We have investigated the processing of \(O^6\)-methylguanine (\(O^6\)-MeGua) in plasmid DNA by extracts of human cell extracts. The in vitro DNA synthesis was non-semiconservative and depended on the presence of \(O^6\)-MeGua in the substrate. The involvement of DNA polymerase \(\delta\) or \(\epsilon\) and proliferating cell nuclear antigen but not single-strand binding protein was indicated by partial fractionation, inhibitor, and antibody studies. Processing of \(O^6\)-MeGua is not via the UV nucleotide excision repair pathway since additional component(s) are apparently required to perform repair synthesis on the methylated substrate. This is the first direct demonstration of DNA repair synthesis provoked by \(O^6\)-MeGua in DNA. Since \(O^6\)-MeGua is not excised from DNA by Mex cells, it represents a novel type of processing of the methylated base that may be involved in its cytotoxicity.

\(O^6\)-Methylguanine (\(O^6\)-MeGua) in DNA is the major contributor to the biological effects of methylating carcinogens or chemotherapeutic agents. The relative efficiencies of incorporation of cytosine and thymine opposite \(O^6\)-MeGua of DNA polymerses in vitro are consistent with its role in inducing the G:C to A:T transitions observed in the absence of repair by MGMT, Mex- cells do process the methylated base (for review, see Ref. 8). Transfection of the cloned human MGMT cDNA has demonstrated that resistance to \(O^6\)-MeGua either by MGMT or by an alternative pathway, but instead is apparently a consequence of the ability to tolerate \(O^6\)-MeGua in DNA. Indeed, these tolerant cell lines have provided evidence that cytotoxicity may not be due directly to the presence of the \(O^6\)-MeGua in DNA but rather to the cell's attempts to process the methylated base (for review, see Ref. 8).

The possible pathways by which \(O^6\)-MeGua in DNA might be processed by cells have not been systematically investigated but there is some evidence from cellular studies that in the absence of repair by MGMT, Mex cells do process the methylated base. Sedimentation velocity data on nucleoids from Mex+ or Mex- cells indicated that treatment with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) induces a similar degree of relaxation of DNA. Mex- cells fail to reconstitute rapidly sedimenting nucleoids even after prolonged incubation. This is in contrast to Mex+ cells in which restitution occurs within 2–4 h (9) and indicates that Mex- cells contain persistent discontinuities in their DNA. In addition, treatment with MNNG induces more non-semiconservative DNA synthesis in Mex- cells than in Mex+ cells (4). Since the only known DNA repair defect in Mex- cells is the absence of MGMT, both of these phenomena are related to the persistence of \(O^6\)-MeGua in the DNA of Mex- cells.

Measurement of DNA synthesis in vitro using a modified template and an oligonucleotide primer has defined the abilities of important DNA adducts to block chain elongation by several purified DNA polymerases. In the particular case of alkylating agents, the methylated base 3-methyladenine (3-MeAde) and abasic sites derived from the hydrolytic loss of N-methylated purines, both of which are potentially lethal lesions (10, 11) have each been identified as blocks to DNA elongation in this simplified system (12). Despite its cytotoxicity, \(O^6\)-MeGua is not detected as a block to replication in this assay. In order to investigate whether a block to replication might be produced by the cell, we have examined the processing in vitro of the plasmid pSVori containing a low level of methylation damage by replication-competent human cell extracts. Methylation of the plasmid provoked a form of T antigen-independent DNA synthesis. The DNA synthesis
was not seen with unmethylated plasmid, or with plasmids methylated with an agent that induces very little O'Fl-MeGua, and could be abolished by a prior removal of O'Fl-MeGua with the specific DNA repair enzyme MGMT. It represents a novel in vitro manifestation of a processing event, other than direct demethylation, at O'Fl-MeGua residues in DNA.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLaMR, an MGMT-defective variant cell line, was a gift of J. Thomale (University of Essen, Germany). Cells were routinely cultured in Dulbecco’s modification of Eagle’s medium (Flow Laboratories) supplemented with 10% fetal calf serum (Flow), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cultures were incubated at 37 °C in 5% CO2 and 95% relative humidity.

