Carcinogenesis vol.17 no.11 pp.2449–2454, 1996

Formation and persistence of O^6-ethylguanine in genomic and transgene DNA in liver and brain of λlacZ transgenic mice treated with *N*-ethyl-*N*-nitrosourea

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***LacZ* transgenic mice are suitable for short-term mutagenicity studies *in vivo*. Mutagenicity in these mice is determined in the *lacZ* transgene. Since the *lacZ* gene is of bacterial origin the question has been raised whether DNA-adduct formation and repair in the transgene are comparable to those in total genomic DNA. Mice were treated with *N*-ethyl-*N*-nitrosourea (ENU) and killed at several time points following treatment. Some mice were pretreated with O^6-benzylguanine to inactivate the repair protein O^6-alkylguanine-DNA alkyltransferase (AGT). O^6-ethylguanine (O^6-EtG) was determined in *lacZ* in liver and brain by means of a monoclonal antibody-based immunoaffinity assay. In addition, O^6-EtG and N7-ethylguanine (N7-EtG) were assayed in total genomic DNA of liver and brain with an immunoslotblot procedure. In liver, the initial O^6-EtG level in total genomic DNA was 1.6 times that in *lacZ*. The extent of repair of O^6-EtG during the first 1.5 h after treatment was 2.1 times that in *lacZ*. At later time points, O^6-EtG repair was the same. N7-EtG repair in genomic DNA was evident. In contrast to the liver, little repair of O^6-EtG in total genomic and lacZ DNA occurred in the brain while N7-EtG was repaired. No initial difference in O^6-EtG levels were found in *lacZ* and genomic brain DNA. These findings indicate that in the liver, total genomic DNA is more accessible than *lacZ* to ENU and/or the AGT protein, during the first 1.5 h following treatment. Because the difference in O^6-EtG levels in the transgene and genomic DNA in the liver is restricted to the first 1.5 h after treatment, while the fixation of mutations occurs at later time points, O^6-EtG-induced mutagenesis most likely is also very similar in both types of DNA.**

Introduction

Mammalian short-term *in vivo* mutagenesis studies have become possible with the development of transgenic animals carrying multiple copies of a reporter gene (1–3). The transgenic mouse strain 40.6 (Muta™ Mouse)harbours 80 copies of the λ*gt*10*lacZ* shuttle vector in each somatic cell in the form of two 40-mer concatemers. The *Escherichia coli lacZ* gene located within each λ prophage codes for β-galactosidase and serves as the indicator sequence for mutations.

Whether mutations induced in the transgene DNA reflect those in total genomic DNA is a critical question for assessing the value of transgenic marker mice in mutagenesis studies. The accessibility of foreign DNA sequences for genotoxic agents and repair proteins may be different from that of the rest of the genomic DNA, due to differences in conformation and transcriptional status. This contention is confirmed by the observation that increased levels of alkylation and enhanced repair of certain adducts have been found in the relatively less compact, transcriptionally active chromatin compared to the more condensed, inactive chromatin, both *in vitro* and *in vivo* (4–6). At the gene level, repair of DNA damage induced by agents such as UV has been extensively studied *in vitro*. Bohr *et al.* (1985)(7) first observed the preferential repair of UV-induced pyrimidine-dimers in transcriptionally active DNA versus inactive DNA. The same was shown to occur *in vivo* in the transcriptionally active *hprt* and *ada* genes as opposed to the inactive *c-mos* and *Hp* sequences of mouse epidermal cells (8). This phenomenon is not limited to UV-damage, as illustrated by the preferential repair of cisplatin adducts in the actively transcribed *dhfr* gene compared to the non-transcribed *c-fos* gene (9). Similar observations were made for the mutagenic DNA-alkylation product O^6-ethylguanine (O^6-EtG*) in the actively transcribed β-actin gene in comparison to the inactive *IgE* gene and to total genomic DNA of ENU-treated rat hepatoma cells (10). Not all types of adducts, however, are subject to preferential repair in transcriptionally active genes. The DNA alkylation products N7-methylguanine and N3-methylguanine are repaired with equal efficiency in both the active *dhfr* domain and the inactive sequences further downstream of the gene (11,12).

