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Properties of the chromatin repair activity against O^6-ethylguanine lesions in DNA

Mechanism of the reaction

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Chromatin proteins from rat liver contain a repair activity that removes O^6-ethylguanine from ethylnitrosourea-treated DNA. This activity does not depend on divalent cations and works in the presence of EDTA, but does depend on the presence of free thiol groups. Thus, it is destroyed by *N*-ethylmaleimide and is protected by dithiothreitol. The repair activity on single-stranded DNA is only 20 % of what it is on double-stranded DNA; its half-life at 35 °C is 55 min, but DNA, ethylated or not, affords some protection.

The repair reaction is a transethylation from O^6-ethylguanine in DNA onto two different cysteine residues contained in acceptor proteins. The reaction can be followed by monitoring the appearance of ethylated proteins or by disappearance of O^6-ethylguanine from DNA.

In the preceding paper [1], we have shown that proteins prepared from rat liver chromatin induce the disappearance of O^6-ethylguanine (e^6Gua) from DNA. The e^6Gua did not appear in the incubation medium as a free base nor was it excised in an oligonucleotide.

In this work, we have studied some properties of the repair activity. We have shown that the ethyl group of the DNA e^6Gua base is transferred onto cysteine residues of acceptor proteins. Similar observations with bacterial proteins [2, 3] or proteins prepared form total cellular extracts from rat liver [4], mouse liver [5] or human liver [6] have been published. Several preliminary papers describing our work have also appeared [7–9, 9a].

MATERIALS AND METHODS

Materials

Calf thymus DNA was purchased from Sigma; proteinase K from Boehringer; hydroxyapatite (DNA grade) from Bio-Rad; thiopropyl-Sepharose, Sephacryl S200 and Sephadex G-150 from Pharmacia Fine Chemicals; Lichrosorb RP18 (5 μm) HPLC columns from Merck; and Ultrasphere ODS (5 μm) HPLC columns from Altex (Beckman). [^{3}H]Ethylnitrosourea was synthesized at a specific activity of 3–5 Ci/mmol by the Institut des Radioéléments (Fleurus, Belgium). DNA-cellulose was prepared according to Alberts and Herrick [10]; 1 ml of gel contained 200 μg DNA. Ethylated DNA-Sephadex was prepared according to Weissbach and Poonian [11]: calf thymus DNA alkylated with ethylnitrosourea (2 e^6Gua/10^3 Gua) and sonicated, was linked to Sephadex G-150 with carbodiimide; 1 ml of gel contained 320 μg of ethylated DNA. HPLC chromatography was performed on an Altex (Beckman) 332 HPLC system equipped with a Perkin-Elmer LC 75 spectrophotometer.

Preparation of chromatin proteins

Rat liver nuclei were purified and the chromatin was prepared according to Thibodeau and Verly [12]. The chromatin proteins were extracted as described by Renard and Verly [7]: the chromatin was dissociated with heparin-Sepharose and the resulting DNA-protein–heparin-Sepharose complex was extracted with 0.3 M KCl, 10 mM K_2HPO_4, 10 mM Tris/HCl, pH 8.0. The extract was dialyzed against buffer A (1 mM EDTA, 0.2 mM dithiothreitol, 20 mM Tris/HCl, pH 8.0); the final protein concentration was about 250 μg/ml.

Substrate

Calf thymus DNA was alkylated with [^{3}H]ethylnitrosourea at 45 °C in a cacodylate buffer, pH 7.3. The ethylated DNA was partially depurinated according to Karran et al. [13] to provide a DNA substrate that contained 16 % of its radioactivity as O^6-ethylguanine residues; this substrate had about 10 e^6Gua/10^6 Gua. It was kept at 4 °C in 0.15 M NaCl, 0.015 M sodium citrate, 1 mM EDTA, pH 7.0, containing 1 % benzyl alcohol. The solution was extensively dialyzed against buffer B (20 mM Tris/HCl, 1 mM EDTA, pH 8.0) before use and adjusted to a final DNA concentration of 1 mg/ml.

Assays of the repair activity

The chromatin protein solution (100 μl; about 25 μg protein) was incubated with the [^{3}H]ethylated DNA solution

Abbreviations. e^6Gua, O^6-ethylguanine; CTAB, cetyltrimethylammonium bromide; HPLC, high-performance liquid chromatography.

Enzymes. DNase I (EC 3.1.21.1); snake venom phosphodiesterase (EC 3.1.4.1); spleen phosphodiesterase (EC 3.1.16.1); alkaline phosphatase (EC 3.1.3.1); leucine aminopeptidase (EC 3.4.11.1); trypsin (EC 3.4.21.4).

