Direct assay for *O*⁶-methylguanine-DNA methyltransferase and comparison of detection methods for the methylated enzyme in polyacrylamide gels and electroblots

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We describe in detail a direct assay for the substrate-inactivated DNA-repair enzyme, *O*⁶-methylguanine-DNA methyltransferase (*O*⁶-MT), which measures the transfer of radiolabelled methyl groups from a prepared *O*⁶-methylguanine-DNA substrate to the protein fraction of an enzyme-containing cell/tissue extract. This assay, a modification of a previously suggested method for monitoring *O*⁶-ethylguanine-DNA repair [Renard, Verly, Mehta & Ludlum (1983) *Eur. J. Biochem.* **136**, 461–467], is sensitive, highly reproducible, accurate and is, as described here and relative to previously published methods, well suited for use with a large number of test samples. We identified two problems with the *O*⁶-[*Me*-³H]methylguanine-DNA substrate used in the present work and in other reported assays: firstly, that of progressively higher assay backgrounds with increasing age of substrate, which was nullified by once-only purification of the double-stranded substrate by hydroxyapatite chromatography; secondly, a substrate of high specific radioactivity (30 Ci/mmol), made with freshly prepared tritiated methylnitrosourea, behaved as a substrate of 5 Ci/mmol when referenced against radiolabelled *O*⁶-methylguanine-DNA made with either [³H]- or [¹⁴C]-methylnitrosourea at the lower specific radioactivities of 1 Ci/mmol and 61 mCi/mmol respectively. This apparently stemmed from the known instability of high-specific-radioactivity [³H]methylnitrosourea and indicated that an expected increase in sensitivity of the assay does not necessarily result from increasing the specific radioactivity of substrates above approx. 1 Ci/mmol. Although *O*⁶-MT was stable to preincubation at 25 °C, marked losses of activity were observed at 37 °C, and more so at 45 °C. Enzyme lability at the higher temperatures was not, however, seen during preincubation in the presence of its substrate, *O*⁶-[*Me*-³H]methylguanine-DNA, which apparently protected *O*⁶-MT against thermal inactivation. As previously seen with other human cells and tissues, extracts of human spleen in the present study showed wide inter-individual differences in *O*⁶-MT specific activity (18-fold), which spanned the range 50–900 fmol/mg of protein. Cultured human lymphoblastoid Jurkat cells contained approx. 57000 enzyme molecules/cell. Substrate-inactivated [*Me*-³H]methylated *O*⁶-MT was analysed by SDS/PAGE and electroblotting. The different but similarly sized forms of this enzyme that we previously detected in human spleen [Major, Gardner, Carne & Lawley (1990) *Nucleic Acids Res.* **18**, 1351–1359] were clearly resolved by fluorography of electroblots, but only at considerable expense of time. As expected, scintillation counting of the protein extracted from gel slices and linear-wire scanning of enzyme-associated radioactivity on electroblots were quicker methods for detecting the [*Me*-³H]methylated inactivated *O*⁶-MT.

INTRODUCTION

Several sites on DNA are modified after reaction with a methylating carcinogen, but of these, *O*⁶-methylguanine is generally considered to be the most important because of its potential for miscoding [1]. The presence of *O*⁶-methylguanine in DNA is positively correlated, in animal models, with both tumour and autoimmune-disease induction [2], and a point mutation of the type expected from *O*⁶-methylguanine was found to cause the malignant activation of the cellular *H-ras* oncogene in methylnitrosourea (MNU)-induced rat mammary tumours [3].

A repair activity for *O*⁶-methylguanine was first described in *Escherichia coli* by Lawley & Orr [4], and subsequently in most species examined (for reviews, see refs. [5–7]). *O*⁶-Methylguanine repair is performed by the enzyme *O*⁶-methylguanine-DNA methyltransferase (*O*⁶-MT; DNA-*O*⁶-methylguanine:protein-L-cysteine *S*-methyltransferase, EC 2.1.1.63), which acts by transferring the methyl group from guanine to an internal cysteine

residue, thereby restoring native guanine in DNA. The resultant formation of *S*-methylcysteine causes the inactivation of this protein, which thus acts only once, and is not regenerated [8,9].

