, and digested with *Pst* I. In each case, complete *Pst* I digestion was obtained (data not shown). The DNA was electrophoresed in a polyacrylamide gel, which was subsequently dried and autoradiographed. Lane 1, undigested amplified DNA. Lanes 2–9, the *Pst* I-digested PCR products from genomic DNA isolated from the G418^r clones derived by transfection of the following DNAs. Lanes: 2 and 3, pKE15- O^6MeGua(B); 4 and 5, pKE15-Gua(B); 6 and 7, pKE15; 8 and 9, pKE15-O^6EtGua(B). The positions of the mutant (292 bp) and wild-type (201 and 91 bp) bands are indicated.

not cleaved by *Pst* I and therefore migrated as the uncut 292-bp PCR product. As shown in Fig. 2, *Pst* I-resistant DNA was present in the amplified DNA from *mex*$^-$ cells transfected with pKE15-O^6MeGua (lane 2) and pKE15-O^6EtGua (lane 8), indicating the presence of mutants. By contrast, very little undigested DNA was detectable in the samples from cells transfected with the control genomes, pKE15-Gua (lanes 4 and 5) or pKE15 (lanes 6 and 7). Furthermore, little or no *Pst* I-resistant DNA was detectable in the amplified DNA from *mex*$^+$ cells transfected with pKE15-O^6MeGua (lane 3) or pKE15-O^6EtGua (lane 9). These data showed that both O^6MeGua and O^6EtGua are mutagenic in mammalian cells deficient in O^6AlkGua-DNA alkyltransferase, but the presence of this enzyme largely prevents mutagenesis, presumably by mediating the repair of the adducts.

Mutation Frequencies of O^6MeGua and O^6EtGua in Repair-Proficient and Repair-Deficient CHO Cells. To determine the mutation frequencies of O^6MeGua and O^6EtGua in *mex*$^-$ and *mex*$^+$ CHO cells, the fraction of amplified DNA resistant to *Pst* I was quantified. This was accomplished by measuring the amount of radioactivity in the *Pst* I-resistant and *Pst* I-digested bands on gels containing radiolabeled amplified DNA, such as that shown in Fig. 2. To establish that this protocol could distinguish different mutant fractions, the method was tested on amplified DNAs containing increasing quantities of *Pst* I-resistant DNA. These were generated by mixing known quantities of a model mutant plasmid, pKE14, with pKE15. pKE14 is identical to pKE15 except that it contains an *Xmn* I site instead of the *Pst* I site (the adduction target in pKE15) and hence is 4 bp smaller than pKE15 (23). The amplified DNA from pKE14 is thus resistant to *Pst* I. The mixtures of pKE14 and pKE15 were amplified by the PCR, and the *Pst* I-resistant fraction of the amplified DNA was measured. As shown in Fig. 3, the protocol was able to distinguish between various fractions of *Pst* I-resistant DNA, indicating that this method was suitable for measuring mutation frequencies.

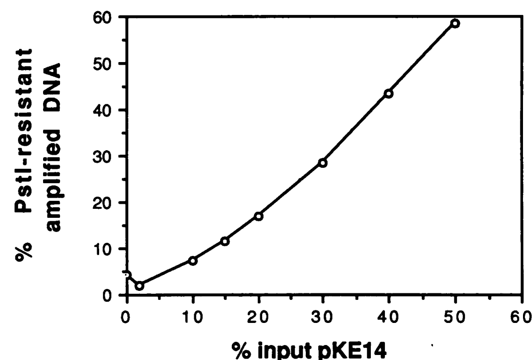

FIG. 3. Validation of the method for measuring mutation frequency by using known fractions of *Pst* I-resistant amplified DNA. Known amounts of a plasmid pKE14 (see ref. 23) were mixed with pKE15 DNA, and the PCR was performed on the mixture. The final concentrations of pKE14 in the mixtures were 0%, 2%, 10%, 15%, 20%, 30%, 40%, and 50%. After PCR, the *Pst* I-resistant fraction of the amplified DNA was determined.