(100 µl; 100 µg DNA) for 0 or 2 h at 37 °C. Two methods are used to determine the amount of repair.

In the 'depurination/HPLC' assay, 50 µl 10 mM HCl containing 25 µg of unlabelled e^6Gua and 26 µl 1 M HCl were added to the incubation mixture and the mixture was then heated 50 min at 70 °C. Then, 25 µl 1 M KH_2PO_4 and 100 µl acetonitrile were added and the mixture was centrifuged for 5 min at 19000 × *g*. 250 µl of the supernatant was then analyzed on a column (250 × 10 mm) of Ultrasphere ODS (5 µm). This column was eluted isocratically at a flow rate of 2 ml/min with 25 mM ammonium phosphate containing 27.5 % acetonitrile adjusted to pH 4 with H_3PO_4. Absorbance at 260 nm was monitored continuously and the e^6Gua peak which appeared at 9.5 min was collected automatically. The e^6Gua recovery was usually around 95 % as calculated from absorbance data; the radioactivity of this fraction was corrected for recovery and the amounts of e^6Gua which are present in DNA before and after incubation with the chromatin proteins was calculated from the known specific radioactivity of the [^{3}H]ethyl-nitrosourea.

In the second method used to determine the amount of repair, which we have called the 'CTAB method', the following were added to the incubation mixture: 200 µl 80 mM EDTA, pH 6.0, containing 200 µg of calf thymus DNA; 140 µl 3 % cetyltrimethylammonium bromide (CTAB) solution in water; and 10 µl of freshly prepared 1 mM $CaCl_2$ containing 50 µg proteinase K. The solution was incubated 1 h at 37 °C and then centrifuged 5 min at 19000 × *g*. Then, the radioactivity of the supernatant was measured on an aliquot.

Sephasorb HP chromatography

This method was described by Renard and Verly [1].

Purification of the repair activity

DNA-cellulose chromatography. The solution of chromatin proteins was poured on a column (18 × 1.6 cm) of DNA-cellulose at a flow rate of 8 ml/h. The column was washed with buffer C (20 mM Tris/HCl, 1 mM EDTA, 0.2 mM dithiothreitol, 10 % glycerol, pH 7.8) and the retained proteins were eluted with a linear 0 – 1 M KCl gradient in buffer C; 4.7-ml fractions were collected. Absorbance at 280 nm and repair activity (after appropriate dilution to give a final KCl concentration less than 0.1 M in the assay) were measured on each fraction.

Hydroxyapatite chromatography. The solution of chromatin proteins was poured on a column (15 × 0.9 cm) of hydroxyapatite at a flow rate of 6 ml/h. The column was washed with 5 mM potassium phosphate, 2 mM dithiothreitol, 10 % glycerol, pH 6.8, and then eluted stepwise with 25-ml volumes of the same buffer at potassium phosphate concentrations of 0.1, 0.2, 0.3 and 0.5 M. Two-ml fractions were collected and the absorbance at 230 nm and repair activity were measured on each.

Thiopropyl-Sepharose chromatography. The solution of chromatin proteins without dithiothreitol was poured on a column (10 × 0.7 cm) of thiopropyl-Sepharose at a flow rate of 4.3 ml/h. The column was washed with buffer D (50 mM Tris/HCl, 1 mM EDTA, 0.1 M KCl, 10 % glycerol, pH 8.0) at a flow rate of 9.2 ml/h, and then the retained proteins were eluted with 20 mM dithiothreitol in buffer D at a flow rate of 4.3 ml/h; 1-ml fractions were collected. Protein concentration was measured in each fraction by the method of Peterson [14] and repair activity by the CTAB method.

Ethylated DNA-Sephadex chromatography. The solution of chromatin proteins was poured through a Pasteur pipet containing 2 ml of ethylated DNA-Sephadex gel at a flow rate of 4 ml/h. The column was washed with 50 mM Tris/HCl, 0.1 mM EDTA, 0.2 mM dithiothreitol, 50 mM NaCl, pH 8.0 and eluted with 4-ml volumes of the same buffer containing increasing concentrations of KCl (0.1, 0.2, 0.5 and 1 M); 1-ml fractions were collected for determinations of repair activity.