Given the fact that transgenic DNA sequences present in the *lacZ* transgenic mice are not actively transcribed, it is necessary, to determine possible differences between transgene and genomic DNA with respect to adduct formation and repair. This will make interpretation of mutagenesis data collected with these mice more reliable. Here, we address this question with respect to O^6-EtG in *lacZ* transgenic mice.

O^6-EtG, together with O^2- and O^4-ethylthymine, are premutagenic lesions formed *in vitro* upon exposure of cells to ethylating agents. These adducts are thought to exert their mutagenic effect by mispairing during DNA replication (13–15). Repair of O^6-alkylguanine in DNA is accomplished largely by the O^6-alkylguanine-DNA alkyltransferase (AGT) protein. The alkyl group is transferred from the O^6-atom of guanine to a cysteine residue in the active site of the AGT protein. This leads to the restitution of the original guanine base and inactivation of the AGT protein (16). AGT is also capable of

*Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; ISB, immunoslotblot; Mab, monoclonal antibodies; N7-EtG, N7-ethylguanine; O^6-BzG, O^6-benzylguanine; O^6-EtG, O^6ethylguanine.

interacting with the base analogue O^6-benzylguanine which also leads to the inactivation of the protein. Pretreatment of cells with O^6-benzylguanine (O^6-BzG) results in the depletion of the pool of cellular AGT (17). This allows the study of the initial repair of O^6-EtG in DNA in the absence of repair during the treatment period.

To determine whether a difference in O^6-EtG formation and repair exists between transgene DNA and total genomic DNA, we applied a recently developed method (18), using genomic DNA digested with *Eco*RI and mixed with a known amount of internal standard DNA (linearized ethylated pSV2*gpt* plasmid). O^6-EtG containing DNA fragments are isolated by use of anti-O^6-EtG monoclonal antibodies (Mab), followed by the quantification of the *lac*Z and *gpt* DNA by quantitative PCR. O^6-EtG levels at *lac*Z loci were determined in liver and brain DNA of transgenic mice at various time points after treatment with ENU, and in mice pretreated with O^6-BzG followed by exposure to ENU. In addition, the O^6-EtG and N7-ethylguanine (N7-EtG) contents in total genomic DNA were determined with immunoslotblot (ISB)(19). Liver and brain DNA was analysed because these tissues exhibit large differences with respect to their rates of DNA synthesis (20) and O^6-EtG repair (21).

Materials and methods

Transgenic mice and treatment

Female transgenic mice (strain 40.6) were obtained at 14 weeks (±2 weeks) of age from the TNO Centre for Animal Research (The Netherlands). The mice were injected intraperitoneally (i.p.) with ENU (Sigma, St. Louis, MO, USA), 150 mg/kg body weight (b.w.) dissolved in DMSO (Merck, Darmstadt, Germany), while the control animals received DMSO (2 ml/kg b.w.). In the ENU-treated group a total of four animals were killed after 1.5 h and two animals each after 24 and 72 h and 14 d. Control animals were killed after 1.5 h and 14 d. Two hours prior to ENU treatment, four mice were injected i.p. with 10 mg/kg b.w. O^6-BzG dissolved in DMSO. At various time points after treatment animals were killed by CO_2 asphyxiation after which the organs were collected, placed on dry ice and stored at –20°C.

DNA isolation

High molecular weight DNA was isolated from liver and brain in accordance with a protocol described by Roggeband *et al.* (1993)(22), except for the incubation of the isolated chromatin, which was for 16 h at 20°C in the presence of proteinase K (50 µg/ml; Merck). In the case of DNA isolation from the brain, the tissue was directly homogenized in the sucrose/EDTA/Tween-20 buffer. The remaining steps were the same as those for liver DNA. The DNA was finally dissolved up in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4). After incubation with RNase A (0.1 mg/ml; Sigma) and T1 (100 U/ml; Boehringer Mannheim, Mannheim, Germany) for 2 h at 37°C, the DNA was once more extracted with phenol/chloroform and washed with chloroform prior to alcohol precipitation. The purified DNA was dissolved in TE buffer.