The mutation frequencies for the O^6MeGua- and O^6EtGua-containing vector sequences amplified from the G418^r clones are presented in Table 1. The frequency of the mutations from O^6MeGua in *mex*$^-$ cells was ≈19% in each of two independent experiments when the transfected genome contained only covalently closed circular DNA [pKE15-O^6MeGua(B)]. The frequency was slightly lower for pKE15-O^6MeGua(A) (≈13%), where the nicked circular DNA was not eliminated prior to transfection. A possible explanation for the lower mutation frequency may be that the presence of the nick enhanced the susceptibility of the vector to nucleases (repair or otherwise), which may have removed the adduct prior to replication of the adducted sequence. The mutation frequency of O^6EtGua was ≈11% in *mex*$^-$ CHO cells, which is approximately one-half the mutation frequency of O^6MeGua under the same conditions (transfected as covalently closed circular DNA). It should be noted that the actual mutation frequencies induced by the adducts are twice those reported in Table 1, because the adducts were present in only one strand of the vector and the unadducted strand presumably engendered only wild-type progeny.

The mutation frequencies of O^6MeGua and O^6EtGua were substantially reduced in *mex*$^+$ cells, such that, in all except one experiment, no induced mutants were detected. This result demonstrates that the mammalian O^6AlkGua-DNA

Table 1. Mutation frequencies at the *Pst* I site of pKE15-O^6MeGua, pKE15-O^6EtGua, pKE15-Gua, or pKE15 transfected into *mex*$^-$ or *mex*$^+$ CHO cells

Transfected DNA	*mex*$^-$ or *mex*$^+$ CHO cells	MF, % (clones) Exp. 1	Exp. 2
pKE15-Gua(A)	+	1.8 (280)	ND
pKE15-Gua(A)	−	1.9 (120)	ND
pKE15-O^6MeGua(A)	+	2.0 (250)	2.9 (290)
pKE15-O^6MeGua(A)	−	13.2 (200)	ND
pKE15-Gua(B)	+	2.5 (160)	1.7 (170)
pKE15-Gua(B)	−	2.5 (40)	1.9 (140)
pKE15-O^6MeGua(B)	+	5.2 (190)	2.4 (310)
pKE15-O^6MeGua(B)	−	19.7 (160)	18.6 (120)
pKE15-O^6EtGua(B)	+	2.1 (200)	1.5 (250)
pKE15-O^6EtGua(B)	−	ND	10.8 (170)
pKE15	+	2.1 (280)	2.3 (330)
pKE15	−	2.2 (230)	2.0 (180)
pKE15 (PCR Control)	NA	3.2 NA	

MF, mutation frequency; ND, not determined; NA, not applicable. Numbers in parentheses represent approximate number of clones.

alkyltransferase is likely to be the major mode of O^6MeGua and O^6EtGua repair *in vivo*. We cannot rule out, however, a contribution by another O^6AlkGua repair system since the maximum mutation frequency observed (19%) was less than the theoretical maximum (50%) for a highly mutagenic adduct in the complete absence of repair. It is noteworthy that in one sample from *mex*$^+$ cells transfected with pKE15-O^6MeGua, a detectable mutation frequency above background was observed (5.2%), possibly because some adducts had escaped repair. This could occur if the number of vectors taken up by the *mex*$^+$ cell during the transfection protocol exceeded the number of alkyltransferase molecules (>2500 per cell; data not shown). The background mutation frequency of 2–2.5% in the control samples may be due to (*i*) mutations induced *in vitro* by the polymerase during the PCR or (*ii*) background radioactivity in the polyacrylamide gel from incomplete cleavage by *Pst* I. The background was probably not due to spontaneous mutations at the *Pst* I site arising in the CHO cells since the pKE15 PCR control had a similar mutation frequency (Table 1).

Sequence Analysis of Mutants Induced by O^6MeGua and O^6EtGua in *mex*$^-$ CHO Cells. The nature of the mutations induced by the adducts in *mex*$^-$ CHO cells was determined by DNA sequencing. From both O^6MeGua and O^6EtGua, the mutations were almost exclusively G → A transitions and were targeted at either of the two possible adduction sites (Table 2). The mutations were uniformly distributed between the two sites in the vector at which the adducts were located (i.e., one site in the W strand and one in the C strand; see Fig. 1). This result suggested that the mutation frequency of each adduct was the same in either strand of the vector. Two G → T transversions were also observed, one each from the O^6MeGua and O^6EtGua vectors. The transversions were targeted at potential adduct sites, but their frequencies were low (1/15 for O^6MeGua and 1/21 for O^6EtGua) so it is possible that they were not induced by the adducts but arose spontaneously.