Sephacryl S200 chromatography. The solution of the chromatin proteins (1 ml) was poured on a column (60 × 1.6 cm) of Sephacryl S200 at a flow rate of 6 ml/h. The column was eluted with 50 mM Tris/HCl, 1 mM EDTA, 0.1 M KCl, 0.2 mM dithiothreitol, 10 % glycerol, pH 8.0; 1.07-ml fractions were collected for repair activity determinations. Blue dextran was eluted in fraction 41 and ribonuclease in fraction 83.

Cesium chloride gradient centrifugation; analysis of DNA

The analysis of [^{3}H]ethylated DNA on CsCl gradient after incubation with chromatin proteins is described by Mehta et al. [9]. High-density fractions that contained DNA were pooled, dialyzed against 50 mM ammonium formate and lyophilized. The DNA was then hydrolyzed to deoxynucleosides using DNase I, snake venom and spleen phosphodiesterases, and alkaline phosphatase (see [9, 9a] for details).

Deoxynucleoside analysis

Two methods were used.

Paper chromatography. The enzymatic hydrolysate of ethylated DNA was adjusted to pH 7.0 with HCl, then heated at 100 °C for 5 min; the protein precipitate was discarded by centrifugation. The supernatant was applied to a Whatman 3 MM paper which was eluted during 5 h with a mixture of *n*-butanol/ethanol/water (80:10:25, by vol). The paper was dried, then cut in 1-cm pieces which were eluted with the developing buffer for radioactivity measurements. The O^6-ethyldeoxyguanosine spot also contained O^2-ethyl and O^4-ethyl derivatives of deoxythymidine [15].

HPLC. This method was described by Mehta et al. [9, 9a].

Protein analysis

Trypsin digestion and peptide separation. The low-density protein-containing fractions from the CsCl gradient were pooled, dialyzed against 50 mM Tris/phosphate, pH 8.1, and lyophilized to dryness. The protein was then digested with trypsin and analyzed by HPLC as described by Mehta et al. [9, 9a].

Proteinase K digestion and peptide separation. The ethylated proteins in 50 mM Tris/phosphate, pH 8.1 (1.2 ml; 760 µg protein; 1600 dis./min) were incubated at 37 °C with 40 µl 80 mM EDTA, pH 6.0, and 110 µl 1 mM $CaCl_2$ containing 150 µg proteinase K. After 1, 2, 4 and 24 h, 300-µl aliquots were taken, adjusted to pH 1.6 with 10 % trifluoroacetic acid, and centrifuged at 12000 × *g* for 10 min. The supernatant (250 µl) was chromatographed on an HPLC column (250 × 4.6 mm) of Lichrosorb RP 18 (5 µm). The elution was done at a flow rate of 0.7 ml/min with a linear 0 – 50 % acetonitrile gradient in 0.1 % trifluoroacetic acid. Starting 3 min after the injection, 80 fractions of 0.35 ml were collected. Absorbance at 230 nm was monitored continuously and the radioactivity of each fraction was measured.

Tryptic peptide analysis

The fractions from HPLC containing the tryptic peptides were pooled and submitted to further digestion with pronase and leucine aminopeptidase. The resulting amino acids were analyzed directly or after preparation of the *o*-phthaldialdehyde derivatives. Details have been described by Mehta et al. [9, 9a].

EXPERIMENTS AND RESULTS

Some properties of the chromatin repair activity for e^6Gua lesions in DNA

Influence of Mg^{2+}, ATP and ionic strength. Ethylated DNA (69 µg; 7.82 e^6Gua/10^6 Gua) in 10 mM Tris/HCl, pH 8.0, was mixed with 100 µl of chromatin proteins (22 µg) in 50 mM Tris/HCl, 0.2 mM dithiothreitol, pH 8.0; to this mixture were added 50 µl of 10 mM Tris/HCl, pH 8.0, containing different concentrations of EDTA, $MgCl_2$ or ATP before a 15-min incubation at 37 °C. The e^6Gua content of the DNA was then analyzed. The repair system was active in the presence of 5 mM EDTA; neither 0.1 mM ATP nor 1 mM $MgCl_2$ had any effect although a higher Mg^{2+} concentration was inhibitory. Because of these observations the standard assay was performed without Mg^{2+} and in the presence of 1 mM EDTA to reduce the parasitic nuclease activity. The repair activity was highly sensitive to ionic strength: addition of KCl is inhibitory with the inhibition being complete at 0.3 M.

N-*Ethylmaleimide and dithiothreitol.* The repair activity was completely inhibited with 0.1 mM *N*-ethylmaleimide. On the other hand, addition of dithiothreitol enhanced the activity. These results indicate that a thiol group is involved in the repair reaction and the chromatin protein solutions were always protected by at least 0.2 mM dithiothreitol.

