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Intermediates in Excision Repair by Human Cells: Use of S_1 Nuclease and Benzoylated Naphthoylated Cellulose to Reveal Single-Strand Breaks†

Peter Karran,‡ N. Patrick Higgins,§ and Bernard Strauss*

ABSTRACT: Treatment of human lymphoid cells with methyl methanesulfonate (MMS) results in discontinuities in cellular DNA. Discontinuities can be detected by sedimentation through neutral sucrose gradients after digestion with S_1 nuclease on top of the gradient and by benzoylated naphthoylated diethylaminoethylcellulose (BND-cellulose) chromatography. DNA with discontinuities is sensitive to shear which produces single-stranded regions and causes the DNA to adhere to BND-cellulose. The shear-sensitive and S_1-sensitive sites produced by MMS treatment are probably identical. Treatment of cells with acetoxyacetylaminofluorene (AAAF) does not result in detectable discontinuities either at doses which produce the same number of adducts as observed after MMS treatment or at doses which inhibit DNA excision repair. Addition of aromatic adducts to DNA can also result in adherence to BND-cellulose but such DNA is not S_1 sensitive. We conclude that MMS- and AAAF-induced damage are repaired by different pathways in human cells and that DNA with discontinuities is a readily detectable intermediate only in the repair of MMS damage by the apurinic pathway.

There are at least two modes of DNA excision repair in human cells. "Long patch" repair is the typical response to ultraviolet-light-induced damage and results in repair segments of the order of 100 nucleotides (Regan and Setlow, 1974). "Small patch" repair is induced by treatment of human cells with ionizing radiation and at low doses results in patch sizes of 3–4 nucleotides (Painter and Young, 1972; Regan and Setlow, 1974).[1] A variety of chemical agents combine with DNA

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‡ Present address: Department of Chemistry, Karolinska Institutet, Solnavagen 1, S 104 01 Stockholm 60, Sweden.
§ Present address: Department of Biochemistry, University of Chicago, Chicago, Illinois 60637.

[1] "Long patch repair" has been given a different meaning for bacteria and refers, in such organisms, to an excision repair process which introduces patches of about 1000 nucleotides and requires the *rec* gene products as well as protein synthesis (Cooper and Hanawalt, 1972). This paper deals with excision repair in the sense defined for human cells by Regan and Setlow (1974).

and the adducts are recognized by enzymes present in human cells and removed by excision repair processes. The lesions induced by such compounds may be characterized on the basis of the size of the repair patch which they induce. MMS[2] induces lesions, the great majority of which are repaired by a small patch mechanism (comparable to ionizing radiation), whereas AAAF is repaired by a process involving insertion of larger patches (Regan and Setlow, 1974).

The distinction between chemical lesions on the basis of the size of the repair patch might be fortuitous and based, for example, on the relative proportion of repair enzymes and lesions. Alternatively, the distinction might indicate the operation of different excision repair pathways. In this paper, we report that MMS treatment, in contrast to treatment with AAAF, induces readily detectable interruptions in cellular DNA. This distinction between the effects of MMS and AAAF may reflect a basic difference in the pathways by which damage induced by these compounds is repaired in human cells.

Materials and Methods

Cell Culture and Labeling. Cell line Raji, a human lymphoblastoid line established from a Burkitt's lymphoma patient (Pulvertaft, 1964), was maintained in RPMI 1640 medium (Gibco) containing 20% fetal calf serum, penicillin, and streptomycin. (The cell line used was free of mycoplasma as determined by Microbiological Associates at the conclusion of our studies.) Cellular DNA was labeled by overnight incubation in the presence of either 0.5 μCi/mL of [^{3}H]methylthymidine (11–15 Ci/mmol) or 0.03 μCi/mL of [^{14}C]thymidine (Schwarz/Mann, N.Y.). Labeled cells were chased for at least 2 h by incubation in the presence of 1 μg/mL of unlabeled thymidine before further treatment.

Chemicals. Methyl methanesulfonate (MMS; Eastman Organic Chemicals) was redistilled under vacuum and diluted in phosphate-buffered saline immediately prior to use. *N*-Acetoxy-*N*-acetyl-2-aminofluorene (AAAF), generously supplied by Dr. James A. Miller, and 7-bromomethylbenz(α)anthracene (BMBA), a gift of Dr. Peter Brookes, were dissolved in sterile dimethyl sulfoxide before use. [^{14}C]Methyl methanesulfonate (52 μCi/μmol) was obtained from Amersham/Searle and [9-^{14}C]AAAF (21 mCi/mmol) from ICN Corporation. [^{3}H]-7-BMBA (2 mCi/mg) was a gift of Dr. P. Brookes.

BND-Cellulose Chromatography. Chromatography on BND-cellulose has been described in detail elsewhere (Scudiero et al., 1975). Cells were treated, washed free of unreacted, unbound, chemicals by centrifugation with PBS, suspended in SSC, and then lysed with 0.2% sodium dodecyl sulfate. The lysates were digested for 1 h at 37 °C with 50 μg/mL of pancreatic ribonuclease and then for an additional hour with 1.5 mg/mL of heat-treated Pronase. The lysates were sheared and applied to a small (1.5 × 1.1 cm) column of BND-cellulose in 0.3 M NET. The columns were step eluted with 10 mL of 0.3 M NET, 10 mL of 1.0 M NET, and 10 mL of 50% formamide in 1 M NET; 2.5-mL fractions were collected. Acid-insoluble radioactivity was determined by precipitation with 5% Cl_3CCOOH in the presence of 100 μg/mL of bovine serum albumin. The precipitates were collected on nitrocellulose filters and the radioactivity on the dried filters was determined after the addition of 5 mL of a toluene-based scintillation fluid containing Permablend (Packard).