Chemicals and Reagents—MNU (Sigma) was dissolved in dimethyl sulfoxide or 10 mM potassium acetate, pH 5.0, and stored at −20 °C. Methyl methanesulfonate (MMS) was diluted in dimethyl sulfoxide or phosphate-buffered saline immediately before use. Ribonuclease A was obtained from Boehringer, Proteinase K from Sigma, and the restriction endonuclease DpnI from New England Biolabs. Endonuclease IV was purified from BW9109 xthA by published procedures (13). A recombinant MGMT comprising the human cDNA sequence fused to a fragment of the bacteriophage λ protein A was purified to essential homogeneity as described (14). MGMT treatment of methylated plasmid was carried out in 100 mM Hepes-KOH, 1 mM EDTA, 0.1 mM dithiothreitol (DTT). SV40 T antigen was prepared as described by Simanis and Lane (15) from recombinant SV40-SVR111-infected 293 cells. T antigen was immunoaffinity purified on a monoclonal anti-T antigen antibody (PAb 419) by published procedures (16). A recombinant MGMT comprising the human HeLaMR cells adapted for growth in suspension following a procedure of the provided by R. Wood (Imperial Cancer Research Fund, Clare Hall Laboratories). Antibodies 7OC and 34A against the 70- and 34-kDa subunits of human single-stranded DNA-binding protein (RP-A) were made available by M. K. Kenny, University of Dundee, and R. Wood.

Preparation of Cell Extracts—Cell extracts were prepared from human HeLaMR cells adapted for growth in suspension following a modification of the procedure of Li and Kelly (16). Briefly, cells were grown in spinner culture in Eagle’s minimal essential medium supplemented with 10% fetal calf serum. Approximately 5 × 107 cells in mid-log phase were harvested by centrifugation (1000 g, 5 min). The cell pellet was washed in 250 ml of ice-cold hypotonic buffer (20 mM Hepes-KOH, pH 7.5, 5 mM MgCl2, 0.5 mM DTT) containing 0.25 M sucrose followed by 250 ml of ice-cold hypotonic buffer alone. The washed cell pellet was suspended in a minimal volume of hypotonic buffer and the cells allowed to swell on ice for 30 min. Aprotinin (Sigma) (0.5%, v/v) was added and cells were disrupted with 10 strokes of a tightly fitting pestle in a Dounce homogenizer. After 30 min on ice, the suspension was centrifuged for 20 min at 10,000 × g at 0 °C. NaCl was added to the supernatant to a final concentration of 100 mM and extracts were centrifuged at 100,000 × g for 1 h at 0 °C. The supernatant was removed, aliquoted, snap frozen in ethanol/dry ice and stored at −70 °C. The protein content of the extracts as determined by the Coomassie Blue method (17) was usually 15–25 mg/ml.

Whole-cell extracts were prepared as described (18).

Enzyme Assays—3-Methyladenine-DNA glycosylase activity of cell extracts was determined by measuring release of ethanol-soluble material from DNA treated with [methyl-3H]dimethyl sulfate (1.1 Ci/mmol) as described (19). Briefly, extract was incubated with methylated DNA in 100 μl of 70 mM Hepes-KOH, pH 7.8, 1 mM EDTA, 10 mM DTT for 60 min at 37 °C. The reaction was stopped by the addition of carrier DNA, NaCl to 0.2 M, and 300 μl of ethanol. After removal of precipitated material, the radioactivity in the supernatant was quantitated by scintillation counting.

Plasmid Methylation—The plasmid pSVori (20) (a gift of R. Posenti, Istituto di Neurobiologia, Consiglio Nazionale delle Ricerche, Rome) was prepared from E. coli DH5 by the alkaline lysis procedure and purified by CsCl-ethidium bromide centrifugation. The purified plasmid comprised >95% form I monomer DNA. Purified pSVori (10 μg) was methylated with varying concentrations of MNU for 30 min at 37 °C in TE, pH 7.5. O'Fl-MeGua is the most stable methylated purine, whereas 3-Me Ade and 7-methylguanine (7-MeGua) are susceptible to spontaneous hydrolytic release from DNA at significantly increased rates. We used the increased lability of the N-methylated purines in DNA to estimate the amount of O'Fl-MeGua produced by MNU treatment of pSVori. The plasmid was methylated with concentrations of MNU in the range 0.05–0.5 mM for 30 min at 37 °C To quantitate the number of methylated bases, the treated plasmid was analyzed by agarose gel electrophoresis. MNU treatment alone did not introduce nicks into the plasmid (Fig. 1a). Following partial depurination of an aliquot by hydrolysis at neutral pH (30 min at 70 °C), the number of induced apurinic/apyrimidinic (AP) sites was quantitated by scintillation counting. The radioactivity in the supernatant after removal of precipitated material, the radioactivity in the supernatant was quantitated by scanning densitometry of the photographic negative of the gel (omitting lane 5) shown in a, right panel.