Quantification of O^6- and N7-ethylguanine in genomic DNA

The quantification of O^6- and N7-EtG in total genomic DNA was carried out by means of the ISB procedure (19). For the assay of N7-EtG, DNA was pretreated as follows: DNA was diluted in potassium phosphate buffer (10 mM; pH 7) to a concentration of 50 µg/ml and sonicated for 5 s (Ultrasonics W-370, USA, with microtip; output level 2.5) To 200 µl DNA, 4.7 µl 3 M NaOH was added and the solution was incubated at 37°C for 30 min to convert N7-EtG bases into their ring-opened form. The reaction was stopped by the sequential addition of 6 µl 1 M K_2HPO_4 and 15 µl 1 M HCl. The pH of the mixture was checked to be 7.4. To this mixture 275 µl phosphate-buffered saline (PBS) was added, and the solution was heated to 100°C for 10 min to denature the DNA and placed on ice for 5 min. After addition of 500 µl 2 M ammonium acetate the DNA concentration was determined spectrophotometrically by measuring absorbance at 260 nm. For determination of O^6-EtG, DNA was sonicated, heated to 100°C for 10 min and placed on ice for 5 min. After addition of an equal volume of 2 M ammonium acetate the DNA concentration was determined. For both the O^6- and the N7-EtG assay, 1 µg genomic DNA isolated from the liver or brain from each animal, along with calibration standards of *in vitro* ethylated DNA of known O^6- and N7-EtG content, were blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using the Manifold II blotting device (Schleicher and Schuell). All slots were washed with 200 µl 1 M ammonium acetate and the membranes were baked for 2 h at 80°C. Pre-incubation of the nitrocellulose blots was carried out for 1 h, under gentle agitation, in 16 ml PBS containing 0.5% (w/v) skimmed milk and 0.1% (v/v) Tween-20, at room temperature. The blots were washed twice in PBS/0.1% Tween-20 and then incubated with Mab ER-6 (23) specific for O^6-EtG (60-fold diluted culture supernatant) or Mab N7E-026 (24) specific for ring-opened N7-EtG (25 000-fold dilution of 100 µg/ml protein A-purified antibody) in 16 ml PBS/0.1% Tween-20/0.5% skimmed milk, for 2 h at room temperature. The blots were washed 3×5 min in PBS/0.1% Tween-20 followed by incubation with the peroxidase-labelled second antibody (ER-6: rabbit anti-rat; N7E-026: rabbit anti-mouse; Dakopatts a/s, Denmark) in PBS/0.1% Tween-20/0.5% skimmed milk for 1 h at room temperature. The blots were washed again 3×5 min in PBS/0.1% Tween-20, and treated with the ECL western blotting reagents (Amersham, Buckinghamshire, England). Chemiluminescence was detected with sensitive film (Hyperfilm-ECL, Amersham) and evaluated by densitometry (Ultroscan XL, LKB, Brommo, Sweden).

O^6-EtG content in the lacZ transgene

The procedure for the gene-specific measurements has been described previously (18); it consists of the following steps:

Enrichment of O^6-EtG-containing DNA fragments

Isolated genomic DNA was digested with EcoRI which cuts out a 3128 bp fragment comprising all but the first 25 bp of the *lac*Z gene from the concatemers of λ*gt10lac*Z shuttle vectors. Completeness of digestion was checked by separating aliquots of the digests on a 1% agarose gel. One-microgram aliquots of digested DNA were placed in separate siliconized 1.5-ml Eppendorf tubes and 1 pg pSV2*gpt* plasmid containing an average of 1 O^6-EtG per plasmid molecule was added to each tube. The plasmid served as an internal calibration standard during the entire procedure.

After drying the samples *in vacuo*, the DNA was dissolved in 70 µl STE (50 mM Tris pH 7.5, 100 mM NaCl and 1 mM EDTA). To each sample, 30 µg of Mab ER-6 (1 µg/µl) was added and the mixture incubated for 45 min at room temperature. Antibody-DNA complexes were separated from non-complexed DNA by passage through a nitrocellulose filter (Protran BA 85, Schleicher and Schuell). Membrane-bound DNA was released and eluted by washing the filters three times with 300 µl 5% (v/v) n-butanol in 5 mM Tris pH 7.5/0.1 mM EDTA. The eluent was dried under vacuum. The dried DNA and constituents were redissolved in 100 µl 0.1 M NaCl containing 30 µg Mab ER-6 and 1 µg of DNase-free BSA.