In addition to the repair of pre-mutagenic DNA lesions caused by methylating carcinogens, increased *O*⁶-MT activity is positively correlated with tumour-cell resistance to alkylating chemotherapeutic agents such as the chloroethylnitrosoureas [7], and decreased enzyme activity with the presence of autoimmune disease in humans [10,11]. In view of its importance and the large level of interest shown in this DNA lesion, many methods have been developed for measuring its repair (for a recent review, see ref. [7]). These methods can be divided into two groups [7]: (a) indirect assays, which measure the removal of *O*⁶-methylguanine, either from the DNA of intact cells following their pretreatment with a methylating carcinogen or from a prepared DNA substrate containing *O*⁶-methylguanine, after reaction with enzyme-containing cell/tissue extract; (b) direct assays, which measure the transfer of radiolabelled methyl groups from a prepared *O*⁶-

Abbreviations used: *O*⁶-MT, *O*⁶-methylguanine-DNA methyltransferase; MNU, methylnitrosourea; CTAB, cetyltrimethylammonium bromide; PBS, phosphate-buffered saline (120 mM-NaCl/2.7 mM-KCl/10 mM-sodium phosphate, pH 7.4).

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methylguanine-DNA substrate to the protein fraction of an enzyme-containing cell/tissue extract (direct assays are not performed with intact cells, as methylating agents are able to alkylate protein as well as DNA).

In indirect assays, *O*⁶-methylguanine removal is usually monitored by h.p.l.c. and/or immunoassay of acid-hydrolysed reaction product DNA [7]. In an attempt to improve sensitivity, several indirect assays have been described which use ³²P-labelled oligodeoxynucleotides containing *O*⁶-methylguanine, where conversion of the methylated into the non-methylated substrate was monitored by h.p.l.c. [12,13] or, more recently, by immunoassay [14,15]. Two substantial problems that need to be overcome before oligonucleotide substrates find general suitability for use in the measurement of *O*⁶-methylguanine repair are, firstly, that extracts prepared from some tissues (colon, spleen and small intestine have thus far been identified) may cause the rapid degradation of the oligodeoxynucleotide substrate [13]. Secondly, the rate of repair increases with increasing oligonucleotide length and is dependent on the nucleotide composition of the substrate [12,13,16]. Additional disadvantages are that oligonucleotide substrates are not generally available, the use of ³²P-labelled oligonucleotide and the short half-life of ³²P necessitating frequent resynthesis of the labelled substrate, and that separation of oligonucleotide product from substrate by h.p.l.c. is relatively laborious and time-consuming [13].

In direct assays, two different approaches have been used to determine radiolabelled methylated protein after its separation from repaired DNA. The first involves either co-precipitation of protein and DNA, followed by either proteinase solubilization of protein and associated radioactivity [17,18] or removal of DNA by acid hydrolysis and repeated washing [19]. The second approach utilizes selective retention of radiolabelled protein on glass-fibre filters after hydrolysis of DNA [20].

All methods described for *O*⁶-methylguanine repair, both direct and indirect, are generally time-consuming and laborious when handling many samples simultaneously. In this paper we report, in detail, a relatively quick and easy method for the direct measurement of *O*⁶-MT, which is a modification of a method originally proposed for the measurement of *O*⁶-ethylguanine repair in tissue extracts [21], but one whose subsequent use by other groups has not been reported. This assay utilizes fewer steps and, as described here, is more suited for use with large numbers of tests than previously reported direct enzyme assays. We recently reported a preliminary description of this assay in work leading to the purification to homogeneity of *O*⁶-MT from human spleen [22]. The present report provides a more detailed account of the assay method and its use. We also present a comparison of methods for the detection of [*Me*-³H]methylated inactivated *O*⁶-MT in SDS/polyacrylamide gels and electroblots.

MATERIALS AND METHODS

Reagents

[*Me*-³H]MNU and [*Me*-¹⁴C]MNU, at specific radioactivities of both 1 and 30 Ci/mmol for the tritiated compounds and 61 mCi/mmol for the ¹⁴C-labelled material, and ¹⁴C-labelled protein *M_r* markers were purchased from Amersham International. High-*M_r* calf thymus DNA and proteinase K (type XI) were from Sigma. Except for reagent-grade EDTA and cetyltrimethylammonium bromide (CTAB), CaCl₂ and all inorganic solvents were purchased as A.R.-grade materials from BDH. High-purity dithiothreitol was from Calbiochem, and Ultrapure-grade Tris and (NH₄)₂SO₄ were from Gibco-BRL. All electrophoresis reagents and prestained and unmodified protein

M_r markers were from Bio-Rad. All other reagents (of analytical grade) and enzymes were from Sigma.