Mutagenesis of O^6MeGua and O^6EtGua *in Vitro*. O^6MeGua in DNA or RNA polymers has been shown to direct misincorporation by DNA or RNA polymerases *in vitro* (11–14). It was of interest to determine whether the site-specific adduct caused misincorporation by *Taq* polymerase during the PCR replication of the template, resulting in mutations. As shown in Fig. 4, amplified DNA using pKE15-O^6MeGua as the PCR template contained 40–50% of *Pst* I-resistant DNA (lane 4), whereas the amplified DNA from pKE15-Gua was completely digested with *Pst* I (lane 2). The *Pst* I-resistant amplified DNA was eliminated by treatment of pKE15-O^6MeGua with purified *E. coli* O^6MeGua-DNA methyltransferase prior to the PCR (lane 5), demonstrating that the O^6MeGua was directly responsible for the PCR-induced mutations. DNA sequencing of 10 mutants revealed the mutations to be targeted G → A transitions, of which half were from the W strand and half were from the C strand. pKE15-O^6EtGua was also found to induce *Pst* I-resistant amplified DNA during the PCR amplification, although to a lesser extent than was observed for pKE15-O^6MeGua (≈10%; data not shown).

Table 2. Summary of point mutations at the *Pst* I site of pKE15-O^6MeGua and pKE15-O^6EtGua transfected into *mex*$^-$ CHO cells

	G → A transition		G → T transversion	
Transfected DNA	W strand	C strand	W strand	C strand
pKE15-O^6MeGua(A)*	8	6	1	0
pKE15-O^6MeGua(B)*	6	5	0	0
pKE15-O^6MeGua(B)†	10	12	0	0
pKE15-O^6MeGua(B)†	10	10	0	1

*Experiment 1.
†Experiment 2.

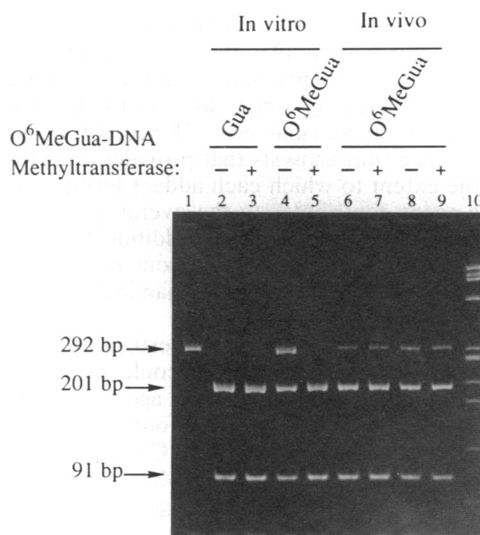

FIG. 4. The effects of *E. coli* O^6MeGua-DNA methyltransferase on the mutation frequency of O^6MeGua *in vitro* and *in vivo*. DNA was used as the template in the PCR that had either been treated (+) or not treated (−) with *E. coli* O^6MeGua-DNA methyltransferase (Applied Genetics, Freeport, NY). One-tenth of the amplified DNA was digested with *Pst* I, electrophoresed in a polyacrylamide gel, stained with 0.5 μg of ethidium bromide per ml, and photographed. Lane 1, undigested amplified DNA. Lanes 2–9, the *Pst* I-digested PCR products of the following DNAs. Lanes: 2 and 3, pKE15-Gua(B); 4 and 5, pKE15-O^6MeGua(B); 6 and 7, genomic DNA from *mex*$^-$ cells transfected with pKE15-O^6MeGua(A); 8 and 9, genomic DNA from *mex*$^-$ cells transfected with pKE15-O^6MeGua(B); 10, markers of φX174 DNA digested with *Hae* III. *In vitro* and *in vivo* refer to whether the *Pst* I-resistant amplified DNA from O^6MeGua arose during the PCR or during passage through the *mex*$^-$ CHO cells, respectively. The positions of the mutant (292 bp) and wild-type (201 and 91 bp) bands are indicated.

