O^6-Methylguanine in DNA Inhibits Replication *in Vitro* by Human Cell Extracts†

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ABSTRACT: To study the effects of methylation damage on DNA replication *in vitro*, the plasmid pSVori containing the SV40 origin of replication was reacted with *N*-methyl-*N*-nitrosourea and used as a substrate for SV40 T antigen dependent replication by HeLa cell extracts. The plasmid was methylated with a range of *N*-methyl-*N*-nitrosourea concentrations that introduced an average of 0.3–2.5 O^6-methylguanine and equal amounts of 3-methyladenine lesions per DNA molecule. When methylated plasmid was incubated with extract of Mex⁻ HeLaMR cells under conditions favoring DNA replication, an impairment of replication was observed as the accumulation of incompletely replicated form II plasmid molecules. These extracts simultaneously performed a T antigen independent, *Dpn*I-sensitive DNA repair synthesis that increased with increasing DNA damage. Subtraction of this repair DNA synthesis revealed that methylation inhibited overall replication. At low levels of methylation (≤ 1 O^6-methylguanine and ≤ 1 3-methyladenine lesion per plasmid), inhibition was transient, while more extensive damage resulted in apparently irreversible inhibition of replication. Removal of O^6-methylguanine by pretreatment of the methylated plasmid with purified human O^6-methylguanine–DNA methyltransferase restored replication to almost normal levels. When the methylated plasmid was replicated by extracts of Mex⁺ HeLaS3 cells proficient in the repair of O^6-methylguanine, a lower level of inhibition and less repair DNA synthesis was observed. The inhibition of DNA synthesis and the stimulation of repair DNA synthesis are thus both largely due to the presence of O^6-methylguanine in DNA. We suggest that the inhibition of DNA replication by O^6-methylguanine may result, in part, from the processing of this lesion during DNA replication.

Methylating agents such as *N*-methyl-*N*-nitrosourea (MNU)[1] are carcinogens able to activate protooncogenes by mutation (Sukumar et al., 1983; Zarbl et al., 1985; Newcomb et al., 1988) and to induce cell proliferation in bone marrow hematopoietic precursors, the target tissue for tumor occurrence (Frei, 1970; Gerson et al., 1987). MNU introduces a variety of lesions in DNA: 7-methylguanine (7-MeGua), 3-methyladenine (3-MeAde), O^6-methylguanine (O^6-MeGua), and methylphosphotriesters together with a number of other minor lesions (Singer & Grunberger, 1983). In bacteria, the lethality of methylating agents has been partly ascribed to the presence of 3-MeAde in DNA, since mutants defective in repair of this methylated base are hypersensitive to killing by methylation damage (Karran et al., 1980; Evensen & Seeberg, 1982; Boiteux et al., 1984). An indication that unrepaired 3-MeAde in DNA might be lethal in mammalian cells comes from recent experiments in which either the *Escherichia coli* 3-MeAde–DNA glycosylase I gene (Habraken & Laval, 1993; Klungland et al., 1992) or the human *N*-methylpurine–DNA glycosylase gene (Ibeanu et al., 1992) was transfected into rodent cell lines. Expression of these repair enzymes conferred resistance to the killing effects of MNU in two cases (Klungland et al., 1992; Habraken & Laval, 1993). Biochemical evidence to support the possibility that 3-MeAde might be lethal comes from *in vitro* experiments with randomly alkylated substrates and purified DNA polymerases. These experiments indicate that, among the major products of methylation, only 3-MeAde directly blocks elongation of DNA (Larson et al., 1985).

One of the other major lesions produced by MNU, O^6-MeGua, directs incorporation of either cytosine or thymine opposite the alkylated base by DNA polymerases *in vitro* (Singer & Grunberger, 1983), and this ambiguous base pairing is consistent with the G·C to A·T transitions observed in mutational spectra of bacterial or mammalian cells exposed to methylating agents (Richardson et al., 1987; Lukash et al., 1991; Palombo et al., 1992). This lesion has been proposed as the critical mutagenic lesion in experimental tumors induced by methylating agents that display G to A activating mutations in K-*ras* or H-*ras* genes (Sukumar et al., 1983; Newcomb et al., 1988; Brown et al., 1990). Direct confirmation of a major role of O^6-MeGua in initiation of tumorigenesis was recently obtained using transgenic mice in which expression of the human repair enzyme for O^6-MeGua, the O^6-MeGua–DNA methyltransferase (MGMT), prevented the formation of thymic lymphomas after MNU treatment (Dumenco et al., 1993).

Human cell lines deficient in removal of O^6-MeGua (Mex⁻) are more sensitive to the killing effects of MNU or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) than their repair-proficient (Mex⁺) counterparts, in which the MGMT is active (Day et al., 1980; Scudiero et al., 1984b) and the Mex⁺ phenotype is independent of the UV damage excision repair capacity of the cells (Sklar & Strauss, 1981). Furthermore, transfection of the *E. coli ada* methyltransferase gene or the human MGMT cDNA into Mex⁻ cells provides striking protection against methylating agent induced killing, thus directly implicating the repair of O^6-MeGua in cellular defense

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[1] Abbreviations: MNU, *N*-methyl-*N*-nitrosourea; 7-MeGua, 7-methylguanine; 3-MeAde, 3-methyladenine; O^6-MeGua, O^6-methylguanine; MGMT, O^6-MeGua–DNA methyltransferase; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; NAP, apurinic.

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against these agents (Samson et al., 1986; Brennand & Margison, 1986; Kataoka et al., 1986; Kaina et al., 1991; Wu et al., 1992). DNA lesions that have been determined to be cytotoxic generally block DNA replication (Strauss, 1985); thus the inability of O^6-MeGua to inhibit the progression of purified polymerases in the *in vitro* system (Larson et al., 1985) is paradoxical. Since O^6-MeGua contributes substantially to the lethality of important chemotherapeutic agents such as procarbazine or triazines (Hayward & Parsons, 1984; Gibson et al., 1986; Fong et al., 1992), to which acquired resistance is a common clinical feature, we have addressed the unresolved problem of the mechanism of its cytotoxicity.