Cells and preparation of extracts

Jurkat cells, a human lymphoblastoid T-cell line, were grown in suspension culture at 37 °C in RPMI-1640 medium containing 10% (v/v) foetal-calf serum. Exponentially growing cells were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS). Broken whole-cell extracts were prepared by disrupting a pellet of 2 × 10⁷ cells in 1 ml of 50 mM-Tris/HCl (pH 8.3)/0.5 mM-EDTA/1 mM-dithiothreitol (buffer A) on ice with a small Dounce homogenizer (5 ml barrel) and 20 strokes of a tight-fitting pestle. The homogenate was centrifuged at 10000 *g*_{max} for 30 min at 4 °C, and the resulting supernatant was removed and kept. The pellet was resuspended as above by homogenization in a further 1 ml of the same buffer, then sonicated for 30 s. The sonicated extract was centrifuged as above, to produce a second supernatant, which was pooled with the first supernatant and stored frozen at -75 °C in batches (if not used straight away). Extracts stored in this manner did not lose *O*⁶-MT activity over several months, but did so if repeatedly thawed and refrozen.

Human spleen extracts

Human spleen samples from patients undergoing splenectomy for idiopathic thrombocytopenia purpura or Hodgkin's or non-Hodgkin's lymphoma were obtained by courtesy of Dr. R. L. Carter, Department of Pathology, Royal Marsden Hospital, Sutton, U.K., and usually within 30 min of organ removal. The spleens were sectioned, frozen on solid CO₂ for transportation to the laboratory, then stored at -75 °C if not used straight away. By a slight modification of the method of Pegg *et al.* [9], whole tissue extracts were prepared, working on ice as far as possible, by blending 2 g of frozen spleen in 10 vol. of buffer A, for two 20 s periods separated by a 5 s pause, using a Virtis 23 homogenizer (Virtis, New York, NY, U.S.A.) at full speed, or until a smooth homogenate was obtained (usually within 1 min). The homogenate was centrifuged at 12000 *g*_{max} for 20 min at 4 °C, and the supernatant produced was harvested and kept. The homogenate particles were resuspended in 5 vol. of the same buffer by Dounce homogenization using 20 strokes of the loose pestle in a large barrel (30 ml), then sonicated for 2 × 30 s, separated by a 30 s interval. The sonicated extract was centrifuged at 31000 *g* for 30 min at 4 °C, and the resulting supernatant was pooled with the first supernatant to produce a crude extract. *O*⁶-MT activity was concentrated in this extract by precipitation with (NH₄)₂SO₄ at a final saturation of 80%. Precipitated protein, which contained all of the *O*⁶-MT extract activity, was collected by centrifugation at 31000 *g* for 30 min at 4 °C. The resulting pellet was redissolved in 1 vol. of buffer A, and dialysed extensively over 24 h against the same buffer. The concentrated dialysed crude extract was stored in batches at -75 °C, and, similarly to the above cell extracts, displayed no appreciable loss of *O*⁶-MT activity upon storage, with the exception of repeated thawing and refreezing, which caused about a 10% decrease in enzyme activity through each cycle of thawing and refreezing.

Extracts were also made on a much larger scale, from 70 g of human spleen, for previously described *O*⁶-MT purification work [22]. These extracts were prepared similarly to the above, except that *O*⁶-MT activity was concentrated and partially purified by precipitation with (NH₄)₂SO₄ at a final saturation of 50%.

Radiolabelled methylated DNA substrate

DNA containing *O*⁶-methylguanine with a radiolabel in the

methyl group was prepared for use as a substrate in the direct assay of *O*⁶-MT by the method of Demple *et al.* [23] and, briefly, as follows: high-*M_r* calf thymus DNA was methylated by reaction at 37 °C for 1 h at pH 7.5 with either [*Me*-¹⁴C]MNU or [*Me*-³H]MNU, then heated at 80 °C for 16 h at pH 7.1 to liberate *N*-methylpurines and enrich the proportion of radioactivity associated with *O*⁶-methylguanine in the DNA. Liberated *N*-methylpurines and other low-*M_r* compounds were removed by dialysis and the substrate was prepared for use in the direct enzyme assay by making the final dialysis for 16 h at 4 °C against 200 vol. of buffer A, changed twice. Substrate DNA was subjected to methylpurine analysis by cation-exchange h.p.l.c. (after hydrolysis of the DNA in 0.1 M-HCl) by the method of Lawley *et al.* [10], and was routinely found to contain 55–75 % of its radioactivity in association with *O*⁶-methylguanine.