Optimum pH. Chromatin proteins (22 µg) in 100 µl of buffer A, 10 % glycerol were added to 100 µl of ethylated DNA (69 µg; 7.82 e^6Gua/10^6 Gua) in buffer B; the final pH was adjusted with 0.2 M HCl or NaOH from 6.0 to 9.0 (the pH was measured at 21 °C on control mixtures) before a 15-min incubation at 37 °C. Duplicates were performed at each pH value. The loss of e^6Gua by the ethylated DNA was estimated by HPLC analysis of the purines after acid hydrolysis. The results (not shown) indicated a broad pH optimum range between 7.5 and 8.5.

The substrate. The repair activity of the chromatin proteins on ethylated DNA denatured by a 10-min prewarming at 90 °C was 20 % of that measured on the usual double-stranded substrate.

Influence of temperature on the rate of the reaction and the stability of the repair activity

The repair rate of e^6Gua lesions in DNA is 35-times smaller at 4 °C than it is at 37 °C.

The chromatin proteins (43 µg) in 100 µl of buffer A were prewarmed at 37 °C for various times up to 120 min; addition of 50 µl of buffer B containing the ethylated DNA (340 fmol e^6Gua) was followed by a further 10-min incubation at 37 °C. The DNA was then analyzed. The logarithm of the enzyme activity (fmol e^6Gua removed from the ethylated DNA in 10 min) plotted as a function of prewarming time gave a straight line, the slope of which indicated a half-life of 55 min for the free enzyme at 37 °C.

The stability of the enzyme-substrate complex was investigated by the Selwyn test [16]: if the enzyme is stable in the complex, the substrate disappearance is a function of the product of incubation time by enzyme concentration whatever this concentration. Three different amounts of chromatin proteins (10, 20 and 40 µg) from the same preparation were used in otherwise identical incubation media; the curves obtained (see [8]) were different, indicating that the enzyme is not stable in the complex. Lateral displacement of the curve enables us to calculate a half-life of 85 min. The presence of ethylated DNA thus slightly stabilizes the enzyme; a similar stabilization is also observed during preincubation in the presence of ordinary double-stranded DNA.

Purification of the repair activity of the chromatin proteins

Five different methods of purification were tried. The description of the chromatographic procedures is given in Materials and Methods. Fig. 1 shows that the repair activity emerged as a single peak from the DNA-cellulose, hydroxyapatite and thiopropyl-Sepharose columns. The purified activity was very unstable so that the yields were variable and quite low; the purification factors were also low and it was impossible to use successive chromatographies in order to isolate the repair activity against e^6Gua DNA lesions. The activity was lost when the chromatin proteins were chromatographed on Sephacryl S200 or on ethylated DNA-Sephadex.

Mechanism of the repair reaction

It was shown in the preceding paper [1] that during incubation of ethylated DNA with chromatin proteins, the e^6Gua that disappears from DNA is not released in the medium as a free base or in an oligonucleotide. Another possibility is that e^6Gua is converted into another ethylated residue which remained attached to the sugar-phosphate backbone of the DNA molecule.

To test this hypothesis, 1 ml of solution containing 300 µg of chromatin proteins was incubated for 1 h at 37 °C with 1 ml of solution containing 27 µg of ethylated DNA (128 fmol e^6Gua; 7.82 e^6Gua/10^6 Gua) and 250 µg of untreated DNA. Two such assays were run in parallel as well as two controls without proteins. The DNAs of one control and one assay were depurinated and the purines analyzed by chromatography on Sephasorb HP; 93 % of the e^6Gua had disappeared during the incubation with the chromatin proteins. The DNAs of the other control and the other assay were enzymatically hydrolyzed to nucleosides which were separated on Whatmann 3MM paper according to Singer [15]; in this latter case, the peak of O^6-ethyldeoxyguanosine also contains the O^2-ethyl and O^4-ethyldeoxythymidine. From 30 % of the total radioactivity in the control, this peak was reduced to 16 % when the ethylated DNA was incubated with the chromatin proteins; this difference agrees with the result of the purine analysis. No new radioactivity peak appeared anywhere in the chromatograms and the size of the other peaks was essentially unchanged. The conclusion is that the e^6Gua is not transformed into another ethylated residue remaining in DNA; the ethyl group of e^6Gua has been lost during the repair of DNA (for details, see [7]).