FIG. 1. Quantitation of methylation damage in pSVori DNA. a, pSVori DNA (10 μg) was treated with MNU at final concentrations of 0, 0.05, 0.1, 0.25, or 0.5 mM (lanes 1–5, respectively) for 30 min at 37 °C. Aliquots were analyzed by agarose gel electrophoresis in the presence of ethidium bromide (left panel). Aliquots of MNU-treated DNA (lanes as left panel) were partially depurinated by heating at neutral pH for 30 min at 37 °C and digested with excess purified E. coli endonuclease IV prior to analysis on the gel (right panel). b, 1 μg of pSVori was treated for 30 min at 37 °C with MNU at 0 (lanes 1, 2, and 7), 0.12 (lane 3), 0.24 (lane 4), or 0.48 mM (lanes 5 and 8). After recovery of the plasmid by ethanol precipitation, all samples were incubated with endonuclease IV (8 units) for 30 min at 37 °C either before (lanes 1–5) or after (lanes 7 and 8) heating at 70 °C and neutral pH for 30 min. Aliquots (300 ng) of the plasmid were analyzed by agarose gel electrophoresis in the presence of ethidium bromide. c, quantitation of MNU + heat-induced AP sites. The proportion of supercoiled plasmid molecules remaining after heat and endonuclease IV digestion was determined by scanning densitometry of the photographic negative of the gel (omitting lane 5) shown in a, right panel.
DNA Repair Synthesis at $\mathrm{O}^6$-Methylguanine

determined by gel electrophoresis after digestion with excess (8 units (Ref. 13)) purified E. coli endonuclease IV. Digestion with endonuclease IV without prior heating confirmed that detectable AP sites were not introduced directly by MNU treatment (Fig. 1b). The combined action of heat and AP endonuclease produced an MNU concentration-dependent increase in the proportion of form II molecules, and at the highest concentration of MNU (4 mM) no detectable form I DNA was observed (Fig. 1a, lane 5, right). The average number of nicks per plasmid molecule was determined from the Poisson distribution after quantitation by scanning densitometry, correction for differential binding of ethidium bromide, and subtraction of the AP sites together with about $10^7$ MeGua residues/plasmid. The pSVori plasmid DNA treated with 0.24 mM MNU that was routinely used to measure DNA synthesis therefore contained about 1 $\mathrm{O}^6$-MeGua residue/molecule and was introduced into the cell-free extract in a preparation almost entirely superoiled form (20% cell I form).

Analysis of plasmids treated with MMS was carried out in an identical fashion. A concentration of 0.12 mM introduced sufficient methylation to yield 2–3 AP sites per plasmid after neutral thermal hydrolysis. This corresponds to about 10 $\mathrm{Me}^7$-Gua and 1 $\mathrm{Me}^3$-Ade bases per plasmid after methylation to yield 2–3 AP sites per plasmid molecule (22), we calculate that this level of depurination of 130 kJ/mol (22), we calculate that this level of depurination of 130 kJ/mol (22), we calculate that this level of depurination of 130 kJ/mol (22), we calculate that this level of depurination of 130 kJ/mol (22). Taking $\omega$ values for depurination of 3-MeAde and 7-MeGua as 26 and 155 h, respectively, at pH 7.5 and 37°C (21), and an activation energy for depurination of 130 kJ/mol (22), we calculate that this level of depurination reflects an initial 3-MeAde level of 0.5–1 per plasmid.

Since MNU produces methylated DNA purines in the ratios of 1:0.12:0.1 for $\mathrm{Me}^7$-Gua:$\mathrm{Me}^3$-Ade:$\mathrm{O}^6$-MeGua (23), this concentration of MNU introduces, on average, at most one $\mathrm{O}^6$-MeGua and 3-MeAde together with about 10 $\mathrm{Me}^7$-Gua residues/plasmid. The pSVori plasmid DNA treated with 0.24 mM MNU which was routinely used to measure DNA synthesis therefore contained about 1 $\mathrm{O}^6$-MeGua residue/molecule and was introduced into the cell-free extract in a preparation almost entirely superoiled form (20% cell I form).

UV-irradiated DNA enriched for pyrimidine dimer photoproducts (18, 24) was provided by M. Biggerstaff (Imperial Cancer Research Fund, Clare Hall Laboratories).