Double-stranded DNA elutes from BND-cellulose with 1.0 M NET. DNA with single-stranded regions requires 50% formamide in 1 M NET (or caffeine in 1 M NET) for elution. *We define DNA fragments as adhering to BND-cellulose if they are absorbed in 0.3 M NET, retained on washing with 1.0 M NET, and eluted by washing with 50% formamide or with caffeine in 1.0 M NET.*

Sucrose Gradient Sedimentation with S_1 Nuclease. Two types of gradient were used: (a) shelf gradients [a 4.2-mL linear 5–20% sucrose gradient in 2 M NaCl was formed above a 0.6-mL shelf of 60% sucrose saturated with CsCl (ρ = 1.8)]; and (b) linear gradients consisted of 4.8 mL of 5–20% sucrose in 2 M NaCl. Both types of gradient were formed in cellulose nitrate tubes (1/2 × 2 in.; Beckman Instruments). Shelf gradients were centrifuged for 60 min at 30 000 rpm in the SW 50.1 rotor of a Beckman L2 or L3 ultracentrifuge. Linear gradients, calibrated with phage T4 DNA (mol wt 1.1 × 10^8), were centrifuged at 40 000 rpm for 2 h or as indicated in the figure legends. Gradients were fractionated through a hole pierced in the bottom of the tube and two-drop fractions were collected on Whatman 3 MM, 2.3 cm, filter paper circles. The acid-insoluble radioactivity in each fraction was determined by counting the dried filters after washing in cold 5% Cl_3CCOOH, ethanol, and acetone.

Cells were lysed directly above the gradient in 100 μL of lysis mixture (0.2% Sarkosyl, 5 mM $ZnSO_4$, 40 mM sodium acetate, pH 4.6) which was separated from the top of the gradient by a layer of 100 μL of 1% sucrose solution in 0.1 M sodium acetate, pH 4.6, in order to avoid the precipitation which occurred if the zinc/detergent mixture came in contact with the high sodium chloride concentration of the gradient. S_1 nuclease (Miles Chemical Co., 50 μL of a solution of 200 μg/mL protein) in 5 mM $ZnSO_4$–40 mM sodium acetate, pH 4.6 (Vogt, 1973), was added into the lysis layer along with 20 μL of a cell suspension containing 10^6 cells/mL. Lysis and S_1 nuclease digestion were allowed to proceed in the dark for 60 min at room temperature. S_1 nuclease retained 90% of its activity toward denatured DNA under these conditions of digestion.

Number average molecular weights (Charlesby, 1954) were calculated using the relationship: $M_n = \Sigma c_i / \Sigma c_i/M_i$, where c_i is the radioactivity in the ith fraction and M_i is calculated from the relationship $M = k(d/\omega^2 t)^{1/a}$ using the value of a as determined by Studier (1965) and k, a constant determined by calibration of a gradient with phage T4 DNA.

Chemical Binding to DNA. Samples of approximately 10^7 cells in growth medium containing serum were treated with different concentrations of radioactive compound at 37 °C. Cells were washed, resuspended in 1 mL of SSC, and lysed in 0.2% sodium dodecyl sulfate. Lysates were taken through three cycles of freezing in dry ice–ethanol and thawing at 37 °C and digested with RNase and Pronase as for BND-cellulose chromatography. After shearing by passage five times through a 20 gauge needle, the volume of the lysate was brought to 5 mL and its density to 1.7 g/cm^3 by the addition of CsCl and SSC. The resulting solution was centrifuged for 40 h at 20 °C and 30 000 rpm in Beckman SW 50.1 rotor. Gradients were fractionated and the absorbancy (A_{260}) and acid-insoluble radioactivity in the region of density 1.70 g/cm^3 were determined.

Preparation of Bacteriophage DNA. Bacteriophage T5 were grown by infection of a thymine auxotroph of *Escherichia coli* in the presence of [^{3}H]thymidine (10 μCi/mL). The DNA was phenol extracted from purified phage and had a specific ac-

[2] Abbreviations used: AAAF, *N*-acetoxy-2-acetylaminofluorene; BMBA, 7-bromomethylbenz(α)anthracene; BND-cellulose, benzoylated naphthoylated DEAE-cellulose; MMS, methyl methanesulfonate; 0.3 M NET, 0.3 M NaCl–10^{-4} M EDTA–0.01 M Tris-HCl, pH 7.5; 1.0 M NET, 1.0 M NaCl–10^{-4} M EDTA–0.01 M Tris-HCl, pH 7.5; PBS, phosphate-buffered saline; PCA, perchloric acid; SSC, 0.15 M NaCl–0.015 M sodium citrate.

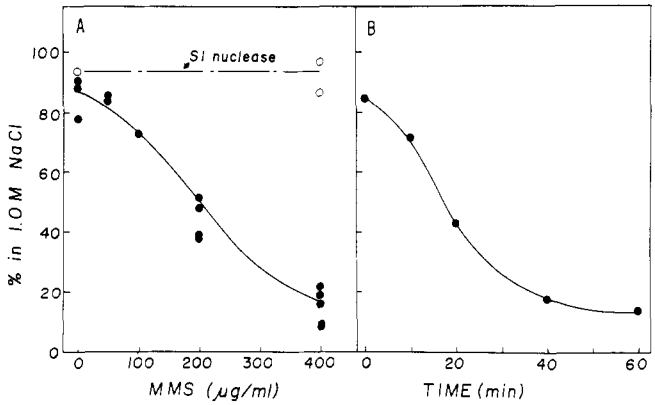

FIGURE 1: The production of adherent sites in cellular DNA after treatment with MMS. [^{3}H]Thymidine-labeled cells (3–5 × 10^5 cells/mL) were treated in growth medium at 37 °C with: (A) different concentrations of MMS for 60 min, or (B) 400 μg/mL MMS for the times shown. After treatment, cells were harvested and the DNA was chromatographed on BND-cellulose columns as described in Materials and Methods. The ordinate values are calculated as a percentage of the radioactivity recovered in the 1.0 M eluate of the columns. Total recovery was 80–100% of the applied radioactivity. For S_1 treatment prior to column chromatography (open symbols), sheared lysates (between 1.5 and 2.5 × 10^6 cells/mL at the time of lysis) were adjusted to 40 mM $NaC_2H_3O_2$–5mM $ZnSO_4$ (pH 4.6) and digested with 10 μg of S_1 nuclease (total volume 1.5 mL) for 60 min at 37 °C before application to the column in 0.3 M NET.