The procedure of antibody-binding and elution was performed three times in total. For the third elution, the butanol/Tris/EDTA-buffer was replaced with 5% (v/v) n-butanol in 5 mM KCl. Standards for the quantification of *lac*Z DNA were prepared by adding various amounts of EcoRI-digested genomic DNA to a constant amount of pSV2*gpt* plasmid. All standards and enriched experimental samples were purified by passage over an octylsepharose (Phamacia) column. The collected eluent was dried and subsequently subjected to quantitative PCR analysis.

lacZ and gpt *amplification by PCR*

PCR conditions for co-amplification of *lac*Z and *gpt* were optimized as to Mg^{2+} concentration and annealing temperature. Co-amplification of short sequences within the *lac*Z (127 bp) and *gpt* (148 bp) DNA, enriched as described above, was carried out in a PCR reaction (25). The PCR conditions were as follows: 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM of each of the dNTP's, 0.11 MBq α-[^{32}P]dCTP, 0.4 µM of each oligonucleotide and 20 U/ml Taq DNA polymerase (Promega, Madison, WI, USA). The PCR profile consisted of 30 s denaturation at 94°C; 1 min primer hybridization at 65°C and primer extension for 3 min at 72°C. This program was run for 20 cycles in a Thermal Cycler (Perkin-Elmer/Cetus). The *lac*Z and *gpt* oligonucleotides used in the PCR reactions were prepared by means of an automated DNA synthesizer (Applied Biosystems). The sequence of the oligonucleotides was as follows:

*lac*Z P1 5′ dTCCGCCGTTTGTTCCCACGGAGAAT;
*lac*Z P2 5′ dACAGATGAAACGCCGAGTTAACGCC;
gpt P1 5′ dCGTTACTGGCGCGTGAACTGGGTAT;
gpt P2 5′ dTCACGAATCGCAACCGCAGTACCAC.

Calculation of O^6-EtG content in lacZ

Fifteen µl of each PCR reaction mixture was run on a 6% non-denaturing polyacrylamide gel. The separate bands were quantified by [^{32}P]-counting using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA). The *lacZ*/*gpt* signal ratio of each sample, as measured from the gel, was compared to a standard curve, an example of which is shown in Figure 1. Interpolation provides the amount of DNA present in the tube prior to PCR. By dividing the ng equivalent of genomic DNA in the PCR mixture by the DNA content

per cell (5 pg) and multiplying this with the number of *lacZ* copies per cell (80), one obtains the number of *lacZ* copies present containing an O^6-EtG adduct. It is reasonable to assume that each antibody-complexed *lacZ* sequence contains 1 O^6-EtG residue. Therefore, the number of O^6-EtG adducts in *lacZ* in the initial 1 µg of genomic DNA at the start of the experiment, is equivalent to the number of bound *lacZ* sequence. Dividing this value by the number of nucleotides making up the *lacZ* loci (1 µg/5 pg per cell×80 copies/cell×6256 nt per *lacZ* molecule) gives the number of O^6-EtG per *lacZ* nt. This is converted into O^6-EtG/10^6 nt. This can also be expressed as O^6-EtG/10^6 G taking into account the 56% GC content of *lacZ*.

Results

PCR analysis

The specificity of the primers was checked by allowing the PCR reaction to reach saturation (40 cycles). No unspecific PCR products were detected when the reaction mixture was analysed by gel electrophoresis (data not shown). A PCR cycle number of 20 was chosen where the two products were still in the exponential phase of amplification.

O^6-EtG in genomic and lacZ DNA

lacZ-transgenic mice were treated with ENU (150 mg/kg). At various time points after treatment, the O^6-EtG and N7-EtG contents in genomic DNA and O^6-EtG in *lacZ* DNA were determined in duplicate 2–4 and 4–9 times, respectively, in liver and brain DNA of each animal. The SD in genomic and *lacZ* DNA was on average ±25%. In total genomic DNA from liver, the average O^6-EtG content found at 1.5 h post-treatment (Figure 2a) was 32.8 ± 14.2 O^6-EtG/10^6 G in the four mice studied. At 24 h, the two mice exhibited 42.5 and 12.0 O^6-EtG/10^6 G in the liver DNA, respectively. O^6-EtG levels at 3 days approached the detection limit of around 1–4 O^6-EtG/10^6 G (1.8 and 4.1 O^6-EtG/10^6 G). Very low levels were measured 14 days post-treatment (0.9 and 0.7 O^6-EtG/10^6 G). In the O^6-BzG pre-treated mice killed at 1.5 h after ENU-treatment, an average O^6-EtG content of 90.6 ± 19.6/10^6 G was determined, i.e. a 2.8-fold increase compared to the mice that received only ENU. At the *lacZ* loci in liver DNA, the level of O^6-EtG was very similar to the corresponding genomic DNA at all time points except for the O^6-BzG-treated mice (Figure 2a). The level detected at the 1.5 h time point amounted to 30.1 ± 13.7 O^6-EtG/10^6 G. No reduction was measured during post-incubation up to 24 h (42.7 and 20.3 O^6-EtG/10^6 G), whereas most O^6-EtG had been removed after 3 d (2.6 and 5.1 O^6-EtG/10^6 G). The levels observed at 14 days post-treatment approached the detection limit.