In Vitro Repair Synthesis by Cell-free Extracts—For repair synthesis, reaction mixtures (50 $\mu$M) contained 30 mM Hepes-KOH, pH 8.0, 7 mM MgCl$_2$, 0.5 mM DTT, 100 $\mu$M each dTTP, dCTP, dGTP, 7.5 $\mu$M [3H]-dATP (3000 cpm/pmol), 4 mM ATP, 40 mM creatine phosphate, and 1 $\mu$g of creatine phosphokinase (rabbit muscle type I; Sigma). Standard reaction mixtures also contained 300 ng of pSVori either unmethylated or treated with MNU. Reaction mixtures for DNA replication were the same except for the addition of CTP, UTP, and GTP (200 $\mu$M each) and a DATP concentration of 36 $\mu$M (670 cpm/pmol). DNA synthesis was initiated by the addition of up to 400 $\mu$g of cell extract and incubation was carried out at 37°C for various times. Reactions were terminated by the addition of EDTA to 10 mM. To determine the extent of DNA synthesis, 50-$\mu$l reaction mixtures were digested onto 1.5 × 1.5-cm DE81 paper squares. The filters were washed 3 times with 500 ml of 0.5 M Na$_2$HPO$_4$, once with 500 ml of water, once with 200 ml of 70% cold ethanol, dried, and the retained radioactivity was determined by liquid scintillation counting.

To prepare samples for agarose gel electrophoresis, 20 mM EDTA and 0.25% sodium dodecyl sulfate were added together with ribonuclease A (20 $\mu$g/ml) and the mixtures were incubated for 15 min at 37°C. Proteinase K (1 mg/ml) was then added and incubation continued for a further 1 h at 37°C. Following extraction once with 500 ml of 0.5 M Na$_2$HPO$_4$, once with 500 ml of water, once with 200 ml of 70% cold ethanol, dried, and the retained radioactivity was determined by liquid scintillation counting.

Nucleotide excision repair of UV-irradiated DNA was assayed by published procedures (18, 24). Briefly, reaction mixtures containing 250 ng of unirradiated pHM14 (14) and 250 ng of UV-irradiated pBluescript KS* in 50 $\mu$l of 40 mM Hepes-KOH, pH 7.8, 70 mM KCl, 5 mM MgCl$_2$, 0.5 mM DTT, 10 $\mu$M each dNTP, [3H]-dATP (0.2 $\mu$Ci/ $\mu$l), 2 $\mu$M ATP, 40 mM creatine phosphate, 2.5 $\mu$g of creatine phosphokinase, and 18 $\mu$g of bovine serum albumin were supplemented with cell extract (0–200 $\mu$g of protein). After incubation for 3 h at 30°C, plasmids were recovered, linearized with BamHI, and DNA separated by electrophoresis overnight in 1% agarose gels containing 0.5 $\mu$g/ml ethidium bromide. Incorporation of dAMP was visualized by autoradiography.

Antibody Inhibition—Limiting amounts of cell extracts were combined with 1 $\mu$g of antibody and the mixture was incubated at 20°C for 15 min. DNA replication or repair synthesis reactions were initiated by the addition of the appropriate reaction mixture containing unmethylated plasmid and $\lambda$ antigen (replication) or MNU-treated plasmid without $\lambda$ antigen (repair synthesis). Incorporation of radioactive DAMP into material retained on DE81 paper was determined as above.

Phosphocellulose P11 Chromatography—Cell extracts (5 ml) containing approximately 80 mg of protein were prepared as outlined above and applied to a column (1.5 cm × 1 cm diameter) of phosphocellulose P11 (Whatman) equilibrated in 50 mM Tris-HCl, pH 7.5, 10 mM DTT, 0.1 M NaCl. The unbound material (10 ml) was retained and after washing with the same buffer, the bound protein was eluted with 10 ml of the same buffer containing 1 M NaCl. All operations were carried out at 4°C.

**RESULTS**

T Antigen-independent DNA Synthesis Associated with $\mathrm{O}^6$-MeGua Residues—With the unmodified template, incorporation of DAMP by extracts of HeLaMR cells was absolutely dependent on exogenous SV40 T antigen (Fig. 2, lanes 1–3). In contrast, when extracts were incubated with plasmids that had been treated with 0.24 mM MNU to introduce around one $\mathrm{O}^6$-MeGua per molecule, DAMP was incorporated even in the absence of T antigen (Fig. 2, lanes 4 and 5). Essentially all the radioactivity incorporated in the absence of T antigen remained sensitive to digestion with DpnI which specifically cuts DNA that is fully methylated at -GATC- sequences (Fig. 3).