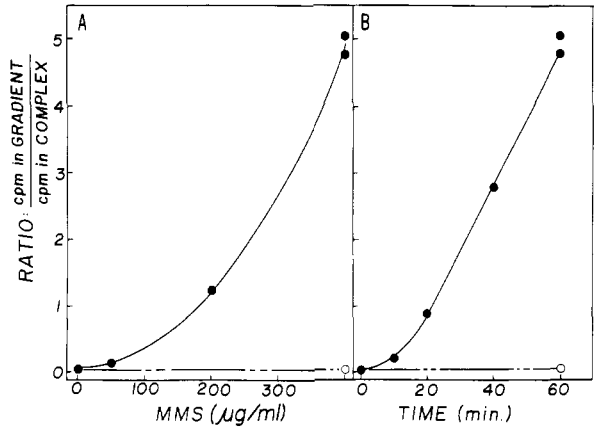

FIGURE 2: Release of MMS-treated DNA from the fast-sedimenting complex by treatment with S_1 nuclease on neutral sucrose gradients. (A) Cells were treated with MMS as in Figure 1A, resuspended in SSC, and 20 μL (1–2 × 10^4 cells) was lysed above a shelf gradient. S_1 nuclease was then gently added and digestion allowed to proceed for 60 min at 20 °C in the dark. (B) Cells were treated with MMS as in Figure 1B. Lysis and S_1 digestion were as described above. Centrifugation was for 60 min at 30 000 rpm in the Beckman SW 50.1 rotor. Gradients were fractionated and the acid-insoluble radioactivity in each fraction was determined. The ratio between the radioactivity which sedimented in the gradient and on the shelf was calculated. (Closed circles) + S_1 nuclease; (open circles) − S_1 nuclease.

tivity of approximately 2000 cpm/μg of DNA and was homogeneous on agarose gel electrophoresis. Phage T5 DNA was sheared at the same DNA concentration and under the same conditions as DNA from cell lysates.

X Irradiation. X irradiation of cells on ice was carried out using a GE Maxitron (250 kVp, 0.25-mm Al filter) at dose rates of 1 or 5 krad/min in air.

Results

Interruptions Induced by MMS: Adherence to BND-Cellulose. Uniformly labeled DNA extracted from Raji cells and then sheared adsorbs to BND-cellulose and is eluted by 1.0 M NaCl (1 M NET). Treatment of cells with MMS results in a DNA preparation which adheres to BND-cellulose. The proportion of DNA which adheres is both dose and time dependent (Figures 1A and 1B). Adherence to BND-cellulose is characteristic of DNA molecules with regions of single-strandedness (Iyer and Rupp, 1971). Addition of S_1 nuclease to the sheared samples before application to the column reduced the adherence (Figure 1A).

Large amounts of acid-soluble radioactivity are not released by S_1 treatment. In our experiments about 1–4% of the radioactivity remained soluble after precipitation of S_1 treated DNA with PCA. This is equivalent to 600–2500 nucleotides per fragment of sheared DNA of the size applied to the columns, but because of the low level of S_1 susceptibility and the problem of S_1 "nibbling" at ends it is clear that these values are only approximate. The effect of S_1 in reducing adherence to the column indicates that sheared DNA obtained from MMS-treated cells contains regions of single-strandedness which are removed by the nuclease treatment.

Discontinuities in Nonsheared DNA. DNA released from treated or untreated cells lysed above a neutral sucrose gradient sediments as a complex (Lehman and Ormerod, 1970). Double-strand breaks are required to release DNA from the complex. The released DNA sediments within the top third of the gradient under the conditions of sedimentation used whereas the complex sediments to the bottom and is retained by the dense CsCl/sucrose cushion over which the gradient is

TABLE I: Effect of S_1 Nuclease Treatment on DNA from MMS-Treated Cells.[a]

Shear	S_1	% of total DNA in 1.0 M NaCl eluate from BND-cellulose
None	−	9
None	+	89
5×, 20-gauge needle	−	19
5×, 20-gauge needle	+	87

[a] Cells were treated and S_1 nuclease was added as described in the legend to Figure 3.

formed (Ormerod and Lehman, 1971). We used this sytem to monitor the accumulation of single-strand discontinuities in DNA, exploiting S_1 nuclease to convert the single-strand lesions into detectable double-strand breaks. Increasing amounts of DNA are liberated from the complex with increasing MMS concentration and time of treatment (Figure 2A,B).

S_1 nuclease treatment can be used to quantitate breaks as seen by the following experiment: cells were treated with MMS (400 μg/mL; 50 min) and extracted under conditions of minimum shear. The DNA from these cells sedimented with a molecular weight of greater than 10^8 (Figure 3A). Treatment with S_1 nuclease prior to sedimentation reduced the molecular weight to an Mn of about 4.3 × 10^7. Shearing DNA from MMS-treated cells produced molecules of about 10^7 daltons (Figure 3B). Further treatment with S_1 nuclease did not further reduce the Mn, although it did remove the single-stranded regions which result in adherence to BND-cellulose (Table I). As an example of the utility of the S_1 methodology we showed that MMS treatment of cells induced a dose-dependent decrease in the average molecular weight of the DNA (Figure 4). DNA from such MMS-treated cells sedimented to the bottom of the gradient without S_1 nuclease treatment.