The discrepancy between the involvement of O^6-MeGua in cell killing and its failure to block DNA synthesis *in vitro* suggested to us that this methylated base might produce subtle effects on DNA replication that would be better revealed in an assay system that more closely resembles the process of replication *in vivo*. We have investigated the effect of a low level of methylation damage on mammalian replication by using an SV40-based *in vitro* assay. A single viral protein, T antigen, is required to supplement soluble cellular proteins in order to carry out complete replication of plasmids containing the SV40 origin of replication. In the initial steps of replication, T antigen binds to specific sequences within the SV40 origin and participates in the extensive unwinding of the template DNA, and the host replication complex is recruited to form the functional replication forks [for reviews, see Stillman (1989) and Challberg and Kelly (1989)]. Methylation, even at low levels, of the plasmid pSVori impaired its ability to serve as a substrate for T antigen dependent replication by cell-free extracts of Mex$^-$ HeLaMR cells and provoked a T antigen independent DNA synthesis. Enzymatic removal of O^6-MeGua from the methylated substrate diminished the T antigen independent synthesis and largely restored the plasmid's ability to undergo replication, indicating that O^6-MeGua in DNA can inhibit the replication process.

MATERIALS AND METHODS

Chemicals and Reagent Enzymes. MNU (Sigma) was dissolved in dimethyl sulfoxide and diluted to the required concentrations immediately before use. Ribonuclease A was obtained from Boehringer; Proteinase K, from Sigma; and restriction enzymes *Dpn*I and *Acc*I, from New England Biolabs. Endonuclease IV was purified from BW9109*xth*A by published procedures (Ljungquist, 1977). A recombinant MGMT comprising the human sequence fused to a fragment of the bacteriophage λ N protein was purified to essential homogeneity as described (Zhukovskaya et al., 1992). MGMT treatment of methylated plasmid was carried out in 100 mM Hepes·KOH, 1 mM EDTA, and 0.1 mM dithiothreitol.

MGMT assays of cell extracts were carried out as described (Harris et al., 1983). One unit of enzyme demethylates 1 pmol of O^6-MeGua under the standard assay conditions.

Preparation of Cell Extracts. Cell extracts were prepared from human HeLaMR (a kind gift of J. Thomale, University of Essen) or HeLaS3 cells adapted for growth in suspension by following a modification of the procedure of Li and Kelly (1985). Briefly, cells were grown in spinner culture in Eagle minimal essential medium supplemented with 10% fetal calf serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). Approximately 5×10^9 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 KCl, 1.5 mM $MgCl_2$, and 0.5 mM dithiothreitol) 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 were 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 lysate was centrifuged for 20 min at 10000*g* at 0 °C. NaCl was added to the supernatant to a final concentration of 100 mM, and extracts were centrifuged at 100000*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 was usually 15–20 mg/mL.

Preparation of T Antigen. T antigen was prepared as described by Simanis and Lane (1985). Briefly, the recombinant virus Ad5-SVR111, which expresses SV40 T antigen, was used to infect 293 cells. T antigen was immunoaffinity purified on a monoclonal anti-T antigen antibody (PAb 419)–protein A–Sepharose column. The pure T antigen was eluted with 20 mM triethylamine, pH 10.8, and 10% (v/v) glycerol; dialyzed against 10 mM PIPES, pH 7.0, 1 mM dithiothreitol, 5 mM NaCl, 0.1 mM EDTA, 10% (v/v) glycerol, and 1 mM PMSF; rapidly frozen in small aliquots; and stored at −70 °C.

Preparation of Replication Substrates. The plasmid pSVori (Decker et al., 1986) (a kind gift of R. Possenti, Neurobiology Laboratory, CNR, 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. The extent of reaction was determined by partially depurinating an aliquot of the methylated DNA by heating at 70 °C for 30 min in 70 mM Hepes·KOH, pH 7.5, and digesting the product with an excess of purified *E. coli* endonuclease IV (8 units) to reveal the number of induced apurinic (AP) sites. The products of digestion were analyzed by electrophoresis on ethidium bromide–1.5% agarose gels and quantified by densitometric scanning of photographic negatives. Since the AP sites are derived approximately equally from heat-induced loss of 3-MeAde and 7-MeGua residues, the overall level of methylation damage can be estimated from the Poisson distribution (Karran et al., 1993). We estimate that treatment with 0.12–1 mM MNU introduced between 0.3 and 2.5 O^6-MeGua, similar levels of 3-MeAde, and 3–25 7-MeGua residues per plasmid molecule. These calculated values were confirmed by direct treatment of the plasmid with radioactive MNU and quantitation of the methylated bases (data not shown). The remainder of the methylated DNA was precipitated with ethanol, redissolved in TE, pH 7.5, and stored at −20 °C. It was used within 1 week as substrate for replication by cell extracts.

In Vitro Replication by Cell-Free Extracts. Reaction mixtures (50 μL) contained 30 mM Hepes·KOH (pH 8.0); 7 mM $MgCl_2$; 0.5 mM dithiothreitol; 200 μM each of CTP, UTP, and GTP; 100 μM each dTTP, dCTP, and dGTP; 36 μM [α-^{32}P]dATP (670 cpm/pmol); 4 mM ATP; 40 mM creatine phosphate; and 1 μg of creatine phosphokinase (rabbit muscle type I; Sigma). Standard reaction mixtures also contained 300 ng of pSVori, either unmethylated or treated with MNU, and 0.5 μg of T antigen. Reaction mixtures were supplemented with up to 400 μg of cell extract and incubated at 37 °C for various times. To determine the extent of DNA synthesis, 10 μg of tRNA was added to the samples and 50 μL of each reaction mixture was spotted onto 1.5 × 1.5 cm^2 3MM paper squares. The filters were washed three times with 500 mL of 5% cold trichloroacetic acid and once with