A problem that we encountered in the use of this substrate for the direct assay of *O*⁶-MT (see below) was that assay background increased as a function of age of substrate. This problem, which caused a progressive decrease in assay sensitivity and precision, was not satisfactorily resolved by either regular precipitation of the substrate with ethanol or storage under a variety of different conditions, but was completely obviated by the use of the following modification of a standard method for the purification of high-*M_r* DNA by hydroxyapatite column chromatography [24] to generate purified double-stranded methylated DNA substrate. DNA-grade hydroxyapatite (20 g) from Bio-Rad was de-fined according to the manufacturer's instructions, equilibrated in 10 mM-sodium phosphate buffer, pH 6.8 (buffer B), and packed in a Pharmacia XK 26 column. After dialysis according to Demple *et al.* [23] above, the DNA substrate was dialysed against buffer B and applied to the column at a flow rate of 1.0 ml/min. The column was washed with 50 ml of buffer B, then eluted with a 10–480 mM-sodium phosphate buffer, pH 6.8, gradient (360 ml), at 22 °C using the same flow rate. The collection of column eluate, in 10 ml fractions, was commenced after sample application, 12 min into the column wash with buffer B. Fig. 1 shows that double-stranded substrate DNA, which comprised about 70 % of the radioactivity, was eluted by 300 mM-sodium phosphate, whereas the remainder of the applied radiolabelled material appeared in the initial column-wash eluate (approx. 20 %; not shown in Fig. 1), and in a presumably single-stranded methylated DNA peak that was eluted ahead of the double-stranded material (approx. 10 %). Double-stranded DNA substrate was dialysed overnight at 4 °C against 200 vol. of buffer A and then stored frozen in batches at –75 °C. Thus we now routinely purify our DNA substrate in this manner, after the 80 °C heating step. Once purified and stored in this manner, the substrate is stable and requires no further pretreatment before its use in the methyltransferase assay.

Direct assay for *O*⁶-MT

*O*⁶-MT activity was measured by a modification of a previously suggested, but mostly uncharacterized, method for monitoring the activity of an enzyme in rat liver chromatin that repairs *O*⁶-ethylguanine in DNA [21]. The assay, which is an adaptation of Schneider's method [25] for the measurement of DNA after its precipitation by CTAB in the presence of proteinase K, the latter removing proteins in association with DNA, monitors the transfer of radiolabelled methyl groups from the *O*⁶ atom of guanine in DNA to methyltransferase protein.

The assay incubation mixture contains enzyme extract made to 200 μ l with buffer A and 10 μ l of double-stranded radiolabelled methylated DNA substrate (routinely used, unless otherwise indicated, at 2000–5000 d.p.m./10 μ l). Although the majority of the repair reaction occurred within the first 5 min (see Fig. 2), assay incubations were performed, unless otherwise indicated, in

Fig. 1. Purification of *O*⁶-[*Me*-³H]methylguanine-DNA assay substrate by hydroxyapatite chromatography (f.p.l.c.)

See the Materials and methods section for further details. The *A*₂₅₄ trace for this procedure (not shown) closely followed the ³H (d.p.m.) trace, especially with regard to relative peak abundance. ●, ³H radioactivity; ----, sodium phosphate gradient.

a water bath at 37 °C for 90 min to ensure completeness of reaction. EDTA, pH 6.0 (300 μ l of 80 mM concentration) containing 200 μ g of calf thymus DNA was mixed, on ice, with each test. Addition of carrier DNA/EDTA was found to stop the enzyme reaction effectively. Total DNA, including repaired and any residual substrate, was then separated from methylated radiolabelled protein by precipitation of the former with the addition and mixing of 200 μ l of 3 % CTAB. Radiolabelled protein remaining in association with DNA (variably between 6 and 15 % of total methylated protein produced) was solubilized during a subsequent incubation with 10 μ l of freshly prepared 1 mM-CaCl₂ containing 50 μ g of proteinase K (Sigma type XI; DNAase-free) for 60 min at 37 °C. DNA was then pelleted by centrifugation at 15000 *g* for 15 min in a microcentrifuge, and radioactive methyl groups associated with protein were determined on a 650 μ l sample of the resultant supernatant, which was added to 2 ml of water, mixed with 10 ml of Pico-Aqua scintillation cocktail (Canberra-Packard) and counted for radioactivity in a scintillation counter to provide a measurement of d.p.m. A 'blank' assay reaction containing no enzyme extract produced about 45 d.p.m., approx. 1 % of the substrate radioactivity in an assay test. This value was subtracted from test d.p.m. measurements to give 'net d.p.m.'.