In another experiment, 1.68 ml of chromatin protein (250 µg) solution and 240 µl of ethylated DNA (290 µg; 2030 fmol e^6Gua; 11.4 e^6Gua/10^6 Gua) solution were incubated

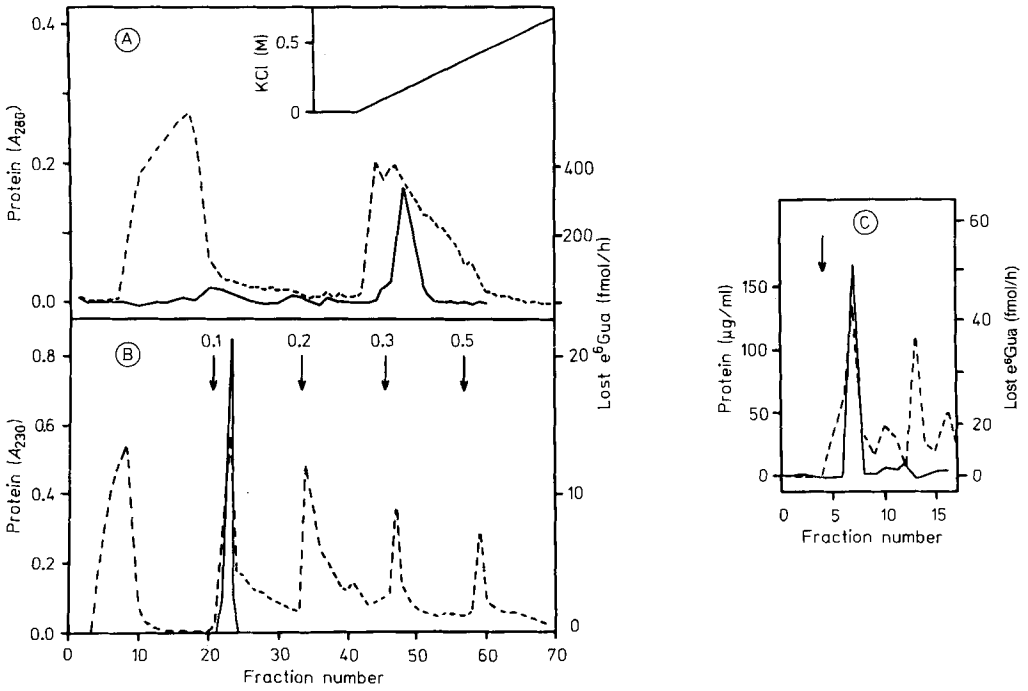

Fig. 1. *Purification of the repair activity of the chromatin proteins.* The chromatin proteins were chromatographed: (A) on DNA-cellulose using a 0 – 1 M KCl gradient (insert) to elute the repair activity; (B) on hydroxyapatite with an elution by steps (arrows) using potassium phosphate of increasing molar concentrations; (C) on thiopropyl-Sepharose using 20 mM dithiothreitol (arrow) as eluent. Details can be found in Materials and Methods. The dotted lines represent absorbance at 280 nm (A), 230 nm (B) or protein concentration measured by the method of Peterson [14] (C). The continuous lines follow the repair activity per fraction (fmol e^6Gua lost/h)

2h at 37 °C. A control without protein was run in parallel. Assay and control were submitted to isopycnic CsCl centrifugation. The 254-nm absorbance profile showed a good separation of proteins and DNA. The radioactivity profiles indicated that all the radioactivity of the control was in the DNA band, whereas, in the assay, part of the radioactivity was in the protein band (see [9, 9a]). DNA and proteins were subsequently analyzed separately.

The fractions containing the DNA were pooled, dialyzed against ammonium formate and lyophilized. The residues (control and assay) were enzymatically digested to nucleosides which were separated on an HPLC reverse-phase column. In the control, numerous radioactivity peaks were observed, one of them corresponding to the position of an O^6-ethyldeoxyguanosine absorbance marker. This peak had almost completely disappeared in the assay incubated with the chromatin proteins; there was little change of magnitude of the other peaks and no new peak had appeared (see [9, 9a]). These observations are essentially the same as those of the preceding experiment, but the much higher resolution of the HPLC analysis brings more weight on the conclusion.

The radioactivity found in the protein band is not very different from the radioactivity lost by the e^6Gua bases of the ethylated DNA. The fractions containing radioactivity in the low-density region of the CsCl gradient were pooled, dialyzed against 50 mM Tris/PO_4, pH 8.1, and lyophilized. The residue, dissolved in water, cannot be eluted from an HPLC reverse-phase column; however, after digestion with trypsin, nearly all the radioactivity was eluted in two peaks in a region characteristic of oligopeptides (see [9, 9a]).