Effect of Shear. Shearing double-stranded DNA may result in fragments with single-stranded ends, depending on the conditions (Pyeritz et al., 1972). However, shearing with a

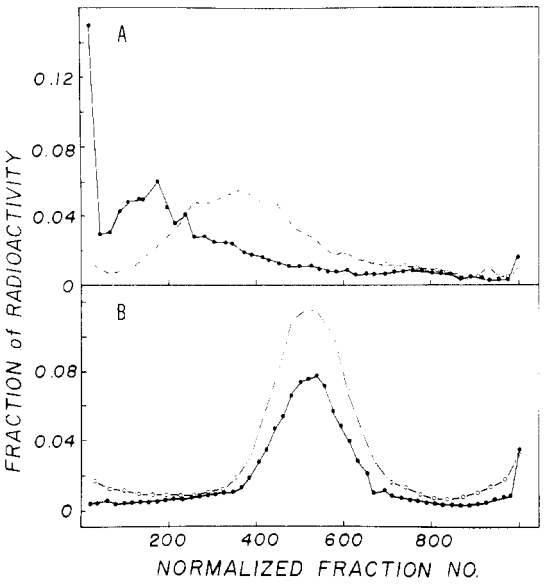

FIGURE 3: The relation between S_1-sensitive and shear sensitive sites produced in DNA by MMS treatment. Labeled cells were treated with 400 μg/mL MMS for 60 min at 37 °C, washed free of untreated drug, and lysed in SSC containing detergent. After digestion with RNase and Pronase, lysates were sheared once through an 18-gauge needle. Half the lysate was further digested with 10 μg of S_1 nuclease in 40 mM $NaC_2H_3O_2$–5 mM $ZnSO_4$ (pH 4.6) for 60 min at 37 °C. Twenty-microliter aliquots of lysate (0.1–0.2 μg of DNA) were layered on linear 5–20% sucrose gradients (2 M NaCl). (A) Limited shear. (Closed circles) Sheared once through an 18-gauge needle. No S_1 digestion. (Open circles) Digestion with S_1 nuclease after shear. Centrifugation was for 2.5 h at 40 000 rpm. (B) Increased shear. (Closed circles) DNA sheared five times through a 20-gauge needle. No S_1 digestion. (Open circles) Digestion with S_1 nuclease after shear. Centrifugation was for 2 h at 40 000 rpm. Sedimentation is from right to left. Gradients are normalized to 1000 fractions.

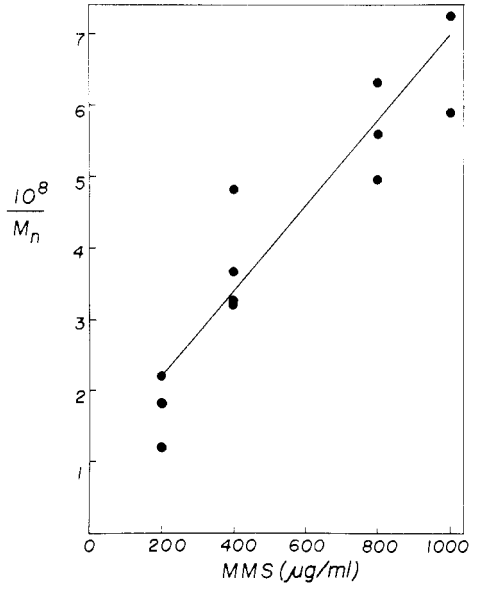

FIGURE 4: Dose dependency of the accumulation of S_1-sensitive sites in DNA. Labeled cells were treated for 60 min with the concentrations of MMS shown on the abscissa. Treated cells ($1-2 \times 10^4$) were lysed above a 5–20% neutral sucrose gradient containing 2 M NaCl. S_1 nuclease digestion was carried out above the gradient for 60 min at 20 °C before centrifugation for 2 h at 40 000 rpm. The number average molecular weight (M_n) was calculated for each gradient.

20-gauge needle in SSC containing detergent does not result in shredding of noninterrupted DNA such as was found by Pyeritz et al. (1972) when DNA was sheared with a 28-gauge needle at 37 °C (Figure 5). We determined the effect of shear on DNA with discontinuities in order to understand the conditions which led to the retention of such DNA on BND-cellulose columns. Bacteriophage T5 DNA has five ligase-sensitive interruptions in a molecule of 8.3×10^7 daltons. These nicks are preferentially sensitive to shear (Hayward, 1974). When T5 DNA in SSC was sheared at room temperature by passing a solution five times through a 20-gauge needle, a significant portion of the fragments adhered to the column and were eluted only at appreciable concentrations of caffeine (Figure 5). Since the caffeine concentration at which DNA is eluted from BND-cellulose is a function of the degree of single-strandedness (Iyer and Rupp, 1971), this result shows that shearing DNA with interruptions produces fragments with extensive single-stranded regions. Sheared DNA from MMS-treated cells adheres to BND-cellulose and the adherent fragments also eluted only at higher caffeine concentrations as would be expected if this DNA contained interruptions before shear in contrast to DNA from untreated Raji cells (Figure 5).