![Fig. 2. Replicative and repair DNA synthesis on pSVori by cell-free extracts of HeLaMR cells. Lanes 1–3, DNA replication. 200 $\mu$g of HeLaMR cytoplasmic extract and 300 $\mu$g of unmethylated pSVori in the presence or absence of 0.5 $\mu$g of purified T antigen as shown were incubated for 60 min (lane 2) or 90 min (lanes 1 and 3) in the standard in vitro DNA replication mixture. The reaction products were isolated, digested with DpnI, and analyzed by agarose gel electrophoresis followed by autoradiography. Lanes 4–8, DNA repair synthesis. 200 $\mu$g of cytoplasmic extract was combined with either untreated or MNU-treated (0.24 mM MNU) pSVori DNA as shown and incubated in DNA repair synthesis reaction mixture for 60 min at 37°C. DNA was recovered and analyzed by agarose gel electrophoresis, either with or without prior digestion with DpnI as indicated, followed by autoradiography. The MNU-treated plasmid (300 ng) substrate in lane 6 was preincubated with a purified recombinant human MGMT (0.5 units for 15 min at 37°C as described under "Experimental Procedures") prior to its incubation with cell extract.](image-url)
2, lanes 7 and 8). These data indicate that the incorporated nucleotides were probably in patches of non-conservative DNA synthesis. We will refer to this incorporation as repair DNA synthesis without implying that functional repair has occurred. The incorporated radioactivity was associated with both form I and form II molecules and at higher MNU concentrations, 540% of labeled plasmids were restored to a closed superhelical form. Direct demethylation of the DNA occurred. The incorporated radioactivity was associated with DNA synthesis. We will refer to this incorporation as repair DNA synthesis without implying that functional repair has occurred.

In view of its low abundance and its relatively poor ability to serve as a substrate for MGMT, it is possible that this methylated base also provokes repair synthesis. In the presence of other than O'-MeGua in the plasmid DNA, O'-MeThy which comprises <1% of the methylation is also a substrate for MGMT. It is possible that this methylated base also provokes repair synthesis. All the radioactivity remaining after MGMT treatment was associated with form I plasmid molecules, suggesting that this particular methylated base in the plasmid DNA, O'-MeGua in the methylated substrate by pretreatment with a purified human MGMT considerably reduced the amount of incorporated radioactivity (Fig. 2, lane 6) indicating that the DNA synthesis is associated with the presence of this particular methylated base in the plasmid DNA. O'-MeGua in the substrate (23) is also a substrate for MGMT. The incorporated radioactivity was associated with form I1 plasmid molecules. The proportion of form I1 molecules increased with increasing MNU concentration.

The dependence of the repair synthesis on the presence of O'-MeGua in the plasmid was confirmed in a series of experiments in which plasmids treated with either MNU or MMS were compared. Treatment for 30 min with 0–1.6 mM MMS methylated the plasmid to similar overall extents as 0–0.96 mM MNU for 30 min as determined by the kind of analysis shown in Fig. 1 (data not shown). Increasing incorporation into the plasmid treated with MNU was observed at concentrations of 0.06–0.96 mM (Fig. 3a). In contrast, MMS-treated plasmid stimulated a very low level of repair synthesis that was independent of dose. The labeled MMS-treated plasmids were essentially all recovered as form I molecules, whereas MNU-induced repair synthesis was associated with nicked, form II, plasmid molecules. The proportion of form II molecules increased with increasing MNU concentration.

The T antigen-independent DNA repair synthesis was also measured as incorporation of radioactivity into material retained by DE81 paper and a comparison of this method and agarose gel analysis is presented in Fig. 3b. Cell extracts incorporated up to 4 pmol of dAMP into the MNU-treated plasmid, whereas treatment with MMS stimulated incorporation of <0.2 pmol of dAMP without an apparent dose dependence. After treatment with 0.24 mM MNU, the concentration used for the standard assays, the maximal incorporation was around 2 pmol. Since the overall methylation of the plasmids is similar in the dose ranges used but MMS introduces 20-fold less O'-MeGua than MNU, the data confirm that the repair synthesis is dependent on the presence of O'-MeGua in the substrate.

**Fig. 3. DNA repair synthesis on MNU- and MMS-treated plasmids.**

a) agarose gel analysis of products. 300 ng of pSVori DNA that had been treated with 0, 0.06, 0.12, 0.24, 0.48, and 0.96 mM MNU (lanes 1–6) or with 0.15, 0.25, 0.35, 0.4, 0.8, and 1.6 mM MMS (lanes 7–12) for 30 min at 37°C was incubated with 200 μg of HeLaMR extract for 60 min in repair synthesis reaction mixture. DNA was recovered and analyzed by agarose gel electrophoresis followed by autoradiography.

b) adsorption of reaction products to DE81 paper. Left panel, MNU-treated plasmid. 300 ng of pSVori DNA treated with 0 (□), 0.03 (●), 0.12 (○), 0.24 (△), 0.48 (●), or 3.0 mM MNU (▲) was incubated with the concentrations of HeLaMR extract shown in a 50-μl repair synthesis reaction mixture. After 30 min at 37°C, 50-μl aliquots were applied to 1.5 X 1.5-cm squares of DE81 paper (Whatman). The bound radioactivity that was resistant to washing in 0.5 M NaHPO4 was determined by scintillation counting as described under "Experimental Procedures." Right panel, MMS-treated plasmid. Incorporation into 300 ng of pSVori DNA treated with 0 (□), 0.1 (○), 0.3 (○), or 1.0 mM MMS (●) was determined as above.
seen with the undamaged plasmid.