Performance of this assay was greatly facilitated by the use of multiple-sample dispensing pipettes (which hold reagent in a reservoir and dispense precise volumes repetitively) for the addition of substrate DNA, carrier DNA, CTAB and proteinase K. In this manner, several hundred assay tests can be performed and prepared for radioactivity measurement by one operator in a day.

Protein assay

Protein was determined by the method of Bradford [26], using a Bio-Rad protein assay kit and BSA as a calibrating protein standard.

SDS/PAGE, electroblotting, fluorography and gel slicing

Tritiated methylated O^6 -MT, which was prepared by incubation of cell or tissue extracts with O^6 -[Me- 3 H]methylguanine-DNA substrate as for the assay of the enzyme described above, was subjected to SDS/PAGE by the method of Laemmli [27] using 15% polyacrylamide gels. After electrophoresis, gels were electroblotted on to nitrocellulose (0.1 μ m, from Schleicher and Schuell) by the method of Towbin *et al.* [28], by using a 20 h electrotransfer at 4 °C, but half the suggested strength of transfer buffer to lessen heating. Radiolabelled protein bands on electroblots were detected by fluorography by the method of Roberts [29] and indicated exposure times to Fuji RX film at -75 °C. This film was pre-exposed to filtered flashlight to an absorbance of 0.4 at 540 nm, to increase the sensitivity of 3 H detection [29]. In some experiments, test fractions were duplicated in a gel which, after electrophoresis, was sectioned into two halves containing the identical tests. One-half was electroblotted as above, and test lanes in the remaining gel half were sectioned into 3 mm slices. Radiolabelled protein was extracted from these slices and measured by scintillation counting essentially as described previously [30].

Linear-wire detection of radiolabelled protein on electroblots

After electroblotting and Amido Black protein staining [28], radiolabelled protein bands were detected on blots by using a model 200 imaging scanner, kindly on loan from its manufacturers, Canberra-Packard. This scanner, which uses an argon-flushed single-wire detector for radioisotope β -particle counting, was found to be suitable for the detection of radiolabelled proteins bound to the surface of nitrocellulose membranes and was used as follows. Air-dried blots were flattened by being clamped between two glass electrophoresis-gel casting plates and heated in a fan-assisted oven at 80 °C for 60 min. The flattened blot was taped on to the surface of one of the plates and positioned under the linear-wire detector head. As the 3 H β -decay particle displays a relatively small penetrance in air, we used the detector head without the removable aperture plate and with the detector wire in actual contact with the nitrocellulose. The detector head, which views a lane of up to 20 cm in length, was manually positioned so that the detector wire was at right-angles to, and lay over the centre of, stained protein bands on the blot. Detection was performed for indicated times, and the position of detected radioactivity was computed by the System 200's built-in microprocessor, which also accumulates data during the analysis. Hard-copy graphical output to a printer was produced on a 1:1 scale with the actual sample size, which facilitated location of the radiolabelled material on the blot. In an initial trial to assess the sensitivity of the imaging scanner for 3 H detection, we applied, in approx. 4 mm-diameter spots dried on to a nitrocellulose membrane, graded amounts of tritiated protein, and found that we were able to detect as little as 100 d.p.m. (total amount in one spot) in 30 min.

RESULTS AND DISCUSSION

Assay validation

We describe a direct assay for O^6 -MT activity in cell and tissue extracts which monitors the transfer of radiolabelled methyl groups from the O^6 atoms of guanine in DNA to protein. As similarly found by others using different enzyme assays [9,12,15,17,30-33], this transfer at 37 °C is rapid with, in the present study, 50% of the reaction against double-stranded substrate occurring within the first 1 min of incubation. The single-stranded form of this substrate displays a much lower rate of reaction with O^6 -MT [9,34,35], the human spleen enzyme

taking 4 h to reach near-completion (Fig. 2). Fig. 3(a) shows an enzyme reaction to completion with a fixed level of substrate, and that, above a certain level of input enzyme, no further methylated protein is produced. Conversely, in the presence of a fixed amount of enzyme, reaction saturation occurs above a certain level of substrate (Fig. 3b), both observations indicating the stoichiometric nature of the DNA-repair reaction. Fig. 3(b) also shows that substrate concentration has little, if any, effect on the rate of repair and that the enzyme reaction is nearly complete by 60 min.