The two labelled oligopeptides were pooled and further digested with pronase and leucine aminopeptidase, and the digest chromatographed on Sephadex G-10; all the radioactivity was eluted in the amino acid position. Three different methods showed that this radioactivity was in *S*-ethylcysteine: (a) HPLC reverse-phase chromatography of the *o*-phthaldialdehyde-derivatized amino acids; (b) HPLC ion-exchange chromatography after warming at 100 °C and pH 7.8 to hydrolyze any ethylaspartate or ethylglutamate that might be present; (c) by paper chromatography also after warming at 100 °C and pH 7.8 (for details, see [9, 9a]). It is worth mentioning that *S*-ethylcysteamine has a different behaviour from *S*-ethylcysteine in these three different chromatographic systems.

The two radioactive oligopeptides obtained by trypsin digestion of the ethylated acceptor(s) are different ultimate products; in particular, one oligopeptide cannot be converted into the other. Fractions from the chromatography of a tryptic digest containing the radioactive peaks were pooled separately. They were submitted again to the same trypsin treatment and analyzed on HPLC. As is evident from Fig. 2, neither peptide was digested further and neither one showed evidence of turning into the other. Thus, it really does appear that there might be more than one acceptor protein or that the acceptor protein could be ethylated on more than one cysteine residue.

The ethylated acceptor is also sensitive to proteinase K

The proteins obtained after centrifugation on a CsCl gradient were found to be contamined with polynucleotides even after extensive dialysis. Acid hydrolysis followed by HPLC analysis showed that 10 – 30 % of the radioactivity in the protein band was due to this contamination.

The ethylated proteins were digested with proteinase K and aliquots taken after 1, 2, 4 and 24 h were analyzed by HPLC as

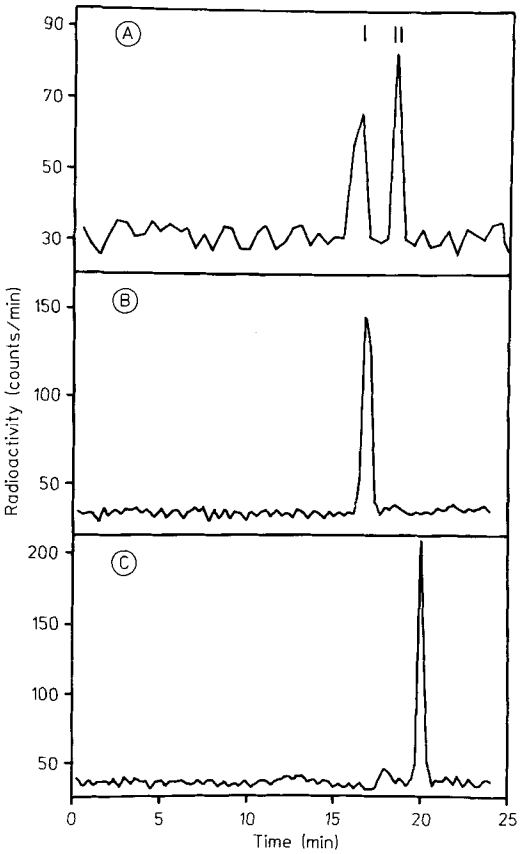

Fig. 2. *The two tryptic oligopeptides from the ethylated acceptor(s) are ultimate digestion products.* The ethylated acceptor protein (1900 counts/min) purified by isopycnic centrifugation was dissolved in 1 ml water. This solution was allowed to stand for 1 h at room temperature and 100 µl was withdrawn for 0 time analysis (not shown). Then 112 µl of trypsin solution (1 mg/ml in dilute acetic acid, pH 3) and 45 µl of 0.2 M $CaCl_2$ were added. The solution was incubated 24 h at 37 °C and 800 µl of this solution was analyzed by HPLC; 1-ml fractions were collected and 125 µl of each was counted for radioactivity (A). Two peaks (I and II) of radioactivity were obtained. Fractions of peak I (B) or peak II (C) were pooled, neutralized, lyophilized, and redissolved in 0.9 ml water. Again, 112 µl of trypsin solution and 45 µl of 0.2 M $CaCl_2$ were added and the incubations carried out at 37 °C for another 24 h; the solutions were then analyzed by HPLC. The graphs give the radioactivity in each fraction from HPLC