Incorporation in the absence of T antigen was absolutely dependent on Mg²⁺ and exogenous ATP together with an ATP-regenerating system (Fig. 4b). This last requirement could be circumvented by the addition of the four ribonucleoside triphosphates which presumably compete for ATPase activities in the extract. Aphidicolin, which is a competitive inhibitor of DNA polymerases α, δ, and ε (28), inhibited incorporation to a maximum level of 70% (Fig. 4b) indicating the probable participation of one or more of these enzymes in the observed repair synthesis. The incompleteness of inhibition by this compound together with the low level of incorporation remaining after MGMT treatment of the methylated plasmid suggests that the residual synthesis might be due to lesions other than 06-MeGua and may be performed by an aphidicolin-resistant DNA polymerase, probably DNA polymerase β. Inclusion of 500 μM dideoxythymidine 5′-triphosphate, an inhibitor of DNA polymerase β, in the standard assay produced ≤10% inhibition of dAMP incorporation (Fig. 4b) confirming that this DNA polymerase makes at most a minor contribution to repair synthesis on an MNU-treated plasmid.

**Partial Characterization of the Requirements of Repair Synthesis**—The replication components present in crude cytoplasmic cell extracts of the type used in these experiments can be partially resolved by chromatography on phosphocellulose P11 (29). Material that remains unbound in the presence of 0.1 M NaCl (designated flow-through) contains the proliferating cell nuclear antigen (PCNA) and RP-A (also known as RF-A or HSSB). The fraction that is adsorbed to the column and requires 1.0 M NaCl for elution contains DNA polymerases α, δ, ε, and β. We resolved the HeLa extracts into these two fractions by adsorption to P11 and step elution with buffer containing 1.0 M NaCl. In the experiment shown in Fig. 5, the extract applied to the column (Fig. 5A) contained 54 units of repair synthesis activity (1 unit = 1 pmol of dAMP/min in the standard assay). The flow-through fraction (Fig. 5B) and the 1.0 M NaCl eluate (Fig. 5C) each contained <0.8 units. When the eluate and flow-through fractions were combined (Fig. 5D), approximately 12 units (20-25%) of repair synthesis activity were recovered indicating that the repair synthesis on MNU-damaged DNA requires components from both fractions for activity. A similar level of repair synthesis was observed if purified PCNA was substituted for the flow-through fraction (Fig. 5D) indicating the probable involvement of DNA polymerase δ or ε.

**Antibodies Against Replication Proteins**—The identity of the factors participating in DNA repair synthesis was further investigated using antibodies to some of the proteins involved in DNA repair or replication. T antigen-dependent DNA replication by cell extracts was completely inhibited by preincubation with antibodies against either DNA polymerase α or the 70- or the 34-kDa subunit of RP-A. In contrast, no significant inhibition of T antigen-independent repair synthesis was observed when extracts were preincubated with the same antibodies either singly or in combination (Fig. 6). In agreement with the observations obtained with crude extracts, preincubation of the phosphocellulose flow-through fraction with either (or both) of the anti-RP-A antibodies did not produce a significant inhibition of the repair synthesis stimulated by addition of the 1.0 M NaCl eluate. All other combinations of antibody and either flow-through or 1.0 M NaCl eluate were equally without effect (data not shown). Taken together with the fractionation properties of the proteins required, it is likely that DNA polymerase δ or ε from the high salt eluate and PCNA, but not RP-A, from the flow-through
fraction are required for MNU-stimulated repair replication.