In the total DNA isolated from the brain tissue, the O^6-EtG level at 1.5 h after ENU-treatment averaged 14.6 ± 4.3/10^6 G for the four animals tested (Figure 2b). No reduction was noticed after 24 h: values of 21.6 and 18.9 O^6-EtG/10^6 G were found in two mice. After 3 and 14 days the average adduct values were 13.8 ± 3.8 and 8.0 ± 3.1 O^6-EtG/10^6 G, respectively. In contrast to the liver samples, brain DNA from the O^6-BzG-pretreated mice showed a smaller increase in genomic O^6-EtG levels (25.9 ± 10.4 O^6-EtG/10^6 G) at the 1.5 h time point compared to non-pretreated mice. The level of O^6-EtG in the *lacZ* loci was again very similar to that in genomic DNA. There was no difference in O^6-EtG content in *lacZ* between animals treated with ENU only (11.9 ± 3.8 O^6-EtG/10^6 G) and those having received O^6-BzG and ENU (13.5 ± 1.2 O^6-EtG/10^6 nt). No significant overall decrease was found to occur during the 14 days post-treatment (1.5 h: 11.9 ± 3.8; 24 h: 20.0 ± 4.6; 3 days: 15.5 ± 4.2 and 14 days: 10.2 ± 1.1 O^6-EtG/10^6 G).

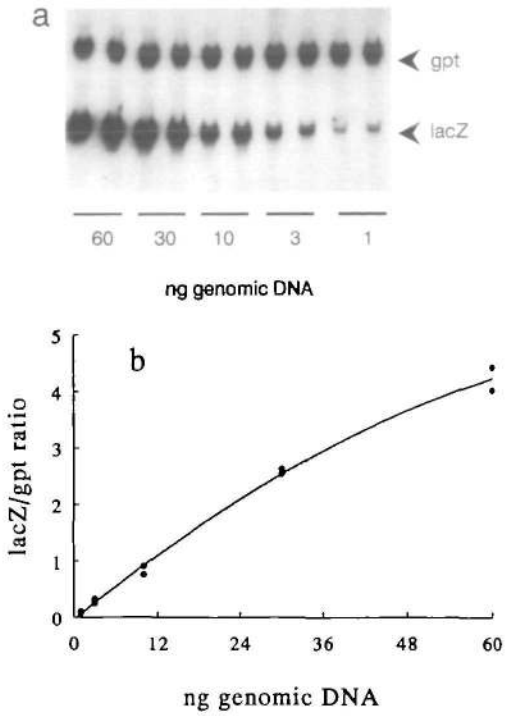

Fig. 1. (a) Autoradiogram of a polyacrylamide gel containing [^{32}P]-labelled PCR products used in the construction of a calibration curve. From left to right of the gel, decreasing amounts of genomic DNA and a constant amount of pSV2*gpt* internal standard were subjected to PCR in duplicate. The radioactivity was quantified by use of a phosphoImager. (b) An example of a calibration curve. Ratios of *lacZ*/gpt signals of the [^{32}P]-labelled PCR products were plotted against the ng genomic DNA input.

