O^6-methylguanine in the SV40 origin of replication inhibits binding but increases unwinding by viral large T antigen

M.Bignami* and D.P.Lane
Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts, EN6 3LD, UK

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ABSTRACT

To study the effect of the potentially cytotoxic base O^6-methylguanine (O^6-meG) on the initiation of DNA replication, double-stranded oligonucleotides corresponding to the SV40 origin of replication were constructed in which O^6-meG replaced guanine in one strand. Out of 14 methylated residues, 10 were present in the Binding sites for T antigen (3 in Binding Site 1 and 7 in Binding Site 2). Binding of purified T antigen to the substituted oligonucleotide was considerably reduced in comparison to the unsubstituted one, as measured by nitrocellulose filter binding. Both the ATP-dependent and ATP-independent binding of T antigen were affected by the presence of the methylated base. Band shift analysis revealed an altered pattern of delayed-migrating complexes of T antigen with the O^6-meG-containing oligonucleotide. Competition experiments, in which unmodified oligonucleotides containing Binding Site 1 or 2 were included in the binding assays, indicated that the affinity of T antigen for the O^6-meG modified sites was reduced. When partially duplex oligonucleotides containing either Binding Site 1 or Site 2 of the origin of replication were used as substrates for the helicase activity of T antigen, the presence of O^6-meG increased the extent of T antigen catalysed displacement of single-stranded DNA fragments.

INTRODUCTION

The mutational spectra of methylating agents in both bacteria and mammalian cells indicate that O^6-methylguanine (O^6-meG) is the main mutagenic base produced by these alkylating agents (1,2). Its mutagenicity is a consequence of the ability of O^6-meG to pair with thymine at replication and thereby produce G:C→A:T transition mutations (3−5). Considerable evidence indicates that O^6-meG also contributes significantly to the cytotoxic effects of alkylating agents towards mammalian cells. Cell lines that are proficient in the removal of O^6-meG from their DNA (Mer^+ or Mex^+) survive treatment with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) better than cell lines in which O^6-meG is persistent (Mer^- or Mex^-) (6). Furthermore, transfection into Mex^- mammalian cell lines of the cloned *E. coli ada* gene that encodes the bacterial O^6-methylguanine-DNA methyltransferase (7), greatly improves their survival after MNNG treatment, thus giving a direct confirmation of the role of O^6-meG in cytotoxicity (8−10). The mechanism by which O^6-meG exerts its cytotoxic effect is, however, obscure. When either *E. coli* DNA polymerase or AMV reverse transcriptase is used on an MNNG-treated template, termination of DNA synthesis occurs only at modified adenine bases, indicating that the major methylated guanines (0^6-meG and N7-methylguanine) are not blocks to DNA synthesis in this *in vitro* system (11).

In apparent contradiction to the central role of O^6-meG in cell killing by alkylating agents is the observation that cell lines selected for resistance to MNNG do not have enhanced capacities to repair this methylated base (12−14). Instead, resistance is the consequence of a less well-defined mechanism that enables the cells to become tolerant to the O^6-meG adducts which remain in the DNA. A recently discovered interesting feature of tolerant cell lines is their cross-resistance to the base analogue 6-thioguanine (6-TG) (15,16). The cytotoxicity of 6-TG is a delayed effect which is evident only during replication of DNA substituted with the analogue (17). In particular, addition of 6-TG to SV40-infected CV-1 cells causes a relatively minor inhibition of net viral synthesis and the major portion is related to inhibition of new rounds of replication (18). This observation of a preferential inhibition of replication initiation by 6TG in conjunction with the cross-resistance of tolerant cells to O^6-meG and 6-TG, led us to ask whether O^6-meG residues specifically placed in a region containing an origin of DNA replication could lead to an impairment of normal initiation functions.

The SV40 replication origin was chosen because it has been widely used as a model system to identify and characterize the different steps of DNA replication in mammalian cells (19,20). Initiation of viral DNA replication requires the presence of specific nucleotide sequences that collectively define the viral origin of replication and the participation of a single virus-encoded protein, the SV40 large T antigen. We have used oligonucleotide sequences corresponding to the SV40 origin of replication to study the interaction between O^6-meG and the T antigen. We report here that the binding to specific sites and the helicase activity of T antigen are both affected by the presence of O^6-meG residues in the SV40 origin of replication.

* To whom correspondence should be addressed

MATERIALS AND METHODS

Oligodeoxynucleotide Preparation

Oligonucleotides were synthesized using an Applied Biosystems Model 380B DNA synthesizer. The purity of each phosphoramidite, including O^6-meG, was reported by the supplier (American Bionetics) to be 100% as analyzed by NMR. The efficiency of polymerization of each phosphoramidite was greater than 97%. Single stranded oligonucleotides were purified by gel electrophoresis using either 5% (109-mer) or 10% (41-mer and 60-mer) polyacrylamide gels (acrylamide/bisacrylamide 19:1 w/v) containing 7 M urea in TBE buffer (89 mM Tris-borate, pH 8.2, 2 mM EDTA). DNA was visualized by UV light and the region of the gel containing the full length oligonucleotide was excised. Oligonucleotides were eluted by soaking overnight in 0.5 M ammonium acetate (pH 8), 1 mM EDTA at 37°C and recovered by precipitation with ethanol. The sequences of the oligonucleotides used in this study are given in Fig. 1 and 4. The purity of the oligonucleotides was checked by gel electrophoresis after 5′ [^{32}P] end labelling.

Construction of 5′-end Labelled Substrates

Single stranded oligonucleotides (1μg) were labelled at the 5′ terminus with T4 polynucleotide kinase (10 U), 20 μCi of γ [^{32}P] ATP in 10 μl of T4 polynucleotide kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol). The polynucleotide kinase was inactivated by heating at 65°C for 15 min and complementary unlabelled oligonucleotides were added in equimolar amounts (in a total volume of 20 μl of kinase buffer). Annealing was carried out for 2 min at 88°C followed by 10 min at 65°C, 10 min at 37°C, and finally 10 min at room temperature. Both O^6-meG-containing and unmodified oligonucleotide duplexes were stable under our experimental conditions.