Comparison with Other Pathways of DNA Repair Synthesis—3-MeAde and 7-MeGua are substrates for a short patch (30) excision repair mediated by a DNA glycosylase (3-MeAde-DNA glycosylase), AP endonuclease (previously demonstrated in cytoplasmic replication extracts (31)), a diesterase (32), and DNA polymerase β. To assess the contribution of short patch excision repair, we measured the level of 3-MeAde-DNA glycosylase in the extracts (19). Fig. 7 shows that this enzyme is present only in low levels in the cytoplasmic extracts. In contrast, the activity of 3-MeAde-DNA glycosylase in whole cell extracts that carry out significant repair synthesis on both MNNG- and MMS-treated DNA is more than 10-fold higher. The standard repair synthesis assay contained 0.1–0.2 pmol of 3-MeAde and incorporation was routinely measured at 0–100 μg of extract (Fig. 3). In this concentration range, 3-MeAde-DNA glycosylase in the extract excised ≤0.03 pmol of methylated base per h from heavily methylated DNA. In addition, the standard 3-MeAde-DNA glycosylase assay is performed at high substrate concentrations. The initial concentration of the methylated base in the repair synthesis assay is 3.10^-8 M. The reported apparent K_m of the human enzyme for 3-MeAde is 3.10^-8 M (33). These data indicate that there is insufficient 3-MeAde-DNA glycosylase activity in the replication extracts to remove a significant amount of the methylated base from the plasmid. Short patch excision repair synthesis at 3-MeAde residues is therefore likely to be limited by the level of the initiating DNA glycosylase and is insufficient to account for the extensive incorporation into MNU-treated plasmid DNA.

To investigate whether the presence of O6-MeGua was stimulating long patch repair synthesis initiated by UV nucleotide excision repair, we tested the ability of cell extracts to carry out DNA repair synthesis on a UV-irradiated plasmid. The UV-irradiated plasmid containing 8–10 pyrimidine dimer photoproducts did not stimulate significant repair synthesis by the cytoplasmic HeLa extract and incorporation was not significantly different from that observed with the untreated plasmid (Fig. 8c). The inability of replication extracts to carry out detectable repair synthesis on UV-irradiated DNA confirms a recent report by Carthy et al. (34). Efficient nucleotide excision repair of UV-damaged DNA is carried out in vitro by whole cell extracts (18). When presented with the MNU-treated substrate, a whole cell extract that was proficient at UV photoproduct excision repair (Fig. 8b) carried out <5% of the synthesis observed with a cytoplasmic replication extract (Fig. 8c). Since whole cell extracts contain high levels of 3-MeAde-DNA glycosylase (Fig. 7), AP endonuclease, and DNA polymerase β, this low level of repair synthesis most likely represents repair of 3-MeAde and 7-MeGua by a short patch mechanism. We conclude that although UV photoproduct excision repair and O6-MeGua-stimulated repair synthesis both require a PCNA-dependent DNA polymerase, essential factors for O6-MeGua-stimulated repair synthesis are present in cytoplasmic replication extracts but not in whole cell nucleotide excision repair extracts.

DISCUSSION

Unrepaired O6-MeGua in DNA is clearly implicated in cell killing by methylating agents and in inhibition of DNA synthesis in vivo but does not have a direct effect on DNA
Fig. 6. Characterization of proteins involved in repair synthesis using antibody inhibition. a, cell extract (70 μg) was combined with the antibodies indicated (0.5 μg) and incubated at 20 °C for 15 min. Controls (−Ab) were incubated in the absence of antibody. Replication reaction mixture containing 300 ng of pSVori DNA and supplemented with 0.5 μg of T antigen was then added and incubation continued for a further 60 min at 37 °C. Incorporation was quantitated by DE81 binding and is expressed relative to the values in the absence of antibody (2 pmol). b, extract (60 μg) was preincubated with antibody as above. Repair synthesis reaction mixtures containing 300 ng of MNU-treated (0.24 nm) pSVori DNA without T antigen was added and incubation continued for a further 60 min at 37 °C. The extreme right hand bar shows incorporation by repair synthesis after simultaneous preincubation with antibodies against both RP-A subunits. Repair synthesis was quantitated by DE81 binding and is expressed relative to the values in the absence of antibody (1.2 pmol).

DNA Repair Synthesis at O6-Methylguanine

O6-MeGua in DNA is not a substrate for the UV excision repair pathway in vivo. However, processing of the methylated base shares some properties with in vitro nucleotide excision repair in that it exhibits a requirement for a PCNA-dependent DNA polymerase. The possibility that O6-MeGua-related repair synthesis reflects excision of O6-MeGua from DNA by the UV repair pathway in vitro can be excluded for a number of reasons. First, unlike UV nucleotide excision repair (35), O6-MeGua-related repair synthesis does not require RP-A and second, whole cell extracts proficient in UV excision repair are essentially inactive on a methylated substrate. In addition, cytoplasmic extracts of the type we used do not contain all the factors necessary to carry out excision repair on a UV-irradiated plasmid.