N7-EtG content in genomic DNA of liver and brain

The level of N7-EtG could only be measured in genomic DNA. In the liver, maximum values were obtained at 1.5 h (90.3 ± 42.8 N7-G/10^6 G) and 24 h (116.1 ± 14.9 N7-EtG/10^6) after ENU treatment (Figure 2a). More than 50% of the adducts had disappeared after 3 days (41.2 ± 7.0 N7-EtG/10^6 G), whereas nearly all N7-EtG had been removed at 14 days. In the BzG-pretreated mice an average of 99.1 ± 17.4 N7-EtG per 10^6 G was measured. In brain DNA, N7-EtG levels were 21.5 ± 9.1 N7-EtG/10^6 G after 1.5 h and averaged 18.6 ± 6.7 N7-EtG/10^6 G at 24 h after ENU administration (Figure 2b). In contrast to O^6-EtG, N7-EtG was removed from brain DNA. About half of the adducts had disappeared by day 3 (7.2 ± 1.2 N7-EtG/10^6 G) and barely detectable levels were found after 14 days (1.0 ± 0.3 N7-EtG/10^6 G).

Discussion

In view of their prokaryotic origin, their repetitiveness and the sheer size of the transcriptionally silent transgene concatemers present in the genome of the transgenic mouse 40.6, it is possible that the configuration of this DNA and its interaction with nuclear proteins is different from that in the rest of the DNA. These factors may have profound effects on the reactivity of an agent towards exogenous versus endogenous DNA.

To address this question a recently developed method for the measurement of O^6-EtG in single-copy genes (18) was employed. The level of O^6-EtG was measured in total genomic DNA and in *lacZ* sequences of liver and brain cells isolated from *lacZ* transgenic mice treated with ENU. As a control N7-EtG was measured in the overall genome of both tissues.

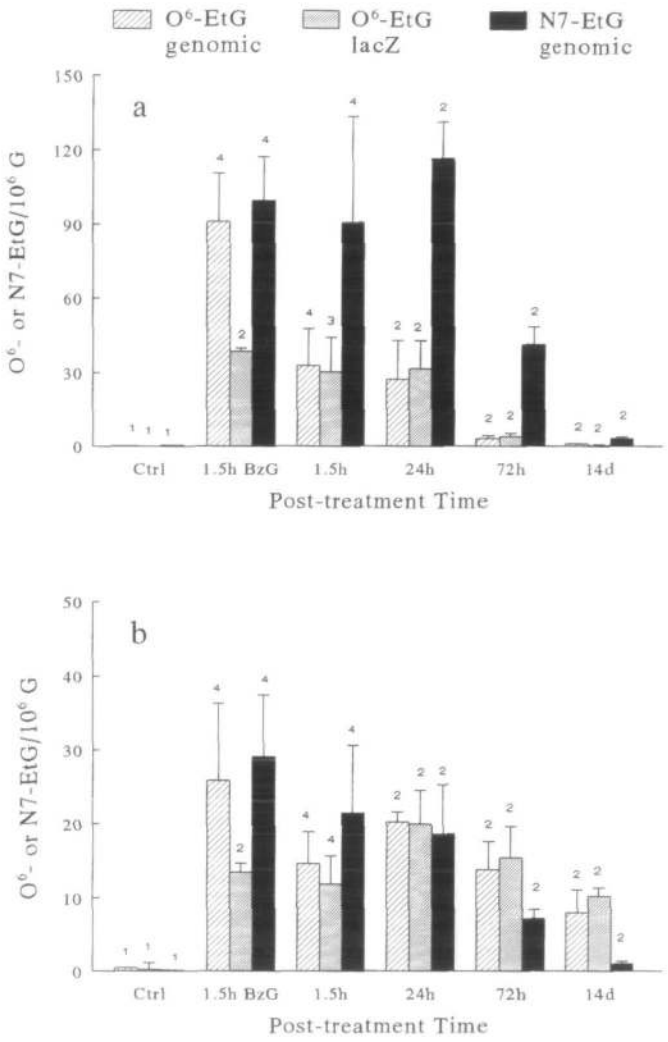

Fig. 2. O^6-EtG levels in genomic and *lac*Z sequences and N7-EtG levels in genomic DNA isolated from (a) liver and (b) brain of mice treated with O^6-BzG/ENU or ENU only The values are expressed as adducts/10^6 G after correction of the genomic and *lac*Z data for their respective GC-content of 40.3 and 56%. The data are presented as means with standard deviations when more than two animals were used and the range when two animals were employed. The numbers above the error bars indicate the number of mice involved.