Preparation of T Antigen

T antigen was prepared as described by Simanis and Lane (21). 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 protein-A sepharose column (PAb 419). The pure T antigen was eluted with 20 mM triethylamine, pH 10.8, 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, 1 mM PMSF, and rapidly frozen in small aliquots and stored at −70° C.

Nitrocellulose Filter Binding Assay

The nitrocellulose filter binding assay was performed essentially as described by Borowiec and Hurwitz (22). Reaction mixtures (50 μl) containing 40 mM creatine phosphate (diTris salt, pH 7.8), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, 0.3 μg poly (dI-dC), 2.5 ng 5′ [^{32}P]-labelled oligonucleotide, 4 mM ATP (when indicated) and T antigen (0−300 ng) were incubated for 15 min at 37°C. The mixture was then passed through a 0.45 μm nitrocellulose filter (Millipore) presoaked in NC buffer (25 mM Hepes/NaOH, pH 7, 5 mM $MgCl_2$). The filter was washed 3 times with 1 ml portions of NC buffer, dried and radioactivity was determined by liquid scintillation counting.

Band Shift Assay

The binding reaction was performed as described above and bound T antigen fixed by the addition of glutaraldehyde to 0.1% and a further 15 min incubation at 37°C. To each sample, 5 μl of loading buffer (50% glycerol, 50 mM EDTA, 0.2% bromophenol blue, 0.2% xylene cyanol) was added and a 25 μl aliquot of the reaction mixture was immediately loaded into a 1.5 mm thick 5% nondenaturing polyacrylamide gel in TBE buffer. Electrophoresis was carried out at room temperature at 140V for 3.5 h with continuous circulation of the buffer. The gels were dried and the DNA visualized by autoradiography.

Helicase Assay

The measurement of T antigen helicase activity with duplex linear DNA oligonucleotides was performed essentially as described by Goetz et al. (23). The substrates were constructed as described above with the exception that the unlabelled 109-mer was added in a 10-fold molar excess. The standard reaction mixture (30 μl) containing 40 fmol [^{32}P]-labelled oligonucleotides, 40 mM creatine phosphate (di-Tris salt) pH 7.8, 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 25 μg/ml creatine phosphokinase, 30 μg/ml bovine serum albumin, 20 mM NaCl, 4 mM ATP, 160 ng *E. coli* single strand binding protein (Ssb) when indicated, and T antigen (0−300 ng) was incubated for 2 h at 37°C. When Ssb was present in the reaction mixture, a preincubation of T antigen with the substrate for 15 min at 37°C was performed before adding Ssb. The reaction was stopped by the addition of 0.1% sodium dodecyl sulfate/20 mM EDTA and the mixture was then treated with proteinase K (10 μg) for 1 h at 37°C. Samples were electrophoresed through 8% nondenaturing polyacrylamide gels in TBE buffer to resolve the displaced oligonucleotides. Helicase activity was determined on the basis of densitometric tracing of autoradiograms with a LKB Ultroscan XL laser densitometer. The percentage of single stranded oligonucleotide in the absence of T antigen or ATP was never higher than 1% and has been subtracted from the results presented.

RESULTS

T Antigen Binding

The oligonucleotides used in this study are synthetic double-stranded 109-mers corresponding to the HindIII-NcoI fragment of the SV40 genome which contains the origin of viral DNA replication (nucleotides 5172 to 37) (Fig. 1A). The oligonucleotide comprises: T antigen Binding Site 1 (nucleotides 5184−5211), the early palindrome (nucleotides 5211−5232), Binding Site 2 (nucleotides 5232−12), and the 3′- AT-rich domain (nucleotides 15−31) (22,24). The top strand (Figure 1B) was synthesized either unsubstituted or with O^6-meG replacing each guanine base; when two guanine bases were consecutive, only the 3′-guanine was substituted by O^6-meG. Out of 14 methylated residues, three were therefore present in Binding Site 1 and seven in Binding Site 2. The unmodified (normal oligonucleotide) or the modified (O^6-oligonucleotide) top strands were then annealed to complementary strands that had been previously 5′-end labelled with [^{32}P]. We used these oligonucleotides to examine the interactions between SV40 T antigen and an O^6-meG-substituted SV40 replication origin.

Binding of T antigen to DNA was measured initially by a nitrocellulose filter binding assay (22). Increasing amounts of T antigen (0−300 ng) were incubated with the [^{32}P]-oligonucleotide at 37° C for 15 min in the presence of ATP. The oligonucleotides complexed with T antigen were collected by filtration through nitrocellulose membranes. Maximal binding was observed at low concentrations of T antigen (75 ng), with close to 90% of the input oligonucleotide retained (Fig. 1C).