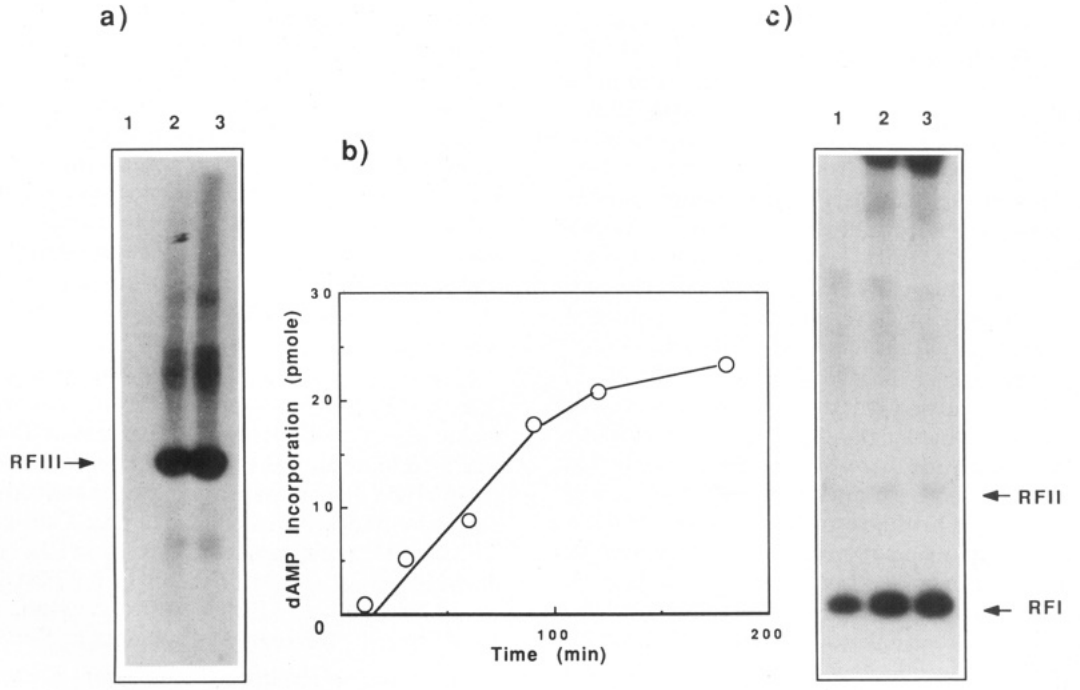

FIGURE 1: DNA synthesis by HeLaMR cell extracts. (a) *Dpn*I sensitivity of replication products. Reaction mixtures containing 400 μg of HeLaMR cell extract and 300 ng of pSVori plasmid in the absence (lane 1) or the presence (lanes 2 and 3) of 0.5 μg of purified T antigen were incubated at 37 °C for 60 min (lanes 1 and 2) or 90 min (lane 3). The reaction products were isolated, linearized by digestion with *Acc*I, and further digested with *Dpn*I. Digested products were analyzed by agarose gel electrophoresis. (b) Kinetics of replication. Reaction mixtures containing HeLaMR cell extract, pSVori DNA, and T antigen were incubated at 37 °C as described in Materials and Methods. The TCA-insoluble radioactivity was determined at the times indicated. (c) Products of replication. Reaction products after 1 h (lane 1), 3 h (lane 2), or 5 h of replication (lane 3) replication at 37 °C as described in (a) were analyzed by agarose gel electrophoresis without prior enzyme digestion. The positions of form I (closed circular), form II (nicked circular), and form III (linear) plasmid DNA were determined from ethidium bromide staining of pSVori markers run in parallel.

200 mL of ice-cold ethanol and dried, and the 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 μg/mL), and the mixtures were incubated for 15 min at 37 °C. Proteinase K (1 μg/mL) was then added, and incubation continued for a further 1 h at 37 °C. Following extraction once with phenol/chloroform and once with chloroform/isoamyl alcohol, the DNA was precipitated with ethanol in the presence of 10 μg of tRNA. Since the processing of samples prior to loading of the gels involves several steps, we routinely added nonradioactive plasmid at the end of the replication period. This served as a marker to monitor the overall recovery by ethidium bromide staining of the gel (Figure 2, inset). The precipitated material was then resuspended in 20 μL of TE, pH 7.8, and, when desired, digested for 2 h with *Acc*I (10 units) and/or 5 h with 5–10 units of *Dpn*I in the presence of 0.2 M NaCl (Wobbe et al., 1985). The plasmid pSVori contains 16 *Dpn*I recognition sites that generate fragments ranging in size from 818 to 8 bp. Five fragments of 573, 341, 272, 258, and 105 bp could be resolved by the conditions of electrophoresis we used. These routinely provided evidence of complete digestion by the enzyme of both experimental and added carrier DNA. The digestion products were separated by overnight electrophoresis in 0.8% agarose gels containing 0.5 μg/mL ethidium bromide. The gels were then dried and autoradiographed with Cronex X-ray film (Dupont) at –80 °C with an intensifying screen. The extent of replication was quantified by densitometric scanning.

RESULTS

DNA Replication in Vitro of Normal DNA by Mex⁻ HeLaMR Cell Extracts. Extracts of HeLa cells, when supplemented with purified viral T antigen, replicate plasmids that contain the SV40 origin of replication (Stillman & Gluzman, 1985). We assessed the ability of extracts from the Mex⁻ line HeLaMR to replicate the plasmid pSVori. Incorporation of dAMP into undamaged pSVori was absolutely dependent on the addition of T antigen (Figure 1a). In this experiment, daughter plasmids were cleaved at the single *Acc*I site to 2.6-kb unit length molecules before gel analysis. *Dpn*I digestion was also performed to discriminate between semiconservative and non-semiconservative synthesis. This enzyme recognizes the bacterial methylation pattern and is unable to digest hemimethylated or unmethylated plasmid molecules that result from semiconservative replication in human cell extract (Li & Kelly, 1985).

More than 90% of the radioactivity incorporated at 1 h or 90 min was present in DNA fully resistant to *Dpn*I since very few fragments were seen below the position of form III DNA (Figure 1a, lanes 2 and 3). After a short lag period, DNA synthesis as measured by TCA-insoluble radioactivity proceeded linearly at a rapid rate for 90 min. Thereafter, the rate of incorporation was slower (Figure 1b). These kinetics are similar to those reported for replication by Mex⁺ HeLa cell extracts (Stillman & Gluzman, 1985). If undigested by either *Acc*I or *Dpn*I, the products of replication of the undamaged plasmid were predominantly covalently closed, form I, or slowly migrating catenated DNA molecules (Figure 1c).