In the present study, human spleen extracts showed wide inter-individual differences in enzyme specific activity (18-fold) which spanned the range 50-900 fmol/mg of protein (700 fmol/mg of protein for the extract depicted in Fig. 3). Similarly large inter-individual variation in extractable O^6 -MT, of overlapping specific activity, was previously observed for human small intestine (40-fold [36]), melanoma biopsy material (20-fold [19]) and peripheral blood lymphocytes (30-fold [37]), and to a lesser extent for human colon and liver (10-fold and 8-fold respectively [36]), bone-marrow T-lymphocyte and myeloid precursor cells (8-fold [33]) and lung (5-fold [38]). That such variation with regard to human spleen from different patients was in any way due to differences in enzyme extraction efficiency was ruled out by our finding of low variation of O^6 -MT specific activity in extracts prepared from separate samples of the same spleen on either the same or different days (G. N. Major & E. J. Gardner, unpublished work).

To ascertain that the direct assay monitored the selective transfer of methyl groups to protein from O^6 atoms of guanine, methylated DNA was analysed for methylpurines by cation-exchange h.p.l.c. after prolonged (90 min) incubation in the presence of excess enzyme. Fig. 4 shows that, under the conditions of this assay, selective removal of 72% of O^6 -methyl groups from guanine in substrate DNA had occurred and the majority of

Fig. 2. [3 H]Methyl group transfer to protein as a function of time in the presence of double-stranded (●) or single-stranded (▲) O^6 -[Me- 3 H]methylguanine-DNA assay substrate

See the assay method for further details.

Fig. 3. [³H]Methyl group transfer to protein as a function of (a) added protein and (b) time and added *O*⁶-[Me-³H]methylguanine-DNA assay substrate

Spleen extract (50 μl) was used in each test in (b) and incubated with 3290 (●), 1650 (■) or 660 (▲) d.p.m. of substrate. See the assay method for further details.

Fig. 4. Cation-exchange h.p.l.c. of *O*⁶-[Me-³H]methylguanine-DNA assay substrate incubated, under conditions of the *O*⁶-MT assay, in either the presence (■) or absence (●) of 200 μl of human spleen extract for 90 min at 37 °C

The major peak at fraction 13 co-migrated with marker *O*⁶-methylguanine. See the accompanying text, the Materials and methods section and ref. [10] for further details.

Fig. 5. (a) Effect of preincubation time and temperature on *O*⁶-MT activity and (b) [³H]methyl group transfer to protein (*O*⁶-MT activity) as a function of time and temperature

(a) Preincubation of 50 μl of human spleen extract, in standard assay buffer not containing *O*⁶-[Me-³H]methylguanine-DNA assay substrate was performed for the indicated times at 25 °C (●), 37 °C (■) or 45 °C (▲), before immediate assay of each test. (b) Spleen extract (50 μl) was incubated under conditions of the enzyme assay, for the indicated times, but at three different temperatures, as in (a).

other methylated products detected in DNA were unchanged. The amount of [³H]methyl groups removed from the *O*⁶ atom of guanine was identical with the amount of radioactivity transferred to the protein fraction. The repair-resistant fraction (28 %) of *O*⁶-methyl groups that were not transferred to protein, previously seen by others [31,39], might represent *O*⁶-methylguanine in association with the high concentration of single-stranded DNA that precedes replication forks [5,34].

As a check of *O*⁶-MT stability during the assay incubation, we investigated the effect on activity of preincubation of the enzyme (in the absence of substrate DNA) before assay. Enzyme preincubation at 25 °C for 120 min had no effect, but marked losses of *O*⁶-MT activity with time were observed at 37 °C and more so at 45 °C (Fig. 5a), indicating that the previously seen decrease in methyl-group transfer to protein with time and in the presence of excess substrate, at 37 °C (Fig. 2), may have occurred as a result of enzyme inactivation. In conflict with this, however, was our subsequent observation in the absence of enzyme preincubation of no difference in the amount of methylated *O*⁶-MT produced at any of these temperatures, under conditions of the assay and in the presence of excess DNA substrate, although initial rates of methyl transfer increased with increasing temperature (Fig. 5b). Taken together, these observations indicate that, although human spleen extract *O*⁶-MT alone is labile at its assay temperature, this is not the case in the presence of substrate DNA, which apparently protects the protein against inactivation, suggesting that, in the course of its reaction with

O^6 -methylguanine, O^6 -MT binds to DNA and that this binding is essential for maintenance of its active conformation at physiological temperatures.