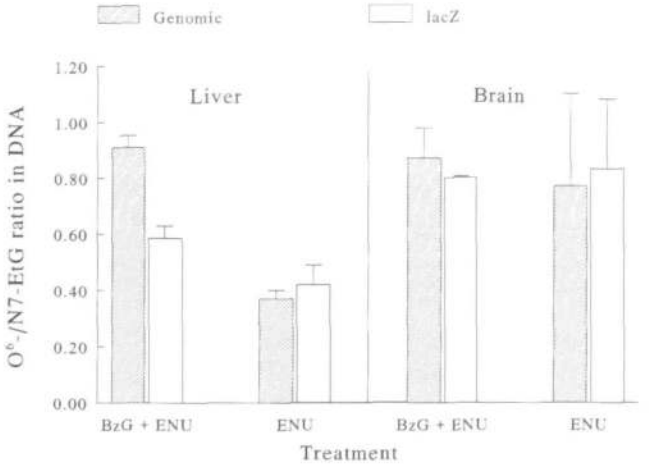

Fig. 3. Comparison of O^6-EtG content in genomic and *lac*Z DNA 1.5 h after treatment with ENU or with O^6-BzG/ENU. O^6-EtG values in genomic DNA have been normalized to the genomic N7-EtG levels to correct for inter-animal variation. The *lac*Z values have been normalized with the genomic N7-EtG content corrected for the GC-content in *lac*Z.

The results demonstrate that 1.5 h after ENU exposure, substantial levels of O^6- and N7-EtG can be measured in liver DNA, which hardly decrease in the subsequent 22.5 h. This indicates a very slow removal of the adducts during this time interval. However, the effect of pretreatment of the mice with O^6-BzG, which inactivates most of the suicidal AGT protein present in the cell (4-fold and 1.7-fold reduction in liver and brain AGT, respectively, determined in cell extracts; data not shown; manuscript in preparation), suggests a very strong reduction, in the absense of O^6-BzG, in the O^6-EtG level shortly after formation of these lesions, attributable to the cellular AGT pool. Evidently, the AGT pool becomes rapidly exhausted and further removal of O^6-EtG is then achieved by *de novo* synthesized AGT. As expected, N7-EtG levels were not affected by O^6-BzG because this lesion is removed by glycosylases and is not a substrate for AGT. The lack of repair of N7-EtG over the first 24 h opened the possibility to adjust the O^6-EtG results with respect to inter-animal variation, which in particular after i.p. dosing can be considerable, by expressing these data relative to the N7-EtG level in the same mouse. The results of this operation for the data at the 1.5 h time point are summarized in Figure 3.

Comparison of the normalized liver data obtained from O^6-BzG-pretreated and ENU- treated mice suggests a more effective initial formation of O^6-EtG in overall genomic DNA than in the *lac*Z genes (1.6×; $P = 0.001$, Student's *t*-test). In non-pretreated mice, 60% ($P < 0.001$) and 28% ($P = 0.06$) lower levels in genomic and *lac*Z DNA are seen, respectively, relative to the pretreated mice, which indicates a 2.1-fold faster repair in genomic versus *lac*Z DNA. These findings indicate that genomic DNA, representing transcriptionally active as well as inactive DNA, is generally more accessible both to ENU and to the AGT repair protein than the inactive and exogenous transgene DNA sequences. This is not entirely in agreement with the result obtained with rat hepatoma cell lines *in vitro* (10). Here, no difference in the initial O^6-EtG content and repair was found between total genomic DNA and transcriptionally inactive IgE gene in cells pretreated with O^6-BzG followed by ENU. On the other hand, the data obtained in the present study at the 24 h time point and later no longer indicate a difference between *lac*Z and total genomic DNA with regard to the efficiency of O^6-EtG repair.

The biphasic repair profile of O^6-EtG in genomic DNA of liver cells, with 60% of the lesions being repaired within the first 1.5 h, and 30% between 24 h and 3 days after treatment, has previously been reported in rats for O^6-methylguanine (26) and O^6-EtG (27). This phenomenon is likely due to a rapid depletion of the cellular reserve of AGT by the suicidal reaction with alkyl groups at the O^6-atom of guanine and the slow recovery of AGT activity over the following days.