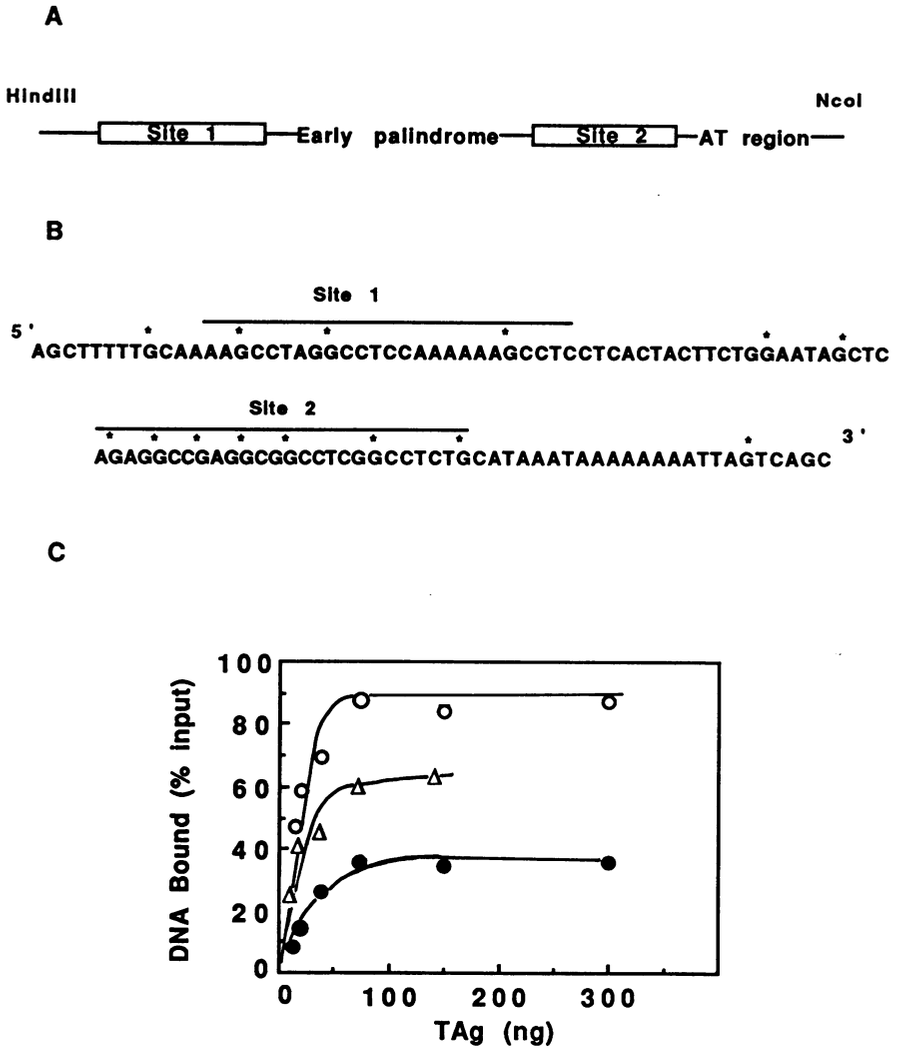

Figure 1. A) Schematic representation of the region of the SV40 origin of replication contained in the HindIII-NcoI fragment. B) Oligonucleotide sequence of the upper strand of the origin region. The positions which were substituted by O^6-meG are represented by *. C) Binding of T antigen to the oligonucleotides containing the SV40 origin. Increasing concentrations of purified T antigen were incubated with 5'-labelled oligonucleotides annealed to the complementary strand. Normal oligonucleotide (○), O^6-meG oligonucleotide (●) or O^6-meG oligonucleotide pretreated with the purified *E. coli* Ada O^6-meG-DNA methyltransferase (△). After 15 minutes incubation, the reaction mixtures were adsorbed onto nitrocellulose filters and the bound radioactivity was determined.

When the O^6-oligonucleotide was used, maximum T antigen binding was reached at the same protein: oligonucleotide ratio as for the normal oligonucleotide but the total amount of oligonucleotide retained was reduced to less than 40%. Addition of higher concentrations of T antigen did not further increase the amount of binding observed. It is possible that the inability of higher concentrations of T antigen to increase the level of binding may be due to the oligomerization of the protein into hexameric forms which are unable to bind DNA (25).

To establish that the effects we observed on large T binding were a result of the presence of O^6-meG in DNA, the O^6-oligonucleotide was pretreated with a two-fold molar excess of the homogeneous *E. coli* O^6-methylguanine DNA-methyltransferase, Ada (a kind gift of B. Sedgwick). After removal of the enzyme by digestion with proteinase K and inactivation of the protease with PMSF, the treated oligonucleotide was used in a nitrocellulose binding assay (Figure 1C). A two-fold increase in the amount of oligonucleotide retained on the filter was observed over a range of concentrations of T antigen after Ada-treatment of the O^6-oligonucleotide. These data show that the reduction in oligonucleotide binding can be largely ascribed to the presence of O^6-meG in the substrate. The inability of the Ada protein to restore completely the T antigen binding capacity suggests that the enzyme may not be 100% efficient in removing O^6-meG residues closely positioned in a GC rich sequence. Consistent with this, it has been reported that repair of O^6-meG can vary at least 3–4 fold depending on the position of the lesion in particular DNA sequences (26).

ATP-Dependence of T Antigen Binding

Two types of binding of T antigen to the SV40 origin have been described. In the presence of ATP, T antigen complexes with the origin in the form of a double hexamer which covers the whole core origin region. In the second type of binding, which is independent of ATP, tetrameric complexes of T antigen bind predominantly to Binding Site 1 (22, 27–28). We analysed to what extent the presence of O^6-meG affected the ATP-dependent or ATP-independent properties of T antigen binding.