We excluded the possibility that extract nuclease activity was contributing to the appearance of CTAB-soluble radioactivity through its degradation of substrate DNA, by substituting radiolabelled DNA not containing O^6 -methylguanine for the usual substrate in the enzyme assay and finding that such control incubations with O^6 -MT-containing extract produced no radioactivity in the final assay supernatant (results not shown). Absence of assay interference by nuclease activity is also suggested by the finding that an enzyme reaction to completion in the presence of excess substrate displayed saturation and selective removal of O^6 -methyl groups.

The accuracy of this assay for O^6 -MT is highlighted by our recently reported use of it in work leading to the isolation in pure form and determination of amino acid sequence from the active site of the enzyme from human spleen [22].

Assay sensitivity and precision

The stoichiometric reaction between O^6 -MT and O^6 -methylguanine in DNA, and knowledge of the specific radioactivity of the MNU used to prepare the DNA substrate, permit the calculation of enzyme molar amount and the number of O^6 -MT molecules (assuming one active site per molecule), using data from the linear portion of an enzyme-activity dilution curve, from an enzyme assay performed to completion in the presence of excess substrate DNA. With substrate prepared from [Me - 3H]MNU at 1 Ci/mmol, a usual specific radioactivity for this compound from commercial suppliers, the present assay was able to measure reliably a minimum of about 80 d.p.m. (gross; about twice the assay background) of [Me - 3H]methylated protein, which corresponds to about 36 fmol of O^6 -MT. For Jurkat cells, this corresponds to about 10 μ l of extract and 3.8×10^5 cells, and a value of about 57000 enzyme molecules/cell. This result is similar to the previously found figure of approx. 65000 molecules/human lymphocyte [10] and indicates that, by using the present method for preparing cell extracts and enzyme assay, O^6 -MT may be measured in very small fractions of extracts of isolated lymphocytes, prepared from about 6 ml of human blood (assuming an average 2.5×10^6 lymphocytes/ml of adult blood).

We attempted to improve the sensitivity of the direct assay by purchasing, at significant expense, a custom synthesis of [Me - 3H]MNU at the higher specific radioactivity of 30 Ci/mmol for the preparation of substrate DNA. Freshly prepared [Me - 3H]MNU was used to make substrate, but we harboured doubts about the stability of this material and thus investigated its suitability. We compared the separate use of 30 and 1 Ci/mmol [Me - 3H]methylated DNA and 61 mCi/mmol [Me - ^{14}C]methylated DNA (results not shown) and we found that the level of O^6 -MT measured with the 30 Ci/mmol substrate was one-sixth that determined with either of the two lower-specific-radioactivity substrates (i.e. it behaved as a 5 Ci/mmol substrate). We have observed lower than expected substrate specific radioactivity with two separate [Me - 3H]MNU custom syntheses. Thus, for the sake of accuracy and unless the prior validation of a higher-specific-radioactivity substrate has been performed, it appears preferable to use tritiated DNA substrate with a [3H]methyl group specific radioactivity of about 1 Ci/mmol. The sensitivity of the direct assay is also a function of assay background and, as outlined in the Materials and methods section, this was kept low by the use of a double-stranded DNA substrate prepared by hydroxyapatite chromatography.

Intra-assay precision was assessed on many occasions, and, in each instance, measurements of enzyme amount (from data near the middle of replicate extract-dilution curves, as seen in Fig. 3),

Fig. 6. Comparison of detection methods for [Me - 3H]methylated substrate-inactivated Jurkat-cell O^6 -MT on nitrocellulose electroblots of polyacrylamide gels run with the enzyme

Radiolabelled O^6 -MT, produced by allowing 30 μ l of cell extract to react with O^6 -[Me - 3H]methylguanine-DNA substrate, was subjected to SDS/PAGE and electroblotting as described in the Materials and methods section. The fluorogram (a) produced from a 2-week exposure to X-ray film, and the linear-wire scanner radioactivity trace (b) from a 40 min scan, are on the same scale. SDS/PAGE tests: (a)i, [Me - 3H]methylated enzyme incubated with 50 μ g of proteinase K (added in 10 μ l of freshly prepared 1 mM- $CaCl_2$) for 30 min at 37 $^{\circ}C$ before electrophoresis, and (a)ii, non-proteinase K-digested [Me - 3H] O^6 -MT; (b) linear-wire detection of radioactivity associated with lane (a)ii. Abbreviations: O, origin; d.f., dye front. See the Materials and methods section and the accompanying text for further details.

displayed standard deviations within $\pm 2\%$. Inter-assay precision in the determination of enzyme amount on different days gave a maximum standard deviation of $\pm 6\%$.