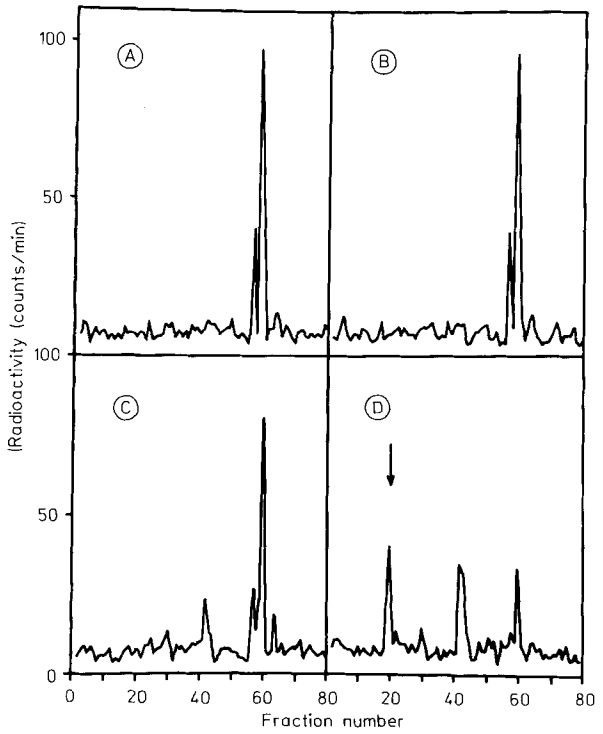

Fig. 3. *Peptide analysis after proteinase K digestion of the ethylated chromatin proteins.* A solution of ethylated proteins purified by centrifugation on a CsCl gradient was incubated at 37 °C in the presence of proteinase K. After different times (A, 1 h; B, 2 h; C, 4 h; D, 24 h), aliquots were taken and analyzed on a reverse-phase HPLC column; 0.35-ml fractions were collected to measure the radioactivity. The arrow in (D) indicates the position of *S*-ethylcysteine

described in Materials and Methods. Fig. 3 gives the results of the four analyses. In Fig. 3A (1 h), 56 % of the injected radioactivity is eluted in two peaks in a region characteristic of oligopeptides (the polynucleotides are strongly retained on the column in the conditions used). The situation had not changed much after 2 h of digestion (Fig. 3B), but, after 4 h (Fig. 3C), two new peaks were eluted earlier. After 24 h (Fig. 3D), the first peaks had almost completely vanished and a radioactive peak had appeared in the position of an *S*-ethylcysteine marker.

A new method to assay the repair activity of chromatin proteins on e^6Gua DNA lesions

The repair activity has been measured so far by the disappearance of the substrate (e^6Gua); now that we know the reaction mechanism, it could be measured by the appearance of the reaction product (ethylated proteins). The detail of the new procedure can be found in Materials and Methods. It is an adaptation of Schneider's method [17] for the determination of DNA in which DNA is precipitated with CTAB in the presence of proteinase K to prevent contamination of the precipitate by proteins.

In exploratory experiments, chromatin proteins and ethylated DNA, after a 2-h incubation at 37 °C, were separated by CsCl isopycnic centrifugation and dialyzed. The two fractions were submitted separately to the proteinase K/CTAB treatment. In the case of ethylated DNA, more than 99 % of the radioactivity was precipitated by CTAB. In the case of the protein fraction, 85 % of the radioactivity remained in the supernatant (the remainder was probably polynucleotides precipitated by CTAB). When the supernatant was analyzed by HPLC, two oligopeptides contained more than 97 % of the injected radioactivity.

In the following experiment, chromatin proteins were incubated with ethylated DNA at 37 °C for 7.5, 15, 30 or 60 min. Duplicates were made for each incubation time; one sample was used to determine the e^6Gua content of the DNA using acid depurination and HPLC analysis; the other was submitted to the proteinase K/CTAB assay. Fig. 4 indicates that the radioactivity in the supernatant of the proteinase K/CTAB assay was not significantly different from the reduction of e^6Gua radioactivity in the ethylated DNA.