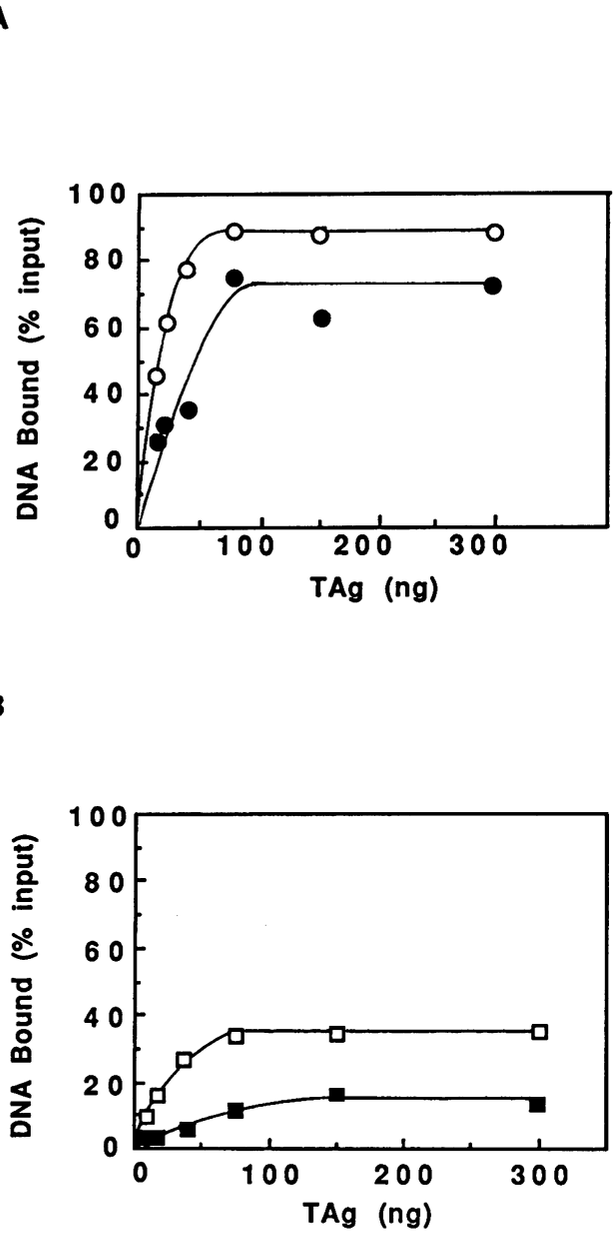

Figure 2. Influence of ATP on the binding of T antigen to normal and substituted oligonucleotides. Nitrocellulose filter binding experiments were carried out as described in the legend to Figure 1. A) Binding of T antigen to the normal oligonucleotide in the presence (○) or in the absence (●) of ATP. B) Binding of T antigen to the O^6-meG-oligonucleotide in the presence (□) or in the absence (■) of ATP.

In the absence of ATP, a slight reduction in the level of complex formation to the normal oligonucleotide was observed at all concentrations of T antigen tested (Fig. 2A). In the case of the O^6-oligonucleotide a more drastic reduction in T antigen binding was observed when the binding reactions were carried out in the absence of ATP. This reduction was more pronounced at lower concentrations of T antigen (Fig. 2B). The preferential inhibition of ATP-independent T antigen-oligonucleotide complex formation suggests that the assembly of the tetrameric T antigen-oligonucleotide complex on Site 1 is most affected by the presence of the methylated base.

Delayed Migration Analysis

The formation of a T antigen nucleoprotein complex reduces the mobility of origin-containing DNA fragments on agarose gels (28). When the labelled normal oligonucleotide was incubated with T antigen under the same conditions as for the nitrocellulose binding assay, fixed with glutaraldehyde, and electrophoresed through a 5% polyacrylamide gel, a shift in the mobility of the oligonucleotide was also observed. In the presence of ATP, multiple delayed-migrating bands were apparent (Figure 3). The intensity of the bands was dependent on the T antigen concentration (Fig 3, lanes 1–4). A different pattern of mobility shift of the oligonucleotide was observed in the absence of ATP (lanes 5–7) reflecting the reduced level of T antigen oligomerization and the bias in binding towards Binding Site 1 (21, 26–27).

In agreement with the nitrocellulose binding assay, the presence of O^6-meG in the oligonucleotide significantly reduced the overall amount of delayed migrating complexes formed in the presence of ATP (Fig. 3A and B, lanes 8–11). In the absence of ATP, very little or no T antigen-DNA complex was observed with the O^6-oligonucleotide (lanes 12–14). These data indicate that as few as three O^6-meG residues in the 28 bp Binding Site 1 are sufficient to abolish the stable binding of T antigen oligomers to this site. Preliminary experiments using an oligonucleotide which contains only the O^6-meG residues in Binding Site 1 indicate a similar degree of altered T antigen binding, thus confirming this conclusion (work in progress).

Competition of T Antigen Binding

The two T antigen binding sites in the origin region can be distinguished by their relative affinity and ATP dependence of T antigen binding. The effect of O^6-meG on the affinity of T antigen for Binding Site 1 or Binding Site 2 was investigated separately by competitive binding experiments. Oligonucleotides containing either Binding Site 1 (oligonucleotide 1, nucleotides 5175–5215) or Binding Site 2 (oligonucleotide 2, nucleotides 5216–32) (Fig. 4A) were synthesized, annealed to their radiolabelled complementary strands and their ability to bind T antigen was determined by the nitrocellulose filter binding assay. Incubation with increasing concentrations of T antigen resulted in the retention of increasing amounts of oligonucleotide 1, up to a maximum of 50% (Fig. 4B). A similar T antigen-dependent binding to oligonucleotide 2 was also observed although the maximal attainable binding was only 20% (Figure 4B).

Inclusion of a double-stranded, non-radioactive oligonucleotide 1 (Binding Site 1) in the incubation mixture with radioactively labelled normal 109-mer and T antigen (75ng) produced a concentration-dependent reduction in the binding of the labelled normal oligonucleotide to nitrocellulose filters; approximately 50% inhibition was observed at a competitor : substrate ratio of 5:1 and 90% at a ratio of 50:1 (Fig. 5A). In contrast, the binding of T antigen to the O^6-oligonucleotide was inhibited at much lower ratios of competitor : substrate oligonucleotide, and >90% reduction in binding was observed at a competitor : substrate oligonucleotide ratio of only 5:1.

Oligonucleotide 2 (Binding Site 2) was a relatively poor competitor of T antigen binding to the normal substrate oligonucleotide but a good competitor for the O^6-meG containing substrate with >80% inhibition of binding achieved at a competitor : substrate oligonucleotide ratio of only 5:1 (Fig. 5B).