Replication in Vitro of Methylated DNA: Analysis of Replication Products. We examined the effects of methylation damage on plasmid replication. Methylation of pSVori with MNU at concentrations of 0.12, 0.24, and 0.48 mM introduces up to a maximum of 1 O^6-MeGua, an equivalent number of 3-MeAde, and between 2 and 10 7-MeGua residues per

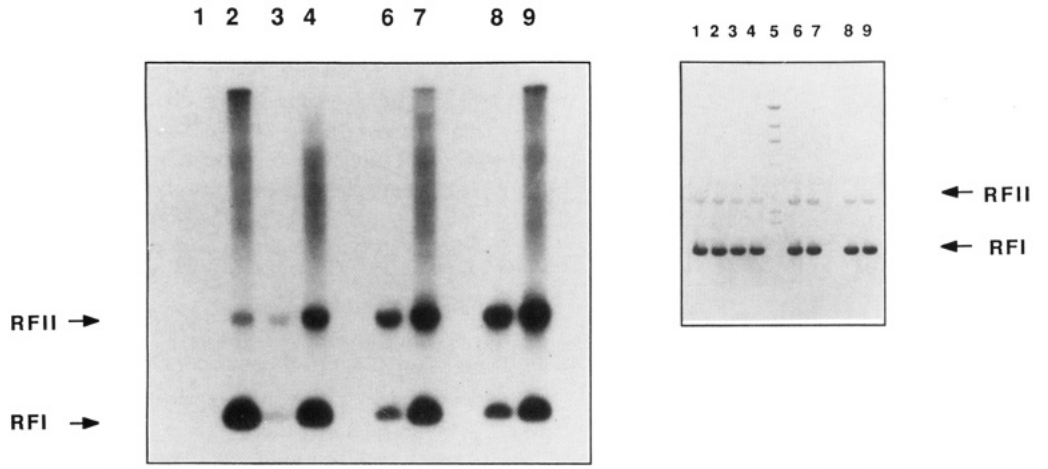

FIGURE 2: Effects of methylation on *in vitro* DNA replication. Reaction mixtures containing HeLaMR cell extract and unmethylated or MNU-treated (0.12, 0.24, or 0.48 mM) pSVori DNA were incubated at 37 °C for 60 min in the absence (lanes 1, 3, 6, and 8) and in the presence of T antigen (lanes 2, 4, 7, and 9). The replication products were supplemented with 2 μg of pSVori to monitor recovery of DNA by ethidium bromide staining (inset, right side). DNA was purified and analyzed by autoradiography of the dried agarose gel as described in Materials and Methods. Lanes 1 and 2, untreated pSVori; lanes 3 and 4, 0.12 mM MNU; lanes 6 and 7, 0.24 mM MNU; lanes 8 and 9, 0.48 mM MNU; lane 5, marker DNA. The positions of form I and form II plasmid DNA are indicated.

plasmid molecule together with a small number of minor methylated bases. The products of replication of undamaged pSVori were predominantly form I or catenated molecules with ≤5% form II DNA (Figure 2, lane 2). These low levels of methylation of the template induced an alteration in the distribution of replication products (Figure 2, lanes 4, 7 and 9). With increasing MNU concentration, the proportion of form II molecules, as determined by densitometry of the total form I + form II DNA, was increased to 25%, 36%, and 55% at 0.12, 0.24, and 0.48 mM, respectively. The amounts of form I and form II (mean and standard deviations) obtained from three independent experiments are shown in Figure 3a.

We conclude that methylation of template DNA inhibits replication and that inhibition is apparent in the impaired conversion of form II replicating intermediates to fully replicated form I products.

Quantitation of Replication Inhibition. In addition to altering the distribution of replication products, methylation of the plasmid inhibited overall DNA replication. Since the extracts also carried out a T antigen independent repair synthesis on methylated plasmids, this incorporation was taken into account. [We refer to this incorporation as repair synthesis to discriminate from DNA replication, and we do not wish to imply that functional repair is taking place (Karran et al., 1993)]. The extent of the repair synthesis was dependent on the degree of methylation of the plasmid and was distributed exclusively between form I and form II DNA (Figure 2, lanes 1, 3, 6, and 8, and Figure 3b). If the amount of repair synthesis on the methylated plasmid was subtracted from the total synthesis, the degree of inhibition of replication was about 60% at the highest MNU concentration (mean of three experiments; Figure 4).

The above treatment assumes that repair synthesis occurs to the same extent during, or in the absence of, DNA replication. In particular, if repair synthesis occurred on replicated plasmids, *Dpn*I sensitivity would be lost. In order to test this, we used digestion with *Dpn*I to discriminate between DNA replication and repair. Repair synthesis, in the absence of replication, is completely sensitive to *Dpn*I digestion (Karran et al., 1993). DNA synthesis was allowed to proceed in the presence of T antigen for 1 h, and incorporation was analyzed with and without *Dpn*I digestion of the samples. Figure 4 shows that similar values for the extent of MNU-induced

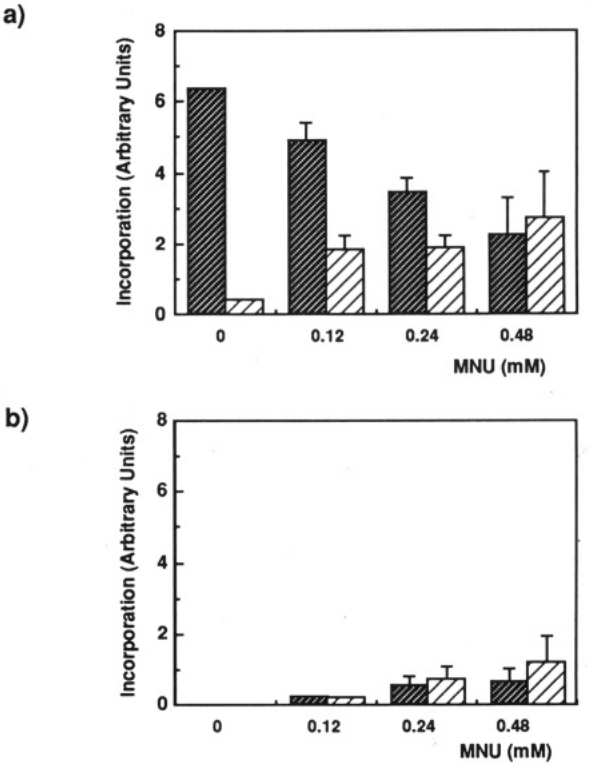

FIGURE 3: Distribution of replication products. form I and form II molecules were quantitated by densitometric scanning of autoradiographs derived from experiments performed as described in Figure 2. Replication products were separated by agarose gel electrophoresis. (a) Incorporation into form I (dark bars) and form II DNA (light bars) in the presence of T antigen. (b) Incorporation into form I (light bars) and form II (dark bars) DNA in the absence of T antigen. Densitometry was performed on exposures of similar intensity, and the values have been normalized to those of a single experiment (for example, values for form I DNA for untreated pSVori were 6.3, 5.75, and 7.3). Average values from three independent experiments together with standard deviations are shown.

inhibition of replication were obtained when the T antigen independent repair synthesis was removed by simple subtraction or by *Dpn*I digestion. These data indicate that the repair synthesis occurs to about the same extent in the presence of plasmid replication as in its absence. Since repair synthesis on a completely replicated plasmid would be resistant to *Dpn*I, our results also suggest that this event is unlikely to occur.