In DNA from brain tissue of the same animals little or no effect of O^6-BzG and no significant reduction in O^6-EtG content was detected over the first 72 h, although there was a clear elimination of N7-EtG (Figure 2). No differences in either O^6-EtG formation or repair were evident from the normalized data (Figure 3). In previous studies carried out on the brain of adult rats, no or very slow repair of O^6-EtG ($t_{\frac{1}{2}}$ 44 days) was found, but a faster repair rate of N7-EtG was measured ($t_{\frac{1}{2}}$ 2.5–16 days) (21,27,28).

The level of O^6-EtG in genomic DNA of the liver, 1.5 h

after treatment, was found to be approximately twice the level detected in the brain. Some difference in O^6-EtG contents of various organs such as the liver, brain, testis and bone marrow was also found by others at 2 h after ^{3}H-ENU treatment (21,29). This difference, together with the inter-animal variation we found, may be ascribed to the route of administration: the local distribution of ENU in the abdominal cavity after administration of the chemical, may play a role. This effect was in part accounted for in our studies, when O^6-EtG values were normalized to the N7-EtG level determined in the total genomic DNA.

The role of cell proliferation should also be considered as the levels of alkylation products in the DNA of replicating cells will be diluted. Cell proliferation in the liver of *lacI* transgenic mice is low with approximately 0.07% of the hepatocytes taking part in the cell-division process (30). Had excessive cell proliferation been induced as a result of ENU treatment, a reduction in adduct levels would have been expected due to DNA replication. However, we found that in the liver approximately 54% of N7-EtG was lost after 72 h compared to 90% of O^6-EtG. With the chemical half-life of N7-EtG, *in vitro*, being ≈. 150–225 h (30), about 20% of the N7-EtG initially formed is lost owing to the inherent instability of this alkylation product in DNA. Therefore, 34% (54–20%) of the N7-EtG must be removed through repair by DNA glycosylases (31) or diluted by cell-proliferation. As O^6-EtG is much more chemically stable in DNA than N7-EtG, the loss of this lesion (more than 90% within 72 h) should be predominantly attributed to repair, whereas the proliferation effect must have been relatively small.

The results presented here indicate that the initial repair (during the first 1.5 h after ENU-treatment) of O^6-EtG in the liver is higher in total genomic DNA than in *lacZ* sequences. Thereafter, further repair of the transgene does not appear to be different from that seen in total genomic DNA. In the brain, no repair of O^6-EtG was detected in either genomic or *lacZ* DNA although brain cells are proficient in removing N7-EtG and contain AGT (data not shown). It is noteworthy to mention that in both the liver and brain DNA, when the O^6-EtG content in genomic and *lacZ* DNA was expressed as O^6-EtG/10^6 nucleotides, it was evident that at all time points after treatment (except for the O^6-BzG pretreated mice) the level of O^6-EtG was slightly but insignificantly lower in genomic DNA compared to *lacZ* DNA. This is due to the difference in GC-content between mouse genomic DNA (40.3% GC) and *lacZ* transgene DNA (56% GC). This insignificant difference is absent after correction for this difference in GC-content.

These findings are of importance to the use of these mice for mutagenicity testing. Since the *lacZ* sequences present in the shuttle vectors is used as an indicator target in which mutations are scored, it is essential that transgene and genomic DNA are similar with regard to formation and repair of DNA adducts. As observed in the present study, the difference between transgene DNA and genomic DNA appears to be restricted to a somewhat different initial O^6-EtG level (i.e. a lower level of O^6-EtG in *lacZ*, the effect of which is mitigated by less efficient repair) at a very early stage in the process of mutation induction. As the fixation of mutations is most likely to occur after the 1.5 h time point, only a small fraction of replication-competent cells are in S-phase during this time period, mutation analysis in the *lacZ* DNA from the livers of 40.6 mice probably provides data that are significant for the vast majority of the genomic DNA where O^6-EtG-mediatied mutations are concerned. Further studies may be necessary to determine the repair of mutagenic lesions in the *lacZ* DNA in comparison to transcriptionally active DNA sequences. Studies are presently being carried out to investigate the repair profiles of DNA lesions in the *lacZ* DNA that are not repaired by AGT.

Acknowledgements

Research supported by Dr Mildred Scheel-Stiftung für Krebsforschung (J.T., M.F.R.). Part of this research was funded by the European Communities (Contract no. EV5V-CT93–0246).

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Received on February 19, 1996; revised on July 18, 1996; accepted on July 26, 1996