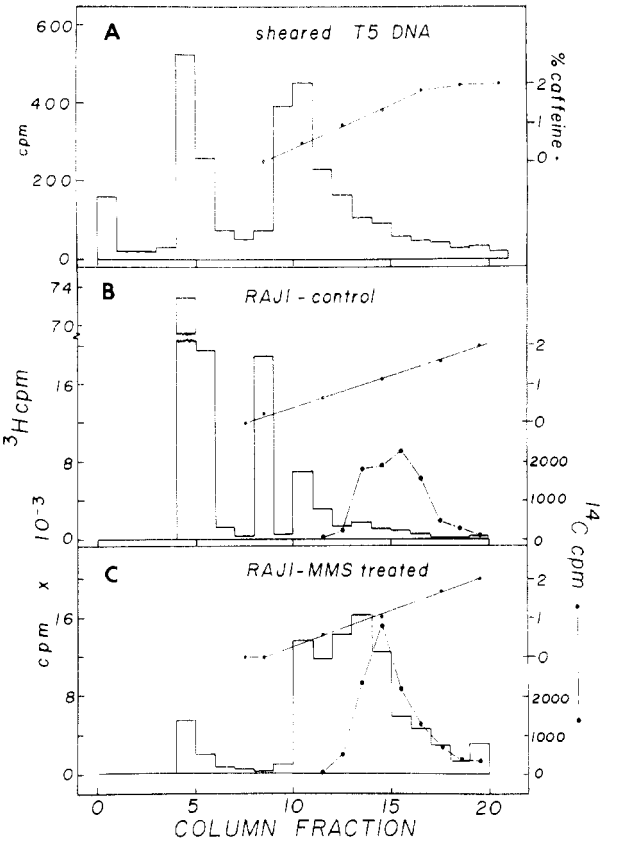

FIGURE 5: Caffeine elution of adherent DNA from BND-cellulose. DNA was applied to columns of BND-cellulose in 0.3 M NET and eluted stepwise with 10 mL of 0.3 M NET–10 mL of 1.0 M NET followed by a gradient of 20 mL of 0–2% caffeine in 1 M NET. Fractions (2.5 mL) were collected. The shape of the gradient was determined by refractometry (●—●) and acid-insoluble radioactivity determined for each fraction. (A) T5 DNA. DNA purified from phage T5 sheared five times through a 20-gauge needle at a DNA concentration of 20 μg/mL in SSC. (B) Control cell DNA. DNA, from untreated Raji cells, prepared by RNase and Pronase digestion of cell lysate was sheared five times through a 20-gauge needle at approximately 20 μg of DNA/mL in SSC containing 0.2% sodium dodecyl sulfate. (C) Treated cell DNA. DNA from Raji cells which had been treated for 60 min with 400 μg/mL MMS was prepared and sheared as in B. The dotted line in panels B and C represents the position of a small amount of heat-denatured [^{14}C]thymidine-labeled DNA (of about 10^7 daltons) cochromatographed with the [^{3}H]thymidine-labeled cellular DNA.

We tested the hypothesis that breaks in DNA can be de-

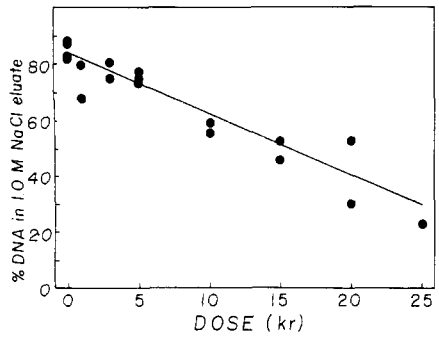

FIGURE 6: The production of adherent sites in cellular DNA by x irradiation. Labeled cells were suspended in 0.15 M NaCl and irradiated in air at 0 °C. The dose rate was 1 krad/min for doses of 0–5 krad and 5 krad/min for higher doses. After irradiation, cells were lysed in SSC containing 0.2% sodium dodecyl sulfate, and the DNA was prepared and chromatographed as in Figure 1.

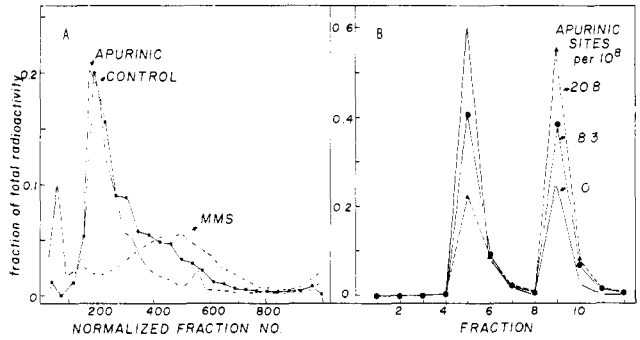

FIGURE 7: (A) Stability of apurinic sites to S_1 nuclease. Raji cells were incubated for 48 h with [^{14}C]thymidine (0.1 μCi/mL; 45 mCi/mM) and chased 16 h in cold medium. The cells were lysed in separate tubes and treated with RNase and Pronase as described in the text. Lysates at pH 7.0 were heated at 70 °C for 0, 3, 5, 10, or 20 min, the pH was lowered to pH 5.5 and the lysates were heated at pH 5.5 for an additional 0, 3, 5, 10, or 20 min to give a total of 0, 3, 5, 9, or 19 apurinic sites per 10^8 (Lindahl and Nyberg, 1972). The lysates were sheared once through an 18-gauge needle and 20 μL was added to the top of an S_1 shelf gradient as described in the text. Cells treated with 400 μg/mL MMS were lysed on top of the S_1 gradient. Centrifugation was for 2 h at 40 000 rpm and 20 °C in the SW 50.1 rotor of the Beckman L3 centrifuge. Only samples heated for 0 and 20 min are shown. Intermediate heating times gave identical results. (B) Shear sensitivity of apurinic sites. Raji cells were labeled by overnight incubation with [^{3}H]thymidine. The cells were chased for 60 min with 1 μg/mL unlabeled thymidine, washed, and lysed with sarkosyl (0.2%) and Triton X-100 (0.2%) in SSC. After Pronase and RNase treatment, the pH was lowered to pH 5.1 and the lysates were heated 0, 2, and 5 min at 70 °C to give 0, 8.3, and 20.8 apurinic sites per 10^8. After incubation with S_1 nuclease and excess Zn ion at pH 4.6 to remove any single-stranded ends, the lysates were sheared, immediately brought to pH 7.2, and chromatographed on BND-cellulose.

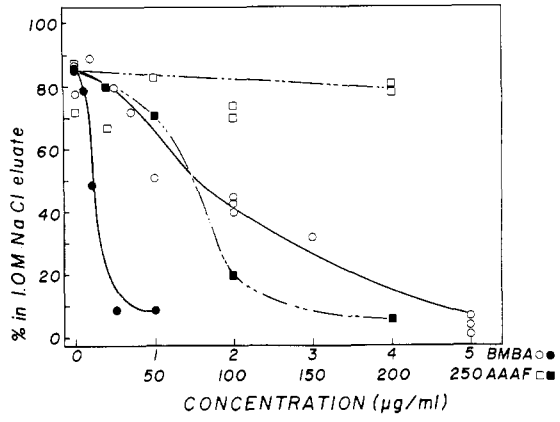

FIGURE 8: Adherence of DNA to BND-cellulose induced by reaction with aromatic carcinogens. (A) In vitro, solid symbols. Radioactive labeled double-stranded DNA was prepared from cell lysates by adsorption to BND-cellulose and elution with 1.0 M NET. After extensive dialysis, aliquots (0.5 mL of 10 μg of DNA/mL) were treated at 37 °C with either AAAF or BMBA at the concentrations indicated. Treatment times were 20 min for BMBA and 60 min for AAAF. After extraction of unreacted carcinogen with ether, the DNA samples were rechromatographed on BND-cellulose. (B) In vivo, open symbols. Labeled cells (3–6 × 10^5 cells/mL in a total volume of 5 mL of medium) were treated for 30 min with either BMBA or AAAF at the concentrations shown. Cells were washed free of unreacted carcinogen and the DNA was prepared and chromatographed as in Figure 1. (Circles) BMBA treatment; (squares) AAAF treatment.