The relative ease with which the binding to the

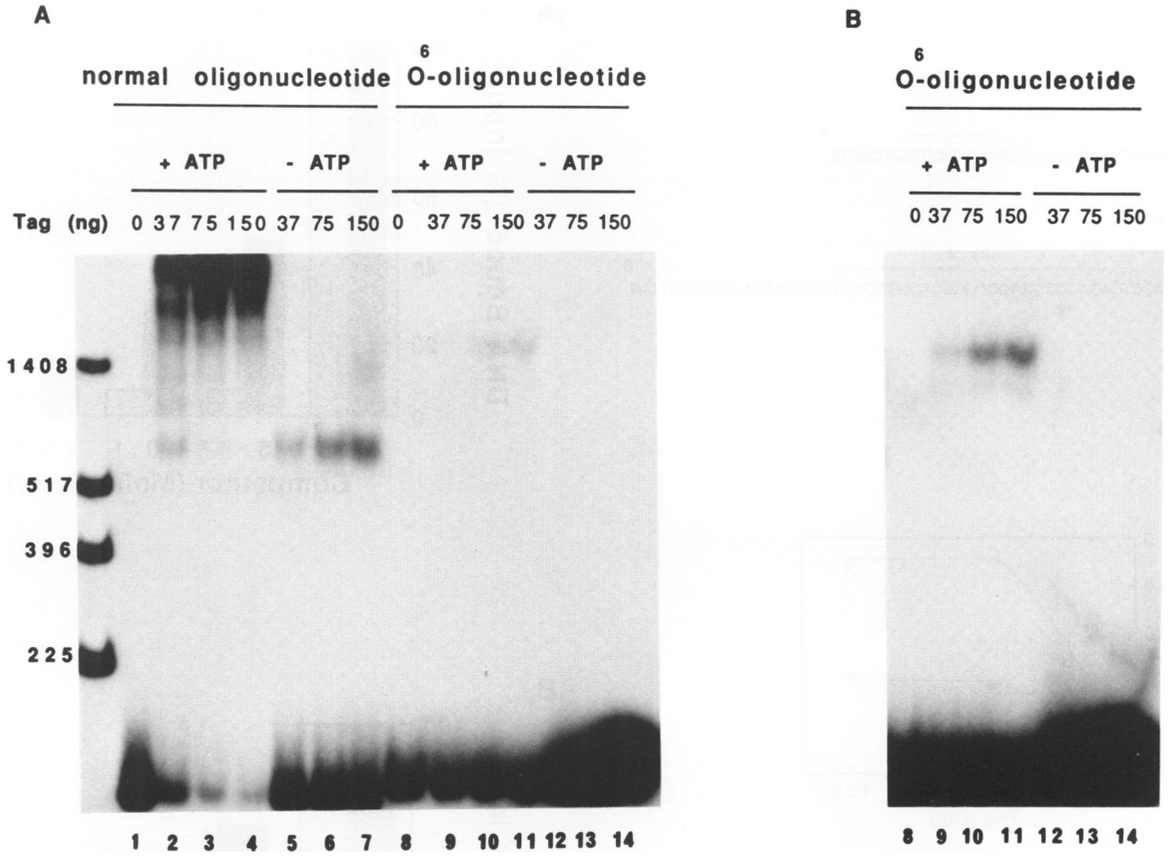

Figure 3. A) Polyacrylamide gel electrophoresis of T antigen-oligonucleotide complexes. Increasing concentrations of T antigen were incubated with the oligonucleotides in the presence or absence of ATP as for the nitrocellulose binding assays. Reactions were carried out at 37°C for 15 minutes and fixed with 0.1% glutaraldehyde for a further 15 min at 37°C. The reaction products were separated on a 5% polyacrylamide gel and analysed by autoradiography. A longer exposure of the part of the gel containing the T antigen complex with the O^6-meG-oligonucleotide is shown in panel B.

O^6-oligonucleotide is reduced by the competitor oligonucleotides, despite the inefficient binding of the latter by T antigen, indicates that not only is the extent of binding of T antigen to the O^6-oligonucleotide reduced by substitution with 0^6-meG, but that the affinity of T antigen for the modified binding sites is also reduced.

Effects of O^6-meG on DNA Helicase Properties of T Antigen

T antigen is a DNA helicase that binds to partially duplex DNA molecules with a 3′ region of single stranded DNA and then translocates along the double-stranded region unwinding the DNA in a 3′ - 5′ direction (29). In the presence of *E. coli* or HeLa Ssb, T antigen also carries out an ATP-dependent unwinding of duplex linear or circular DNA containing an SV40 replication origin (23). We investigated the effect of O^6-meG substitution in the origin of replication on the unwinding activity of T antigen.

The synthetic 60-mer corresponding to Binding Site 2 (the strand complementary to Oligonucleotide 2, Fig. 5A) was radioactively labelled at its 5′ end and annealed to the normal 109-mer. The resulting molecule comprises a 60bp duplex region with a 3′ overhanging region of 5 bases and a 5′ overhang of 44 bases (Fig 6A). When this substrate was incubated with purified T antigen in the presence of ATP and an ATP-regenerating system, the 60-mer oligonucleotide was displaced by T antigen in a dose-dependent manner (Fig. 6B and C, lanes 2−6). The extent of displacement was increased by the inclusion of *E. coli* Ssb (Fig. 6B, 6C, lanes 7−9) and no displacement occurred in the absence of ATP (Fig. 6B and C, lane 10), indicating that the DNA helicase activity of T antigen was being observed.

When the 60-mer annealed to the 109-mer containing O^6-meGua was used as a substrate, a greater displacement of the fragment was observed at all concentrations of T antigen (Fig. 6B and C, lanes 12−16). The extent of the displacement was enhanced dramatically by the inclusion of *E. coli* Ssb (lanes 17−20). Again no unwinding was observed in the absence of ATP (data not shown).

Qualitatively similar data were obtained with a substrate in which the 41-mer Binding Site 1 oligonucleotide was annealed to the 109-mers, although the extent of unwinding was reduced. In this case the substrate comprises a 41 bp duplex region with a 3 base overhanging region at the 5′ end and a 3′ overhang of 65 bases (Fig. 7A). The T antigen-catalysed unwinding of the unmodified substrate was very limited (Fig. 7B). Less than 10% of the 41-mer molecules were displaced in the presence of ATP and up to 300 ng of T antigen. No further increase was observed by increasing T antigen up to 1 μg (data not shown). No displacement was observed in the absence of ATP while the addition of *E. coli* Ssb stimulated unwinding mostly at the lower T antigen concentrations (Fig. 7B).