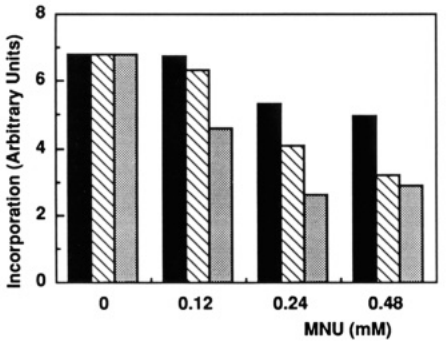

FIGURE 4: Quantitation of replication inhibition. Untreated or MNU-treated (0.12, 0.24, or 0.48 mM) pSVori was incubated with HeLaMR cell extract in the presence or in the absence of T antigen for 60 min at 37 °C. DNA was isolated, and one-half of the samples incubated with T antigen were digested with *Dpn*I and analyzed by agarose gel electrophoresis. The amounts of form I and form II DNA were obtained by densitometry. Shown are the total incorporation in the presence of T antigen (solid bars) (mean of at least two experiments), the net incorporation obtained by subtraction of DNA synthesis occurring in the absence of T antigen (hatched bars) (mean of at least two experiments), and the incorporation remaining after digestion with *Dpn*I (stippled bars).

(The slowly migrating molecules were not included in the summation of replication. The inclusion of this material did not, however, affect the conclusions of the analysis.)

Inhibition of plasmid replication by methylation was consistently observed, although some variation in the extent of inhibition was noted from experiment to experiment. We attribute this to small differences in the extent of methylation by the unstable MNU. In all experiments, however, the true extent of inhibition is only revealed if incorporation due to simultaneous DNA repair synthesis is subtracted from the overall signal.

Kinetics of Inhibition. We investigated the kinetics of inhibition by allowing replication of the methylated plasmid to proceed for up to 3 h (Figure 5). In the experiment shown, we included plasmid treated with 1 mM MNU to extend the range of damage analyzed. We again examined the distribution of replication products between form I and form II plasmid molecules. After 1 h of incubation of the untreated plasmid in the presence of T antigen, more than 90% of labeled form I + form II molecules produced by replication were form I. In contrast, form II molecules were the predominant products with MNU-treated plasmids. Following more prolonged incubation (2–3 h), form I molecules comprised an increasing proportion of the replication products of methylated pSVori (Figure 5a). However, at least 30% of the products remained nicked compared to <10% with an unmethylated template. At 1 mM MNU, the predominant DNA form remained nicked circular even after 3 h.

A similar analysis of the repair synthesis indicated that after 1 h more than 50% of the products of MNU-induced repair synthesis were in form II, and at 1 mM MNU 80% of the label was present as nicked circles. At later times form I DNA predominated at the two lower doses, whereas form II was still the predominant form in the DNA treated with 1 mM MNU.

Incorporation was quantitated by summing the amounts of forms I and II DNA of Figure 5a, and the amount of replication was again determined by subtracting the incorporation due to repair synthesis (without T antigen) from the total incorporation in the presence of T antigen (Figure 5b). In this particular experiment, overall inhibition was somewhat greater than that shown in Figures 3 and 4. Replication of the plasmid was 28% and 32% of the control value after treatment with 0.24 and 0.48 mM MNU, respectively, and this decreased further to 2.5% at 1 mM. The inhibition produced by lower MNU concentrations was reversible, and during more prolonged incubation, replication of pSVori treated with either 0.24 or 0.48 mM MNU approached control levels. Replication of plasmid treated with either of the two lower concentrations was essentially indistinguishable from replication of unmethylated plasmid (≤20% inhibition) at 3 h. In contrast, replication of plasmid treated with 1 mM MNU did not recover significantly, and incorporation remained at <15% of the control value even after 3 h. The slowly migrating forms were difficult to quantitate, but their inclusion supported the general conclusion.

Two phenomena are observed in this experiment. First, methylation at the lower MNU concentrations induces a transient inhibition of replication that is apparent at early times but reversed later. Higher levels of damage induce inhibition that is not reversible within the time scale of our experiments. Second, the inhibition is characterized by an accumulation of incomplete replication products that migrate as form II molecules. During the reversal of inhibition that occurs at later times, control levels of incorporation are not accompanied by a return to the distribution of form I and form II molecules seen with unmethylated DNA. These observations indicate that although the replication apparatus can overcome most of the block to replication resulting from low levels of template methylation, the products of replication contain a higher proportion of nicked (or gapped) circles.

Contribution of O^6-Methylguanine to Inhibition of Replication. To investigate whether O^6-MeGua contributed to the inhibition of replication, we analyzed replication of the methylated plasmid after enzymatic reversal of O^6-MeGua lesions. This was carried out in two ways. In the first set of experiments, methylated plasmid was treated with purified human MGMT protein, and the DNA was reisolated and used as a substrate for replication. In a different approach, we examined replication of methylated plasmid in cell extracts containing active endogenous MGMT. The human enzyme rapidly transfers the methyl group from the base *in situ* and covalently attaches it to one of its own cysteine residues in a stoichiometric reaction (Lindahl et al., 1988). The enzyme is essentially specific for O^6-MeGua in DNA and does not demethylate other methylated bases to any significant extent (Sassanfar et al., 1991).

When pSVori was methylated with 0.48 mM MNU and used directly as a substrate, replication was inhibited to about 30% of control values (Figure 6). In this experiment, which was one of two that produced similar data, the replication products were linearized by *Acc*I followed by *Dpn* I digestion to remove repair synthesis. The proportion of DNA recovered from each of the samples as detected by ethidium bromide staining of the gel was similar (data not shown). MGMT treatment of the methylated plasmid immediately prior to its inclusion in the replication assay restored its ability to act as an effective template, and overall synthesis was restored to 70–80% of the level seen with the untreated plasmid (Figure 6). These data indicate that O^6-MeGua contributes substantially to the inhibition of replication on the methylated substrate.