DISCUSSION

It has been known for some time that DNA containing e^6Gua lesions is repaired in rat liver [18, 19]. In the preceding

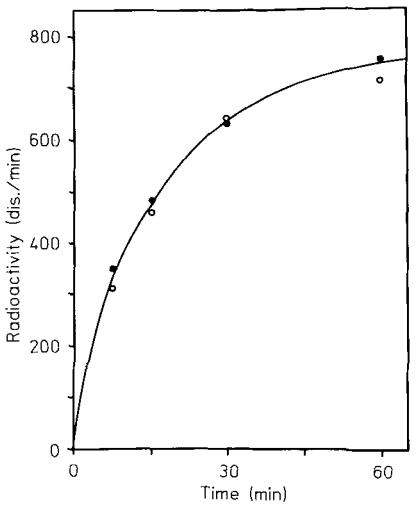

Fig. 4. *Comparison between the depurination/HPLC (○) and proteinase K/CTAB (●) assays of the repair activity.* Tubes containing chromatin proteins and ethylated DNA (final volumes of 200 µl) were incubated at 37 °C. After 7.5, 15, 30 and 60 min, two tubes were used to measure the e^{6}Gua disappearance from DNA by the depurination/HPLC method (○) and three tubes to measure the radioactivity in the supernatant of the proteinase K/CTAB assay (●). Each point is the average of replicates

paper [1], we have shown that all the necessary components of the repair system are in the cell nucleus; moreover, the dissociation of chromatin yielded a solution of proteins able to cause the disappearance of e^{6}Gua from an added ethylated DNA. It is very likely that the chromatin repair activity demonstrated *in vitro* is also responsible for the disappearance *in vivo* of the e^{6}Gua lesions from the nuclear DNA.

Several trials to purify the chromatin repair activity were not particularly successful, but some interesting observations were made when different chromatographic procedures were tried. The retention of the activity on thiopropyl-Sepharose suggests the presence of a thiol group. The loss of activity on ethylated DNA-Sephadex could be due to the exhaustion of a limiting component of the repair system, although the chromatography was performed at 4 °C, a temperature at which the repair activity is practically nonexistent; it could also be due to a very high affinity of the substrate for the repair enzyme. The loss of activity on Sephacryl S200 is difficult to explain although the possibility that the repair activity needs several components of different molecular weights cannot be excluded.

The repair activity has an optimum pH between 7.5 and 8.5; it does not need divalent cations. It is destroyed by *N*-ethylmaleimide and protected by dithiothreitol; the thiol group is not only present in the repair enzyme, but it is also necessary for its activity. The purified activity is very labile; in the crude preparation of the chromatin proteins, the activity has a half-life of 55 min at 37 °C although it can be protected by addition of DNA. The repair is more active on double-stranded than on single-stranded DNA.

In the preceding paper [1], we have shown that isolated nuclei alkylated *in vitro* did not release free e^{6}Gua in the medium. We have also shown that, when chromatin proteins were incubated with ethylated DNA, the e^{6}Gua that disappeared from DNA could not be found as free base or in oligonucleotides, eliminating the possibility that the repair might be due to a DNA glycosylase or the joint action of a specific endonuclease and an exonuclease. In this work, we present evidence that the disappearance of e^{6}Gua from DNA is not the result of transformation into another ethylated base that would remain attached to the sugar-phosphate backbone of the DNA molecule. Instead an amount of radioactivity equivalent to that of the e^{6}Gua lost by DNA appeared in the proteins. That the radioactivity had indeed been transferred into a protein was shown by the sensitivity of the labelled acceptor to trypsin and proteinase K; both treatments resulted in the formation of radioactive oligopeptides.

When the oligopeptides from the trypsin digestion were further hydrolyzed with pronase and leucine aminopeptidase, the radioactivity was found in *S*-ethylcysteine. *S*-Ethylcysteine is also obtained by prolonged treatment with proteinase K.

The repair mechanism is thus a transethylation from e^{6}Gua in DNA onto cysteine residues in acceptor proteins. It is probably a one-step repair, the e^{6}Gua being restored into guanine, as has been shown by Foote et al. [20] in bacteria and Pegg et al. [6] in human liver for the repair of O^6-methylguanine. The trypsin digestion yields two oligopeptides which are resistant to further digestion. This suggests that there are at least two different cysteines able to accept the ethyl group. We do not know whether the two cysteines belong to the same protein or not.

The knowledge of the repair mechanism enabled us to devise a new assay of the activity. Instead of measuring the disappearance of e^{6}Gua from DNA, the appearance of the ethylated protein is followed. The medium containing the chromatin proteins and the ethylated DNA, at the end of the incubation, is treated with proteinase K to digest the proteins and the DNA is precipitated with CTAB. The radioactivity in the supernatant is equal to the radioactivity of the lost e^{6}Gua; this exact correspondence supports the mechanism proposed for the repair of e^{6}Gua in DNA and it also suggests that the contribution of the repair of other lesions, in the ethylated DNA partially depurinated used as a substrate, is not sufficient to disturb the result of the e^{6}Gua repair. The advantage of the CTAB method is its rapidity and simplicity.

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