tected by adherence to BND-cellulose using ionizing radiation. X-rays induce breaks in cellular DNA. If the cells are irradiated at low temperature and immediately lysed, the rejoining of breaks or their enzymatic conversion to "gaps" is unlikely. The efficiency with which single-strand breaks are introduced into cellular DNA of mammalian cells is 50 eV per break in air (Ormerod, 1976). Raji cells were irradiated with a series of x-ray doses calculated to give the range of single-strand breaks observed in our experiments with MMS. A dose of 15 krad applied to cells kept at 0 °C during irradiation and lysed and sheared immediately thereafter corresponds to 200 μg/mL of MMS in its ability to make about 50% of the DNA adhere to BND-cellulose (Figure 6). We calculate that this x-ray dose induced 2.45 breaks per 10^8 daltons of DNA. This value should be compared with 1.2 S_1 sensitive interruptions per 10^8 daltons for DNA from MMS-treated cells, treated at a concentration which makes DNA adhere to approximately the same extent.

Adherence to BND-cellulose and S_1 sensitivity could be due to apurinic sites in DNA. This possibility was tested by assaying the S_1 sensitivity and adherence after shear of high molecular weight DNA extracted from Raji cells and depurinated by heating at 70 °C (Figure 7).

DNA from ^{14}C-labeled Raji cell lysates treated with RNase and Pronase was heated at pH 5.5 for times sufficient to give 3, 5, 9, or 19 apurinic sites per 10^8 daltons (Lindahl and Nyberg, 1972). As a control we used DNA from Raji cells treated with 400 μg/mL of MMS for 2 h and then lysed on top of the S_1 gradient. This MMS dose induces a measured 2.3 breaks per 10^8 (Figure 4). Since the neutral sucrose gradient profiles of depurinated and control DNA after S_1 digestion were identical and very different from the profile of DNA from MMS-treated cells we conclude that S_1 nuclease does not recognize apurinic sites under our conditions. However, depurinated DNA (8–21 apurinic sites/10^8) did adhere to BND-cellulose after shearing through a 20 gauge needle (Figure 7B). Although apurinic sites may contribute to the shear induced adherence of MMS-treated DNA, single-strand breaks must be responsible for all of the S_1-sensitive sites.

Response to Aromatic Adducts. DNA adducts can be produced by treatment of cells with the activated carcinogens BMBA and AAAF. Depending on the compound used, treatment of cells may yield DNA molecules which adhere to BND-cellulose. Treatment of DNA with BMBA leads to adherence at lower concentration than is effective when cells are treated (Figure 8). The efficiency with which different lesions make DNA adhere to BND cellulose can be compared for different compounds (Figure 9). More AAAF than BMBA molecules are required to produce equivalent adherence probably because of greater hydrophobic interaction of BMBA with the column material.

There is a major difference between binding induced by BMBA and by MMS. Although binding produced by treat-

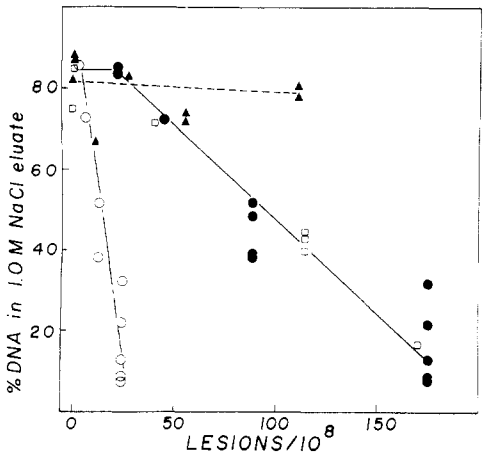

FIGURE 9: Adherence of DNA to BND-cellulose as a function of the level of reaction with different compounds. The level of reaction with cellular DNA was determined with radioactive drugs. Cells were treated with AAAF or BMBA as outlined in Figure 7 with [9-^{14}C]AAAF (21 mCi/mmol), [^{3}H]BMBA (800 mCi/mmol), or with [^{14}C]methyl methanesulfonate (52 mCi/mmol) for 60 min at 37 °C. Cells were washed and the DNA was extracted and banded on CsCl gradients. The extent of reaction was calculated from the absorbancy and acid-insoluble radioactivity which banded at a density of 1.7 g/cm^3. The values for adherence to BND-cellulose (ordinate) are taken from Figures 1 and 7. Symbols: (▲) AAAF treatment; (●) total methylation by MMS; (□) BMBA treatment; (○) methylation by MMS excluding 7-Me-guanine.

TABLE II: Lack of S_1 Sensitivity of DNA from AAAF and BMBA Treated Cells.[a]

Compound	Dose (μg/mL)	Lesions per 10^8	cpm sedimenting: On shelf	cpm sedimenting: In gradient	Ratio: gradient / shelf
None			3920	726	0.18
			4383	1115	0.25
AAAF	100	55	5304	920	0.17
	200	110	5484	995	0.18
BMBA	2	100	4974	1092	0.22
	5	250	6406	1032	0.16

[a] Cells were treated with AAAF and BMBA and then prepared and centrifuged as described in the legend to Figure 2.