Detection of methylated O^6 -MT after SDS/PAGE and electroblotting

As a preliminary to comparative physicochemical studies of the methylated repair protein from a variety of different tissues, we evaluated several methods for the detection of substrate-inactivated [Me - 3H]methylated O^6 -MT after electrophoresis. Detection of [Me - 3H] O^6 -MT on nitrocellulose blots by fluorography was a lengthy procedure, with exposure times against X-ray film being 2–3 weeks for enzyme prepared from '30 Ci/mmol' (probably 5 Ci/mmol; see above) methylated DNA (Fig. 6a) and 3–4 months with 1 Ci/mmol substrate (Fig. 7a). In the latter instance, and owing to the long exposure time, fluorograms of only poor quality were obtained. As expected, methylated enzyme made from the lower-specific-radioactivity substrate was, however, more rapidly detected either by linear-wire scanning the surface of an electroblot using the Bioscan model 200 radioactivity imaging equipment (Fig. 6b) or by scintillation-counting of protein-extracted PAGE gel slices (Fig. 7b). Results were obtained by the gel-slicing procedure in less than 18 h after

Fig. 7. Detection of the different forms (see ref. [22]) of methylated substrate-inactivated *O*⁶-MT in human spleen after SDS/PAGE

*O*⁶-[Me-³H]Methylguanine-DNA substrate was allowed to react with 30 μ l of human spleen extract to produce radiolabelled enzyme which, after SDS/PAGE, was detected in either (a) a fluorogram made from a gel electroblot or (b) gel slices, after protein extraction and scintillation counting (see the Materials and methods section and the accompanying text for further details). The fluorogram (a) and diagrams in (a) and (b) are on the same scale. Fluorography required a 90-day exposure of X-ray film to the electroblot. This long exposure time produced a fluorogram of barely suitable quality for photographic reproduction, hence the inclusion in (a) of a schematic representation of the faint enzyme bands originally seen. ¹⁴C-labelled *M_r* calibration proteins (indicated by arrows): myosin (200 000), phosphorylase *b* (92 500), BSA (69 000), ovalbumin (46 000), carbonic anhydrase (30 000), trypsin inhibitor (21 400) and lysozyme (14 300). Abbreviations: O, origin; d.f., dye front. Recoveries of labelled protein in gel slices routinely ranged between 26 and 35 %.

electrophoresis, and by linear-wire detection in under 2 h after blotting.

By all of these detection methods, and in the majority of our different human spleen and Jurkat-cell enzyme extracts, [Me-³H]*O*⁶-MT was detected as a protein band (abolished by treatment of the enzyme sample with proteinase K before electrophoresis) of *M_r* 24 000 (e.g. Fig. 6a). This finding is in agreement with the generally observed *M_r* of the methylated mammalian enzyme by SDS/PAGE. In contrast with this, we previously reported methylated enzyme forms made from human spleen extracts of *M_r* both higher and lower than 24 000 [22]. An example of the latter, which was obtained by using enzyme from a large-scale human spleen extract (see the Materials and methods section), in which minor enzyme forms of *M_r* less than 24 000 are relatively more abundant than in extracts prepared on a smaller scale [22], is shown in Fig. 7. These minor forms were only resolved by fluorography (using a 90-day X-ray film exposure time), and were not discernible by the gel-slicing approach, emphasizing the greater resolving power of fluorography for visualizing closely migrating protein bands in electrophoresis, albeit at the expense of time. In this regard we were not in a position to compare

fluorography with linear-wire detection, as the equipment associated with the latter was on loan to us for a limited period.

Similarly sized but different molecular forms of the methylated mammalian enzyme have been detected by fluorography of electroblots after SDS/PAGE in only one other study, and were thought to reflect the presence of precursor molecules of *O*⁶-MT in cells [40]. This possibility, and that of multiple catalytically active enzyme forms being generated by endogenous proteinase action, which have previously only been demonstrated from *E. coli*, require further investigation.

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