In order to examine the effect of endogenous MGMT on the replication of the methylated plasmid, extracts of HeLaS3 cells were used to replicate methylated DNA. HeLaS3 cells are Mex^+ and express high levels of MGMT. Direct assay indicated that extracts prepared from the Mex^- HeLaMR cells contained ≤0.05 MGMT unit per milligram of protein,

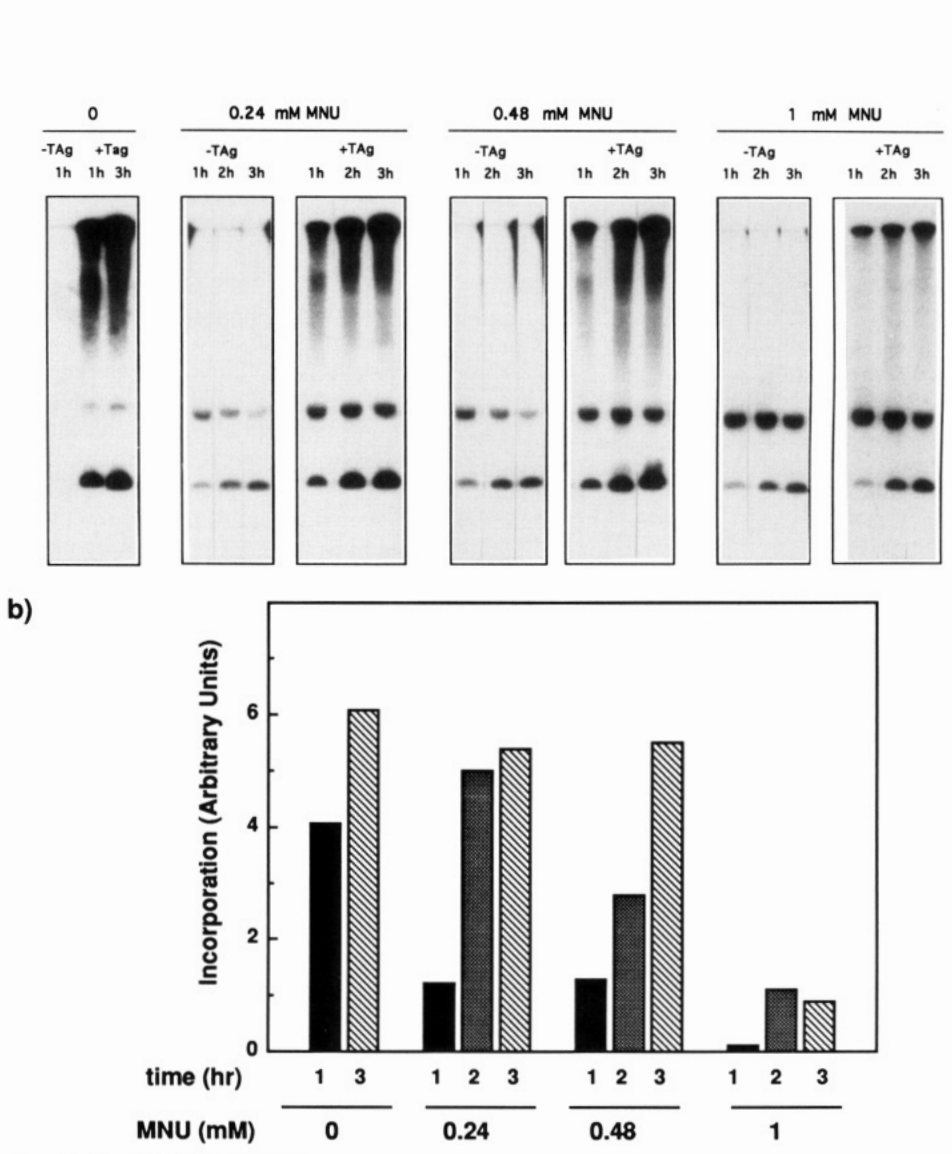

FIGURE 5: Time course of replication inhibition by MNU. (a) Untreated or MNU-treated pSVori (0.24, 0.48, or 1 mM) was incubated for 1, 2, or 3 h with HeLaMR cell extract in the absence or in the presence of T antigen. At the indicated times DNA was purified and analyzed by gel electrophoresis as described in Figure 2. (b) Quantitation of replication products by densitometric scanning of form I and form II DNA shown in (a) after 1 h (solid bars), 2 h (stippled bars), and 3 h of incubation (hatched bars). The data are presented as the amount of incorporation in the presence of T antigen corrected for the incorporation due to repair synthesis in the absence of T antigen.

whereas the MGMT activity of the HeLaS3 extract was 1.7 units per milligram of protein. In order to ensure that MGMT was present in a significant excess over the O^6-MeGua in the template DNA, we used plasmid treated with the slightly lower MNU concentrations of 0.06, 0.12, and 0.24 mM. Since 200 μg of extract was used, we estimate that MGMT was in approximately 3–15-fold excess over O^6-MeGua. The products of 1-h replication were analyzed without linearization or *Dpn*I digestion in order to assess their distribution between form I and form II DNA molecules. Incorporation in the absence of T antigen was monitored in parallel. Figure 7 shows that replication of unmethylated DNA was similar in the two extracts. Even at this low level of DNA damage, an increase in the proportion of form II molecules among the products of replication by HeLaMR extracts was observed (Figure 7, left side). The amount of T antigen independent synthesis also increased in a dose-dependent fashion. In contrast, replication by the Mex$^+$ HeLaS3 extract, in which rapid demethylation of O^6-MeGua residues would have taken place during the 15–20-min lag period before replication started, was much less affected by methylation of the plasmid (Figure 7, right side). This extract also performed considerably less repair synthesis than HeLaMR extracts on the same methylated substrate, and this repair synthesis was present predominantly in form I molecules.

The two separate approaches by which O^6-MeGua was removed from the template, either by prior treatment with purified MGMT or by exposure to the endogenous enzyme present in the cell extract, both indicate that O^6-MeGua is responsible for a large part of the inhibition of replication on the methylated plasmid. The relative absence of form II molecules from the products of replication of methylated DNA by the Mex$^+$ cell extract also indicates that this methylated base is responsible for the accumulation of incompletely replicated molecules that contain discontinuities.