In the presence of ATP, the extent of T antigen catalysed displacement of the 41-mer oligonucleotide from the duplex

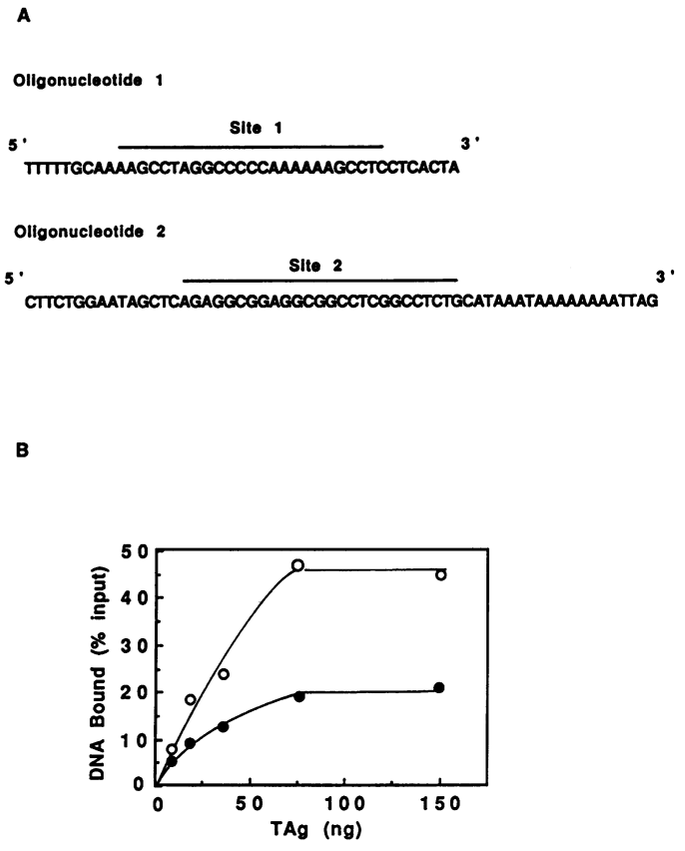

Figure 4. A) Sequences of oligonucleotide containing Binding Site 1 (Oligonucleotide 1) and Binding Site 2 (Oligonucleotide 2). B) Nitrocellulose filter binding assays in the presence of ATP of T antigen to Oligonucleotide 1 (○) and Oligonucleotide 2 (●) annealed to their complementary strands.

region of the O^6-oligonucleotide was again significantly higher than from the unsubstituted substrate (Fig. 7B) and the inclusion of Ssb protein again enhanced 3-fold the efficiency of unwinding.

These data indicate that, despite its slightly greater length and its much reduced 3′ overhanging region, the Binding Site 2 oligonucleotide is displaced more efficiently by the helicase activity of T antigen. In conclusion, although T antigen binding to the O^6 substituted oligonucleotides was greatly decreased, its helicase activity is facilitated by the presence of O^6-meG in the duplex region of either of the helicase substrates.

DISCUSSION

We report in this paper that the presence of O^6-meG in a specific DNA sequence containing the origin of SV40 replication disturbs the complex pattern of interaction between the viral T antigen and its target sequence. The SV40 origin of replication is a well characterized system that represents a model for mammalian DNA replication. T antigen is the only viral protein essential for *in vitro* replication of plasmids containing the SV40 origin and is able to assemble and direct the array of cellular replication proteins present in extracts from permissive human or monkey cells (30). In the SV40 system, T antigen acts both to recognize the origin sequences and to provide the helicase function necessary for its unwinding (31). Under *in vitro* replication conditions, the site-specific binding of T antigen organizes the protein for the bidirectional unwinding of the origin

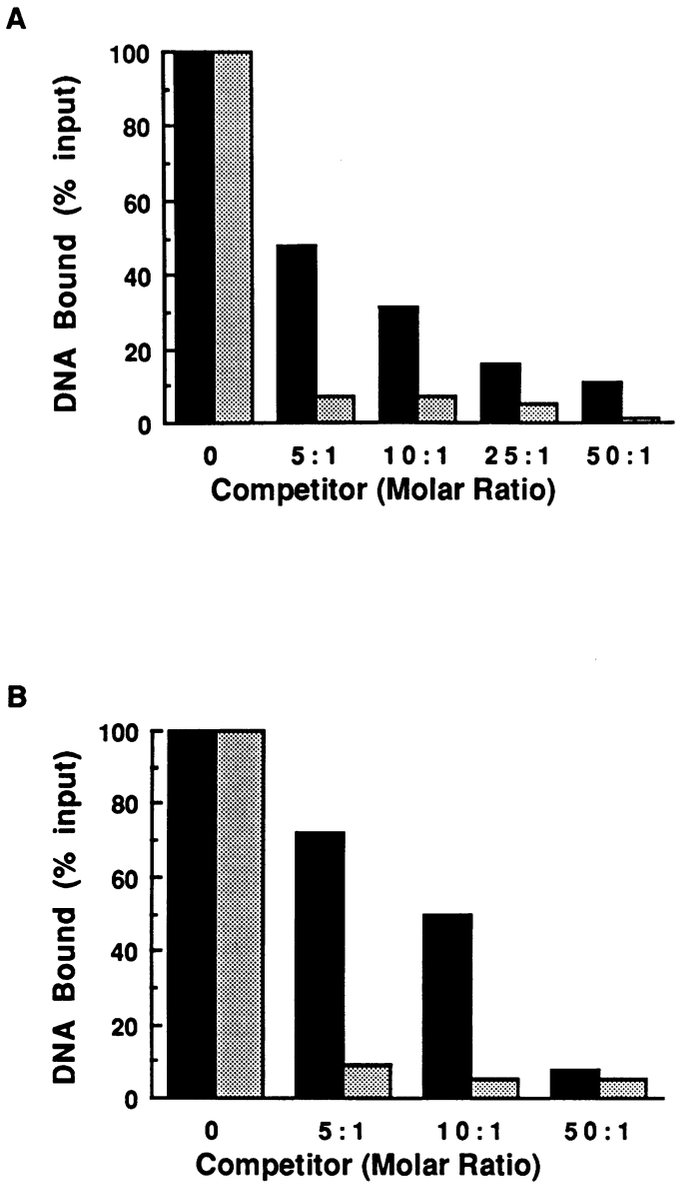

Figure 5. Competition for T antigen binding by non-radioactive oligonucleotide. Nitrocellulose filter binding experiments were carried out using 75ng T antigen in the presence of ATP with the unmodified (Black Bars) or the O^6-meG-substituted oligonucleotide (Shaded Bars) as described except that the reactions were supplemented with unlabelled double-stranded Oligonucleotide 1 (Panel A) or Oligonucleotide 2 (Panel B) at the molar ratios indicated. The level of T antigen binding to the normal or to the O^6-oligonucleotide in the absence of competitor was normalized to 100%.