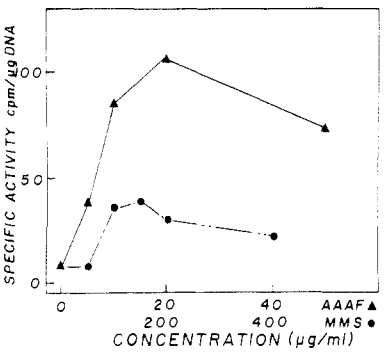

FIGURE 10: Excision repair in Raji cells treated with MMS or AAAF. Exponentially growing cells were preincubated with 10 mM hydroxyurea for 30 min at 37 °C before the addition of MMS or AAAF at the concentrations shown. [^{3}H]Thymidine (10 μCi/mL, 11-17 Ci/mmol) was added simultaneously with the drug and cells were incubated for a further 60 min at 37 °C. After removal of unreacted drug and unincorporated isotope, lysates were prepared and DNA was chromatographed as in Figure 1. Repair synthesis was calculated from the absorbance (A_{260}) and acid-precipitable radioactivity of the material which eluted with 1.0 M NET. (●) MMS-induced repair; (▲) AAAF-induced repair.

ment in vivo with BMBA is as efficient per lesion as is treatment with MMS (Figure 9), we think this agreement is fortuitous. MMS-induced adherence is associated with interruptions in the DNA detectable on neutral sucrose gradients after S_1 nuclease treatment. Concentrations of BMBA which lead to extensive adherence of cellular DNA to BND cellulose do not result in S_1 sensitive sites (Table II). AAAF adducts, equal in number to those produced by MMS treatment (Table III), are also ineffective in inducing S_1 sensitivity at the concentrations and conditions of our assay. The difference between AAAF and BMBA, on the one hand, and MMS, on the other, is even more striking because of the observation (Prakash and Strauss, 1970; Lawley and Orr, 1970) that the major MMS adduct, 7-methylguanine, is ignored by cells even though the product of its spontaneous depurination may be converted into an inactivating lesion. A theoretical curve, corrected for the inert nature of 7-methylguanine by subtraction from the total methylation, is therefore included along with the experimental data of Figure 8. The remaining MMS sites include 3-methyladenine adducts which are rapidly depurinated both spontaneously (Lawley and Brookes, 1963) and possibly by action of the DNA *N*-glycosidase (Lindahl, 1976).

The difference between MMS and AAAF (or BMBA) is not related to their efficiency in inhibiting DNA synthesis (Table III). Concentrations of MMS and AAAF which inhibit DNA synthesis to approximately equal extents result in vastly different adherence to BND-cellulose.

Measurements of the repair of chemically induced damage in DNA by the BND-cellulose method (Scudiero et al., 1975) usually indicate some dose at which maximum repair synthesis occurs. We do not suppose this maximum has any particular theoretical meaning since a number of events could account for the effect, e.g., an increase in permeability at high dose resulting in a loss of the nucleotides required and a consequent decrease in apparent repair activity. However, it is useful to determine the empirical point of maximum repair. Adherence induced by both MMS and AAAF is compared in our experiments at doses well past those which induce maximal repair in intact cells (Figure 10). The differences in detectable strand interruptions after treatment with the two compounds therefore are not likely to be due to differential saturation of the MMS repair system.

Adherence to BND cellulose is the result of at least two different properties: (a) binding of single stranded regions produced by shear at single-strand breaks in the DNA, and (b) binding of aromatic residues directly to the resin. Our results also indicate a difference in the mode of action of different compounds. MMS treatment of cells induces breaks in the DNA. Neither AAAF nor BMBA treatment results in detectable DNA strand interruptions in this human cell line, although excision repair of damage induced by these compounds is readily detected (Scudiero et al., 1975).

Discussion

The discovery of *N*-glycosidases specific for alkylated DNA (Lindahl, 1976; Kirtikar and Goldthwait, 1974) helps to clarify some of the confusing observations on the repair of chemically induced damage. It seems likely that excision repair occurs by two separate pathways: (a) the base excision (apurinic) repair pathway by which reacted DNA depurinates either spontaneously or via the action of an *N*-glycosidase; apurinic DNA is then incised by an endonuclease and repair follows, utilizing exonuclease, polymerase and ligase activities; and (b) the nucleotide excision pathway (Duncan et al., 1976) in which an incision enzyme specific either for pyrimidine dimers,

TABLE III: Comparison of MMS and AAAF Effects.

Compound	μg/mL	Lesions per 10^8 Total	Lesions per 10^8 −7MeG	Interruptions per 10^8 (S_1 sensitivity)	% residual DNA synthesis	% eluting in 1.0 M NaCl
None					100	84.5 ± 5.9
MMS	100	44	6	0.58	41	73
	200	88	12	1.15	17	44 ± 7
	400	176	25	2.3	6	16.5 ± 8
AAAF	50	28			9	83
	100	55		0	5	72 ± 2.8
	200	110		0		79.5 ± 2.1

chemical adducts, or for regions of local denaturation is followed in its action by exonuclease, polymerase, and ligase activities. Free purine derivatives are the first products of the base excision pathway; nucleotides are produced by the second (Duncan et al., 1976). There is no requirement that the exonucleases, polymerases, and ligases be identical in the two pathways.

Our data can be accounted for by the following hypothesis: MMS repair occurs via the apurinic pathway. Endonuclease activity is in excess in the cells we study and DNA with breaks can accumulate as an intermediate. The repair enzymes act in a relatively distributive fashion and the relative amounts of endonuclease as compared with exonuclease, polymerase, and ligase activity determine whether the final result is a repair event or degradation with the production of gaps. The hypothesis predicts that repair patch size after MMS treatment should be related to the dose and be small at low MMS dose when sufficient ligase and polymerase molecules should be available to compete with nuclease and close nicks before they can be extended. However, no data are yet available to test this prediction.