DISCUSSION

Our experiments with MNU-treated pSVori were designed to investigate the effects on replication of a low number (0.5–2) of O^6-MeGua adducts. Because similar amounts of 3-MeAde are introduced at the same time, the situation is similar to UV damage in which more than one potentially blocking lesion is produced. Methylation at these levels

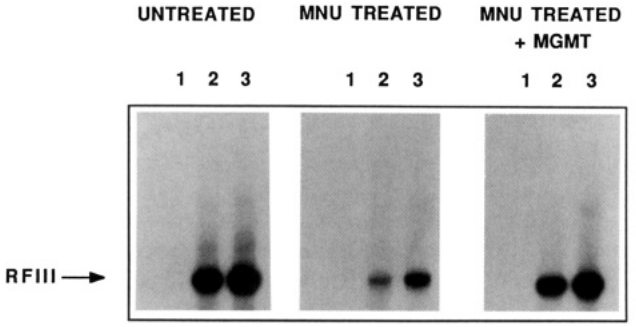

FIGURE 6: Reversal of *in vitro* DNA replication inhibition by removal of O^6-MeGua. Replication mixtures containing untreated pSVori (left panel), MNU-treated (0.48 mM) (middle panel), or MNU-treated pSVori that had been preincubated with 0.5 unit of human MGMT (15 min, 37 °C) (right panel) were incubated with HeLaMR cell extract. DNA synthesis was allowed to proceed for 60 min (lanes 1 and 2) or 90 min (lane 3) in the presence (lanes 2 and 3) or the absence (lane 1) of T antigen. Replication products were isolated, linearized with *Acc*I, digested with *Dpn*I, and analyzed by agarose gel electrophoresis. The position of migration of linear pSVori molecules was determined from ethidium bromide staining and is shown with an arrow.

inhibited replication. Treatment with 0.48 mM MNU introduces between 1 and 2 O^6-MeGua and 3-MeAde bases per plasmid molecule (Karran et al., 1993). This concentration corresponded to a replication block (approximately 37% replication), or two blocking lesions in a circular molecule with two replication forks, which suggests that both of these methylated bases are effective impediments to synthesis. The reversal of inhibition by MGMT pretreatment of the methylated plasmid confirms that O^6-MeGua contributes significantly to the reduction of replication. This conclusion is supported by the much less severe inhibition seen with methylated templates replicated by the Mex$^+$ HeLaS3 extract. Other lesions, such as 7-MeGua, which does not contribute to the biological effects of methylating agents (Newbold et al., 1980; Beranek et al., 1983), and methylation products, such as O^4-methylthymine, which are present in small amounts (Beranek, 1990), are unlikely to make a significant contribution to the effects that we observe.

The amount of methylation damage sustained by the plasmids in the range of MNU concentrations we used is comparable to the levels of UV-induced photoproducts (Gough & Wood, 1989; Carthy et al., 1993) or platinum adducts (Heiger-Bernays et al., 1990) previously used in investigations of inhibition of *in vitro* replication in the SV40 system. The number of bulky DNA adducts in these studies was 1–20 photoproducts or 2–10 platinations per substrate molecule. Between three and six photoproducts per plasmid (Gough & Wood, 1989; Carthy et al., 1993) were necessary to block replication, whereas one or two *cis* or *trans* platinations (Heiger-Bernays et al., 1990) were sufficient. Thus, the relative abilities of UV or platinum adducts and methylated bases to inhibit replication are not dissimilar. The inhibition by methylation damage is fully consistent with the slow kinetics of incorporation and extension (Snow et al., 1984; Singer et al., 1989; Dosanjh et al., 1991) of nucleotides on O^6-MeGua-containing templates and the tendency of 3-MeAde to terminate chain elongation by purified enzymes (Larson et al., 1985). However, the effect of methylation on replication is transient, and at low or intermediate levels of methylation almost normal replication is achieved by 2 h. With two lesions per plasmid molecule, and assuming a Poisson distribution, 60% of the plasmids have at least two adducts, and only 13.5% contain no lesions. It is well established that only a small percentage of undamaged plasmid molecules (around 10%) are replicated more than once by this type of extract (Stillman & Gluzman, 1985; Wobbe et al., 1985; Roberts & Kunkel, 1988; Carthy et al., 1993). It is therefore unlikely that the almost normal levels of replication of the methylated plasmid are due to multiple rounds of replication on the small percentage of undamaged molecules. Our observations are more compatible with a slowing of the replication fork at O^6-MeGua followed by efficient bypass. The level of 3-MeAde–DNA glycosylase in the replication extracts is low (Karran et al., 1993), but nevertheless, the removal of this lesion by excision repair may contribute to the reversal of replication inhibition. The UV-induced block to mammalian DNA replication *in vitro* measured in the same type of assay