DNA when Ssb and topoisomerase I are present (32,33). Both the binding and the unwinding activities were affected by the presence of O^6-meG in the recognition sequence for T antigen.

The reduced level of binding of T antigen to both Binding Site 1 and Binding Site 2 in the O^6-oligonucleotide is in accordance with the predicted importance of guanines in these two regions in the interaction with the T antigen (34,35). All the guanine residues in the top strand of both Binding Site 1 and Binding Site 2 exhibit a reduced reactivity towards dimethylsulfate when T antigen is bound to the origin. In addition, single base pair mutagenesis of Binding Site 2 indicates that all mutations within the pentanucleotide sequence 5′-GAGGC-3′/ 5′-GCCTC-3′

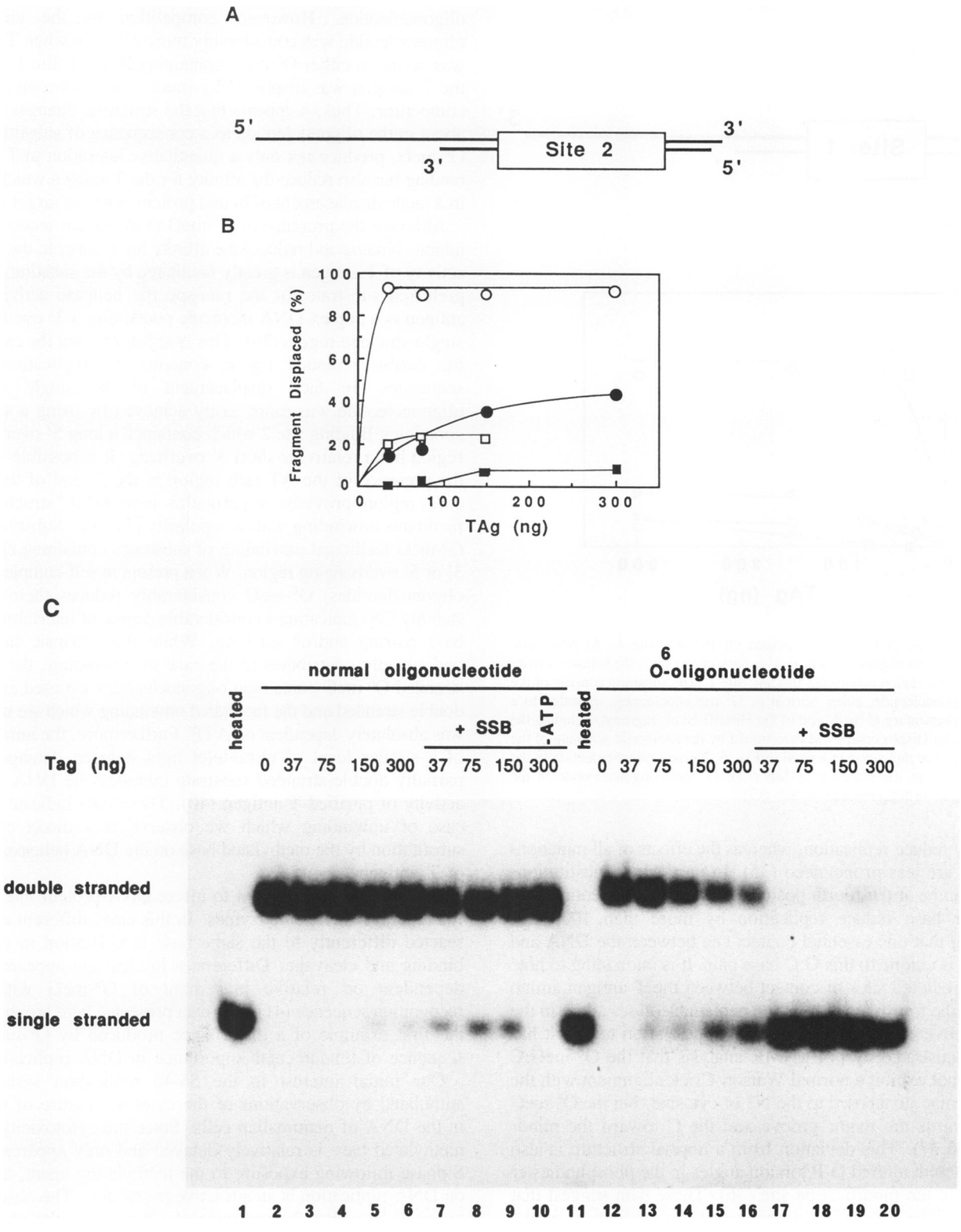

Figure 6. Helicase Activity of T antigen on Binding Site 2. A) Schematic representation of the oligonucleotides used as substrate to study the helicase activity of T antigen in a region containing Binding Site 2. The substrate consists of the 109mer oligonucleotide, either normal or O^6-meG-containing, annealed to a 60mer complementary to the 3' end of the HindIII-NcoI fragment excluding the final 5 bases. B) Displacement of the 60mer single-stranded fragment by T antigen. The partially double-stranded substrates containing the 5'-end labelled 60mer were incubated with increasing concentrations of T antigen in the presence (open symbols) or absence (closed symbols) of *E.coli* Ssb protein. Displacement of the fragment was analysed by acrylamide gel electrophoresis and quantitated by densitometric scanning of the autoradiograms. Normal oligonucleotide (■) (□); O^6-meG-oligonucleotide (●) (○). C) Autoradiogram of a helicase assay using the normal and the O^6-meG oligonucleotides.