We suppose that AAAF damage is repaired in excision competent human cells via the nucleotide excision pathway as is most ultraviolet-induced damage (Regan and Setlow, 1974), although the endonucleases for UV- and for AAAF-induced lesions may not be identical (Ahmed and Setlow, 1977; Amacher et al., 1977). Biological evidence suggests that there is a limiting enzymatic step in this pathway in human cells (Edenberg and Hanawalt, 1973), possibly in the first incision (UV-endonuclease) step. DNA breaks are therefore rare and special techniques, such as alkaline elution which surveys very large molecules, are necessary for their detection (Fornace et al., 1976). We suppose that once an incision is made, exonuclease or exonuclease plus polymerase may behave in a relatively processive fashion so that the repair patches are large and either ligase is not able to compete efficiently for substrate or the timing of the ligation event is controlled in some way. According to this hypothesis the AAAF patches should be long relative to those involved in MMS repair and should be constant in size and independent of the concentration of endonuclease.

There is evidence already published from this laboratory to support the hypothesis of constant AAAF-induced patch size (Scudiero et al., 1976). Unstimulated human peripheral blood lymphocytes are deficient in repair capability relative to concanavalin A stimulated cells or to the Raji lymphoblast line. The rate of removal of AAAF adducts and the rate of repair synthesis differ in unstimulated or stimulated lymphocytes and lymphoblasts. Notwithstanding this variation, the quantitative ratio of the rate of adduct removal to the rate of base insertion was constant for the three cell types indicating a constant patch size. Setlow et al. (1972) have shown that the patch size for UV repair is similar in mouse and human cells even though thymine dimer excision ability in mouse cells is only 10–20% of that in human cells.

Our failure to observe interruptions in the DNA of human cells even at high doses of AAAF implies that any incision is immediately converted into a repair patch, as required by the hypothesis. Since AAAF-induced strand interruptions are not observed even at concentrations which inhibit the repair process (Figure 10), we conclude that either treatment of cells with high AAAF concentrations inhibits endonuclease activity or that there is some regulatory system in human cells which controls endonuclease activity in a way which makes its action at all doses of AAAF coordinate with other activities in the repair pathway; e.g., there is a repair enzyme complex. It has been reported (Kirtikar et al., 1975) that *E. coli* cells contain an enzyme activity which recognizes BMBA-treated DNA and releases alkylated purines. If such an enzyme occurs in Raji cells, it does not appear to result in the accumulation of apurinic sites and breaks.

The hypothesis of a nonprocessive apurinic pathway, not limited by endonuclease activity, accounts for the finding that increased numbers of interruptions are observed at higher MMS doses. The interruptions observed are presumably those which cannot be repaired by a saturated polymerase plus ligase system. This interpretation is supported by the previous finding that more interruptions are observed in unstimulated peripheral blood lymphocytes deficient in repair activity (Scudiero et al., 1976).

The breaks observed in the DNA of MMS-treated cells as compared with AAAF treatment are not related to the greater toxicity of MMS. The 37% survival dose for Raji cells as measured by cloning ability in agarose is about 5.5 μg/mL for AAAF and 250 μg/mL for MMS (unpublished observations). At these doses, 50% of the DNA from MMS-treated cells adheres to BND-cellulose and there are about 1.5 strand breaks per 10^8 daltons after 1 h of treatment as measured by the S_1 sucrose method. AAAF treated cells at both the 37% survival and much higher doses yield DNA which does not adhere and has no breaks. When compared at either equivalent lethality or equivalent number of adducts (Table III), there is a qualitative difference in our ability to detect interruptions in DNA, indicating we think, a difference in the repair pathways.

This investigation also emphasizes the utility of single-strand specific nucleases for the detection of strand interruptions in DNA. This technique was used by Meneghini (1976) using purified DNA and the neurospora endonuclease for the detection of interruptions in newly synthesized DNA. S_1 nuclease has recently been used by Sheridan and Huang (1977) to measure repair by determining the resistance to alkaline denaturation of DNA with varying numbers of breaks. In these studies we have used S_1 nuclease applied to lysates on the top

of a gradient for the analysis of single-strand interruptions induced by chemical carcinogens. In combination with the McGrath–Williams technique (1966) this enzyme treatment is a useful analytical tool for exposing single-strand breaks.

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Addition of Glucosamine and Mannose to Nascent Immunoglobulin Heavy Chains†

L. W. Bergman and W. M. Kuehl*

ABSTRACT: We have investigated the process of protein glycosylation in an attempt to answer the question of whether glucosamine and mannose are added to nascent chains prior to chain completion or only to completed chains after release from the ribosome. The MPC 11 mouse plasmacytoma cell line used in these studies synthesizes a glycosylated γ_{2b} heavy chain which accounts for 12% of the total protein synthesis. Nascent chains were separated from completed chains by ion-exchange chromatography of solubilized ribosomes on QAE-Sephadex. Our results indicate that both glucosamine and mannose are incorporated into nascent heavy chains prior to chain completion and release from the ribosome. Gel analysis of specifically immunoprecipitated nascent chains indicates that the carbohydrate moiety can be added to the nascent heavy chains very soon after the presumptive asparaginyl glycosylation site (CH2 domain) is synthesized on the ribosome.

In recent years, a substantial amount of work has been done to determine the structure and biosynthesis of the carbohydrate portion of immunoglobulin (Uhr, 1970; Potter, 1972; Bevan et al., 1972; Melchers and Andersson, 1974; Nisonoff et al., 1975; Kuehl, 1977). One or more carbohydrate groups appear to be attached covalently to the constant region of virtually all heavy chains and to the variable region of several light chains.

Using cell-fractionation methods the subcellular biosynthesis sites and the order of addition of the various carbohydrate residues to the heavy or light chains of different tumors have been determined (Schenkein and Uhr, 1970; Melchers, 1973; Choi et al., 1971). Several groups obtained evidence which prompted them to suggest that the initial glycosylation event occurs while the polypeptide chain is still bound to the

† From the Department of Microbiology, Jordan Medical Building, University of Virginia, Charlottesville, Virginia 22901. *Received April 28, 1977.* Research funds were provided through a grant from the National Institutes of Health (AI 12525-02) to Dr. W. M. Kuehl.