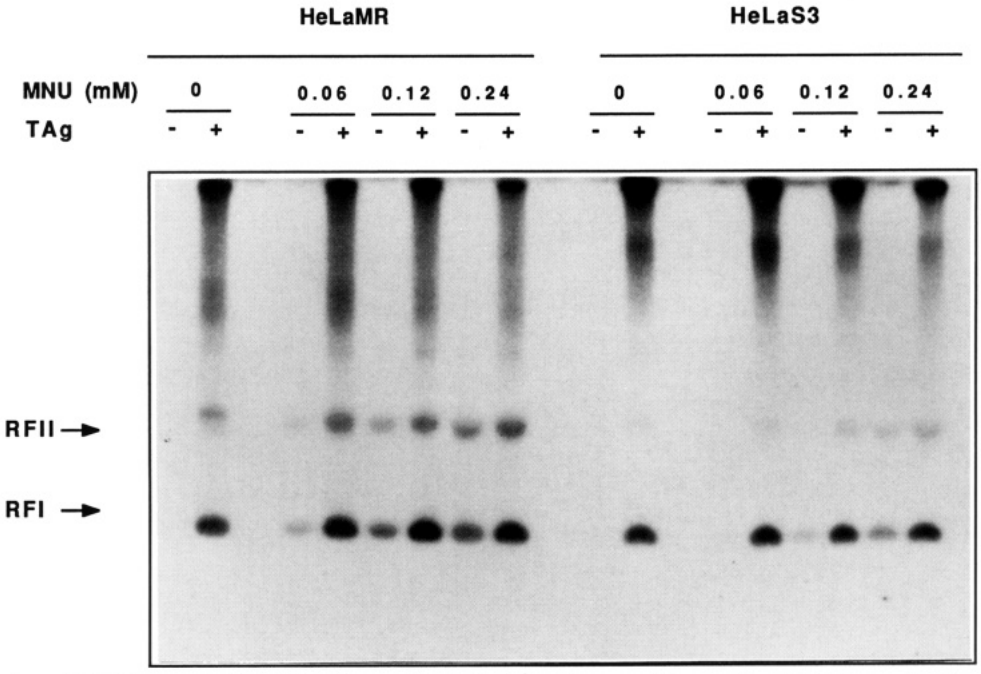

FIGURE 7: Replication of MNU-treated pSVori by Mex$^-$ or Mex$^+$ HeLa cell extract. Untreated or MNU-treated pSVori (0.06, 0.12, and 0.24 mM) was incubated with 200-μg cell extracts prepared from Mex$^-$ HeLaMR (left panel) or Mex$^+$ HeLaS3 (right panel), in the absence and in the presence of T antigen. The products were isolated and analyzed as previously described.

does not change as a function of incubation time (Carthy et al., 1993). It is likely that, in contrast to the situation with the more subtle methylation damage, inhibition resulting from more bulky adducts is not readily reversible. The miscoding properties of O^6-MeGua together with the apparently relative ease of bypass during replication correlate well with the extremely mutagenic nature of this methylated base. The observation that MNU-induced mutations in a gene carried by a shuttle vector in human cells do not differ when O^6-MeGua is replicated by the leading- or lagging-strand polymerases (Basic-Zaninovic et al., 1992) would also suggest that no dramatic differences should be expected on the bypass of this lesion, depending on the strand location.

Of the DNA damage that has been investigated, the effects of O^6-MeGua on replication are unique in that they are complicated by the simultaneous occurrence of a nonsemiconservative DNA synthesis provoked by the methylated base. The properties of this repair DNA synthesis (Karran et al., 1993) indicate that the majority of incorporation is dependent on O^6-MeGua in the DNA, and this is confirmed by the observations reported here of reduced synthesis on methylated DNA by Mex^+ HeLaS3 cell extracts. Repair DNA synthesis occurs more rapidily than replication and, unlike DNA replication, occurs on most plasmid molecules (data not shown). We have also argued that it is unlikely to represent excision repair of O^6-MeGua (Karran et al., 1993). Depending on the concentration of MNU used to treat the plasmid, repair synthesis can comprise up to 30% of the total DNA synthesis in the presence of T antigen. This nonreplicative synthesis is occurring at the same time as replication, and it probably interferes with the movement of replication forks. Alternatively, labeled form II molecules might arise by incision of fully replicated plasmids. Repair synthesis is initiated rapidily by DNA incision, and it is therefore more likely that replication is inhibited by the occurrence of this nonreplicative synthesis.

Since O^6-MeGua-related repair DNA synthesis involves the production of a relatively high proportion of form II DNA molecules and occurs during replication, we would expect that replicating methylated DNA would contain more single-strand nicks than replicating untreated DNA. This is indeed what we observe. MNU treatment of mammalian cells also induces an excess of repair synthesis in Mex^- compared to Mex^+ human cells, suggesting that O^6-MeGua provokes repair DNA synthesis *in vivo* (Scudiero et al., 1984a). The restoration of rapidly sedimenting nucleoids after MNNG treatment of Mex^- cells is also delayed relative to Mex^+ cells, indicating the extended persistence of DNA interruptions in the former (Mattern et al., 1981). It is likely that the discontinuities in replicated DNA that we observe *in vitro* are related to these observations.

The effect of MNU treatment on DNA replication in mammalian cells *in vivo* is unusual in that inhibition is delayed until the second S phase after exposure to the carcinogen (Plant & Roberts, 1971; Schwartz, 1989). The first S phase is apparently of normal duration. Our data indicate that, in contrast, replication of plasmid molecules *in vitro* is significantly slowed by methylation. However, the extent of methylation of the plasmid is about 400-fold higher than the level of methylation required to inhibit replication *in vivo* (1 adduct per 10^5 base pairs), suggesting that the effect *in vivo* would be less dramatic. The induced delay might therefore not result in a significant extension of an S phase that is normally of 8–12-h duration. The role of replication as a source of secondary DNA damage arising from O^6-MeGua in mammalian cells was first proposed by Roberts (Plant & Roberts, 1971; Roberts & Basham, 1990), who suggested that termination and reinitiation of DNA synthesis at O^6-MeGua residues left gaps in the daughter strands. The persistence of these daughter strand gaps was postulated to inhibit replication in the subsequent S phase. In the light of our results, we would modify this model somewhat and suggest that repair synthesis is responsible for the interruptions in daughter strands and that the persistent nicks inhibit initiation of the following S phase. It has also been proposed that O^6-MeGua paired with either cytosine or thymine might be recognized by a mismatch correction system (Karran & Marinus, 1982), and nicking at thymine residues of O^6-MeGua·thymine base pairs in DNA by extracts of human cells has been reported (Sibghat-Ullah & Day, 1992). The repair synthesis we observe might therefore represent unsuccessful attempts to remove the lesion by mismatch correction. The replication extracts are indeed proficient in heteroduplex DNA repair (Thomas et al., 1991).

We have assumed in our experiments that methylation does not directly affect replication initiation. O^6-MeGua in the origin of SV40 inhibits both binding and unwinding by T antigen in a sequence-specific manner (Bignami & Lane, 1990; Bignami et al., 1991). However, at the concentrations of MNU we used, the probability of a methylated base occurring in the replication origin of pSVori is very low, since this region constitutes less than 10% of the 2.6 kb of pSVori. The inhibition we observe is therefore more likely to reflect effects on DNA elongation than on initiation. Considering the strong sequence specificity both in the formation of O^6-MeGua in DNA (Dolan et al., 1988; Richardson et al., 1989; Sendowski & Rajewsky, 1991) and in mutagenesis by MNU treatment (Palombo et al., 1992), it is possible that a sequence specificity in the effects of this methylated base on progression of the fork might occur. The data we report here lay the foundation, by using a single lesion containing plasmid substrate, to elucidate the relationship between effects on the progression of the replicative complex and the relative mutagenic efficiency of O^6-MeGua.

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