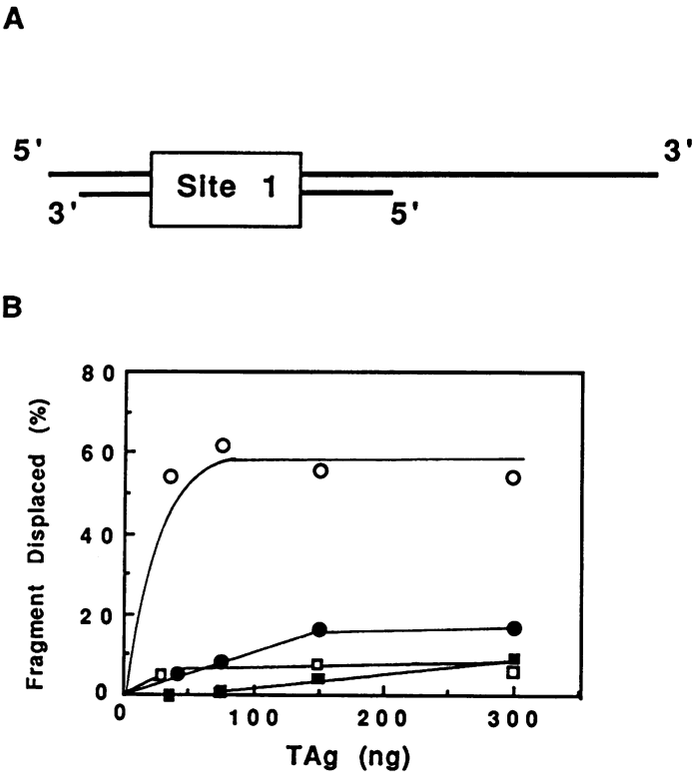

Figure 7. Helicase Activity of T antigen on Binding Site 1. A) Schematic representation of the oligonucleotides used as substrate to study the helicase activity of T antigen in a region containing Binding Site 1. The substrate consists of the 109-mer oligonucleotide, either normal or O^6-meG-containing, annealed to a 41-mer complementary to the 5' end of the HindIII-NcoI fragment excluding the first 3 bases. B) Displacement was determined by densitometric scanning of the autoradiograms as described in legend to Fig. 6. Normal oligonucleotide in the absence (■) or in the presence of Ssb (□); O^6-meG-oligonucleotide in the absence (●) or in the presence of Ssb (○).

drastically reduce replication, whereas the effects of all mutations outside it are less pronounced (35). In particular, substitutions of the guanine at the fourth position of the pentanucleotide with any other base reduce replication by more than 100 fold, suggesting that one essential contact site between the DNA and T antigen is unique to this G:C base pair. It is interesting to note that the predicted sites of contact between the T antigen amino acids and the fourth guanine of the pentanucleotides all lie in the major groove of the DNA helix (35). In relation to this it has been demonstrated by [^{31}P]-NMR analysis that the O^6-meG:C pair does not exhibit a normal Watson-Crick alignment with the N1 of guanine juxtaposed to the N3 of cytosine, but the O^6-meG slides towards the major groove and the C toward the minor groove (36,37). This deviation from a normal structure is also associated with altered O-P torsion angles in the phosphodiester backbone at the modification site (36). These data suggest that the shift of the O^6-meG base pair and the projection of the methyl group into the major groove might be largely responsible for the reduced efficiency of T antigen binding.

The competition experiments in which we investigated the binding of T antigen to the two origin binding sites in the presence of an excess of non-radioactive binding site oligonucleotides, provide information about the relative affinity of T antigen for the normal and substituted origin regions. As expected, inclusion of an excess of unlabelled oligonucleotide reduced the binding of T antigen to its target sequences in the substrate oligonucleotide. However, competition by the unlabelled oligonucleotide was considerably more efficient when T antigen was bound to either O^6-meG-containing Binding Site 1 or 2 and the T antigen was displaced by much lower concentrations of competitor. Thus, it appears that the structural changes brought about in the oligonucleotide as a consequence of substitution by O^6-meG, produce not only a quantitative alteration in T antigen binding but also reduce the affinity for the T antigen which results in a facile displacement of bound protein from the target regions.

Although the presence of O^6-meG in the origin sequence both inhibits binding and reduces the affinity for T antigen, the helicase activity of T antigen is greatly facilitated by the substitution. The preferred substrate for the non-specific helicase activity of T antigen is a duplex DNA molecule containing a 3' overhanging single-stranded region (29). This is apparently not the case when the double stranded region contains the replication origin sequences. In fact, displacement of the single stranded oligonucleotide was more easily achieved by using a substrate containing Binding site 2 which contained a long 5' overhanging region but a relatively short 3' overhang. It is possible that that the presence of the AT rich region at the 3' end of the origin core region provides a particular bent DNA structure that facilitates unwinding with this polarity (31, 38). Substitution by O^6-meG facilitated unwinding of substrates containing either the 3' or 5' overhanging region. When present in self-complementary oligonucleotides, O^6-meG considerably reduces their thermal stability (39) indicating a considerable degree of interference with base pairing and/or stacking. While this intrinsic instability undoubtedly contributes to the ease of unwinding, the double-stranded O^6-meG-containing oligonucleotides we used are stably double stranded and the facilitated unwinding which we observed was absolutely dependent on ATP. Furthermore, the introduction of a similar level of ultraviolet light induced damage into a partially double-stranded substrate inhibited the DNA helicase activity of purified T antigen (40). These data indicate that the ease of unwinding which we observe is a direct effect of substitution by the methylated base on the DNA helicase activity of T antigen.

O^6-meG has been shown to affect DNA-protein interactions for several restriction enzymes. In this case, different enzymes reacted differently to the same base modification in terms of binding and cleavage. Differences in cleavage appeared to be dependent on relative placement of O^6-meG within the recognition sequence (41). The data presented here are, however, the first example of a disturbance produced by O^6-meG in a sequence of fundamental importance in DNA replication.

Our initial interest in the SV40 replication system was stimulated by observations of the cytotoxic nature of O^6-meG in the DNA of mammalian cells. Since the cytotoxicity of this methylated base is relatively delayed and only apparent in the S phase following exposure to the methylating agent, an effect on DNA replication is an attractive possibility. The observation reported here that the presence of O^6-meG on the replication origin enhances the helicase activity of T antigen indicates that its presence in such sequences may interfere with the tight control of DNA replication and allow promiscuous initiation of DNA synthesis. Although the density of methylated bases present in the substrates we used is not likely to be present in DNA after treatment with an alkylating agent, this study is a first approach to dissecting the possible effects of O^6-meG on mammalian DNA replication. The use of oligonucleotides containing single O^6-meG residues in selected positions of the SV40 origin may

extend this approach to identify the molecular sites of G:C pairing fundamental in the binding or unwinding properties of T antigen.

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