Analysis of the products provides some clues to the effectiveness of the processing of different lesions. The majority of labeled molecules formed by processing of the MNU-treated DNA were nicked circular form II molecules. In contrast, the products formed on the same DNA from which O6-MeGua had previously been removed were closed, form I, molecules. Form I molecules were also the predominant product of processing of MMS-treated DNA. It is therefore likely that complete repair of some 3-MeAde or 7-MeGua residues, including ligation of the repaired patch, is carried out by these cell extracts. When supplemented with viral T antigen and undamaged pSVori, these cytoplasmic extracts were able to perform extensive DNA replication and produced fully ligated, form I, molecules. This indicates a high level of DNA ligase activity. The predominance of form II molecules in the products of processing O6-MeGua-containing plasmids suggests that O6-MeGua in the plasmid provokes abortive repair events associated with discontinuities in the DNA that remain unligated for reasons other than DNA ligase deficiency.

While we refer to the incorporation into methylated plasmids as repair synthesis because of its sensitivity to digestion by DpnI, we cannot formally exclude the possibility that a form of aberrant replication is taking place. A possible analog is the DNA damage-induced replication associated with the SOS response in E. coli in which the requirements for a defined replication origin and DnaA protein are relaxed (36). Aberrant replication that involves a re-replication of limited
regions of SV40 DNA in vitro has been reported after MNNG treatment of mammalian cells (for review, see Ref. 37). Unlike the incorporation that we observe, this particular phenomenon retains its absolute dependence on viral T antigen and appears to relate more to the interaction between the drug and the cellular replication apparatus than to direct methylation of DNA. The particular base pairing properties of O^6-MeGua could possibly promote looping out of a section of DNA to form an initiation site for aberrant replication as is seen in single-stranded DNA. All DNA replication systems are, however, dependent on some type of single-stranded binding protein, a requirement that is absent from the observed incorporation into the MNNG-treated plasmids. It seems more likely therefore that O^6-MeGua stimulates repair synthesis in plasmid DNA.

The properties of O^6-MeGua, and in particular its ability to form base pairs in DNA, have led to the hypothesis that DNA mismatch correction may be involved in its processing into a lethal lesion (38, 39). MNU-resistant cell lines in which deficiencies in DNA mismatch binding activities are associated with a spontaneous mutator phenotype lend support to this idea (47). There are two, apparently distinct, pathways of mismatch correction in mammalian cells. A "short patch" repair which exhibits a specificity for G-T mispairs derived from the deamination of 5-methylcytosine in DNA, is initiated by a thymine-specific DNA glycosylase and involves the replacement of a single nucleotide at the mismatched site (40, 41). A more generalized mismatch correction pathway that is active on most other mismatches is associated with a more extensive excision tract in vitro (42). It has recently been reported that the G-T-specific thymine-DNA glycosylase may act at O^6-MeGua-T but not at O^6-MeGua-C base pairs in oligonucleotide substrates (43). This activity is unlikely to contribute substantially to the repair synthesis that we observe since the O^6-MeGua in our substrate is all in the form of O^6-MeGua-C pairs. Furthermore, the observed repair synthesis associated with O^6-MeGua is too extensive to be of the short patch type. We do not know what proportion of substrate molecules undergo repair synthesis in our in vitro assay but if each contains a single O^6-MeGua, a minimum estimate for the extent of the resynthesized region would be 50-100 nucleotides. Therefore it seems more likely that O^6-MeGua is stimulating long patch repair of the type seen in generalized mismatch correction. It has been demonstrated that extracts of this type are able to carry out long patch mismatch repair of heteroduplex DNA substrates (44).

An unresolved question is the extent to which the repair synthesis contributes to a restitution of functional DNA molecules. In the absence of specific demethylation by the MGMT, there is no detectable removal of O^6-MeGua from cellular DNA in vivo. Nevertheless, Mex^- human cells that are unable to remove O^6-MeGua from their DNA perform more MNNG-induced repair synthesis (4, 45) and sustain higher levels of MNNG-induced DNA single-strand breaks (46) than their Mex^+ counterparts. The failure to remove O^6-MeGua suggests that the repair synthesis might be incorrectly

![Graph](image-url)
targeted, perhaps to the complementary strand. It seems likely that our observation of repair synthesis in vitro is a manifestation of the same phenomenon and might represent unsuccessful attempts to remove O\(^\circ\)-MeGua from DNA. Incorrect targeting of mismatch excision repair to the strand opposite O\(^\circ\)-MeGua together with the inability to find a complementary base for the methylated purine, would inevitably be associated with long-lived nicks in the DNA. We suggest that this misguided repair with its associated long-lived DNA strand interruptions contributes to the cytotoxicity of methylating agents.

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