The finding that O^6MeGua induced mutations at a high frequency during the PCR amplification led us to investigate whether the mutations we had observed from O^6MeGua *in vivo* had actually arisen from the *in vitro* replication of residual unreplicated input pKE15-O^6MeGua, which may have been isolated from the CHO cells along with the genomic DNA. Owing to the exponential increase in amplified DNA during the PCR, theoretically only a few unreplicated molecules of pKE15-O^6MeGua would be necessary as a template to produce the observed amplified DNA containing *Pst* I-resistant material. To test this possibility, genomic DNA isolated from *mex*$^-$ cells transfected with pKE15-O^6MeGua was treated with purified *E. coli* O^6MeGua-DNA methyltransferase prior to the PCR. If the observed *Pst* I-resistant DNA were caused by the *in vitro* replication of residual methylated vector DNA, the O^6MeGua-DNA methyltransferase treatment would remove the adduct from the template. The resultant amplified DNA would contain no *Pst* I-resistant DNA. As shown in Fig. 4 (lanes 6–9), O^6MeGua-DNA methyltransferase treatment had no effect on the quantity of *Pst* I-resistant material in the amplified DNA when genomic DNA from *mex*$^-$ cells transfected with pKE15-O^6MeGua was used as the template for the PCR. This result demonstrated that the mutations (Table 1) were indeed fixed during the *in vivo* replication of pKE15-O^6MeGua in the CHO cells.

DISCUSSION

The mutational spectrum of a chemical reveals the locations and types of induced mutations and allows predictions to be made concerning the chemical–DNA adducts that might have

caused those mutations. Mutational spectra rarely, however, enable the unambiguous identification of premutagenic lesions. The system described here permits the investigation of mutagenesis induced by single adducts located within the chromosomes of mammalian cells. Hence, it is an adjunct to mutational spectrum analysis that provides a means to test directly the extent to which each adduct formed by a DNA damaging agent contributes to the overall pattern of mutagenesis induced by that agent. An additional attractive feature of the system is that the vector containing the adduct is replicated within the genome of the mammalian cell, and not as an episome.

We have utilized the system to investigate whether alkylation at the O^6 position of guanine could contribute to the mutations observed from alkylating agents in mammalian cells. O^6MeGua and O^6EtGua were found to induce almost exclusively G → A transitions in CHO cells that were deficient in the O^6AlkGua-DNA alkyltransferase. This mutation is also the major genetic change induced by methylating and ethylating agents in mammalian cells (7–10). Our data therefore support the hypothesis generated by the mutational spectra that O^6MeGua and O^6EtGua are major premutagenic lesions formed by these chemicals in mammalian cells.

The mutagenesis *in vivo* from both O^6MeGua and O^6EtGua was reduced to undetectable levels in cells containing the O^6AlkGua-DNA alkyltransferase (*mex*$^+$), except in one experiment in which the mutation frequency due to O^6MeGua was reduced to approximately one-quarter of that observed in *mex*$^-$ cells. Presumably, the low level of mutagenesis in *mex*$^+$ cells was the result of adduct repair by the alkyltransferase before the adducted sequence was processed into a mutation. Our results indicate that the alkyltransferase strongly modulates the mutagenesis of both O^6MeGua and O^6EtGua. These results, however, do not preclude the possibility that other repair factors can act on these adducts. The methods used to introduce the O^6AlkGua into the vector placed the adduct into only one strand in any duplex pKE15 molecule. Accordingly, the maximum mutation frequency obtainable in our system was 50% because the strand without the adduct should give rise to wild-type DNA upon replication. Consistent with this expectation, when the O^6MeGua-containing vector was replicated by *Taq* polymerase during the PCR *in vitro*, a mutation frequency was observed that was close to 50% (Fig. 4; data not shown). This result suggested that O^6MeGua may be misreplicated virtually every time the polymerase encounters it. These data are in accord with a recent detailed examination of the kinetics of misincorporation opposite O^6MeGua in an oligonucleotide by DNA polymerases *in vitro* (14). We note, however, that the mutation frequency of O^6MeGua in *mex*$^-$ cells *in vivo* was only approximately one-half the mutation frequency *in vitro*. While one explanation for this difference is that *Taq* polymerase differs from mammalian polymerases in its ability to synthesize DNA correctly opposite an O^6MeGua adduct, it may also be indicative of another repair system in the CHO cells that can remove low levels of O^6MeGua, resulting in a lower mutation frequency.

Modified versions of the experimental protocol described above enable the placement of any chemical–DNA adduct in any sequence context (30). Because the adducted sequence becomes part of the host chromosome, it is therefore possible to examine the precise effects of the natural replication and repair systems of the host on the adduct *in vivo*. By using systems such as this one with other DNA adducts, it should be possible to determine to what extent those adducts could contribute to the induction of the types of mutations that arise during the initiation of cancer and other genetic diseases.

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