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Site-Dependent Inhibition by Single O^6-Methylguanine Bases of SV40 T-Antigen Interactions with the Viral Origin of Replication†

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Received October 17, 1990; Revised Manuscript Received December 10, 1990

ABSTRACT: The effects of O^6-methylguanine on the reactions involved in initiation of DNA replication were investigated by measuring the interactions of SV40 T antigen with oligonucleotides substituted with the methylated base. O^6-Methylguanine residues were positioned in either binding site I or binding site II of the SV40 origin of replication. Binding of purified T antigen, measured by both nitrocellulose filter binding and delayed oligonucleotide migration, was unaffected by the presence of seven methylated bases in binding site II. Single substitutions within binding site I were sufficient to inhibit T-antigen binding, and the extent of inhibition was dependent on the position of O^6-methylguanine in the DNA sequence. Unwinding by T antigen was analyzed by measuring displacement of a single-stranded oligonucleotide from similarly substituted, partially duplex substrates. The presence of three O^6-methylguanine residues in binding site I facilitated the helicase activity of T antigen. In contrast, single O^6-methylguanine bases inhibited unwinding. A correlation was observed between the position of the methylated base and the inhibition of both binding and unwinding by T antigen.

Although cells have evolved efficient strategies for the removal of damaged residues from DNA, occasional unrepaired lesions may escape surveillance and interact with cellular DNA replication. This interaction will occur more frequently in cells in which DNA repair enzymes are defective or absent. The specific DNA repair protein O^6-methylguanine (O^6-MeG)–DNA methyl transferase (MT) is not expressed in certain human cell lines [the Mex^- or Mer^- phenotype (Day et al., 1980; Sklar & Strauss, 1981)], resulting in a cellular sensitivity to alkylating agents that introduce O^6-MeG into DNA (Scudiero et al., 1984). Although the mechanism of cytotoxicity

† M.B. was supported in her stay at the Imperial Cancer Research Fund by a grant from the European Science Foundation.
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0006-2960/91/0430-2857$02.50/0

of O^6-MeG has not been defined, it is likely to involve DNA replication. Both O^6-MeG·C and O^6-MeG·T base pairs introduce regions of instability in the DNA helix (Gaffney & Jones, 1989), and kinetic evidence indicates that formation of base pairs with O^6-MeG during replication is much less favorable than a normal base pair (Singer et al., 1989). However, the presence of the methylated base in template DNA does not constitute a complete block for purified DNA polymerases in an in vitro system (Larson et al., 1985).

The structural features that are involved in initiation of replication in eukaryotic cells are beginning to emerge (Burhans et al., 1990), yet viral systems are the best available models with which to study protein–DNA interactions in mammalian DNA replication. Host-cell reactivation of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-treated adenovirus was originally used to define the alkylation-sensitive Mer$^-$ phenotype and implicate O^6-MeG in cytotoxicity (Day et al., 1980). To investigate directly the effects of O^6-MeG on DNA replication, we have used the in vitro SV40 replication system as a model for mammalian cells. A single viral protein, T antigen, is required to supplement soluble cellular proteins that carry out complete replication of plasmids containing the SV40 replication origin (Li & Kelly, 1984). In the initial steps of replication, T antigen binds with high affinity to specific sequences within the SV40 origin (binding sites I and II). Following this targeted DNA binding, the ATP-dependent DNA helicase activity of T antigen participates in the extensive unwinding of the template DNA, and the host replication complex is recruited to form the functional replication forks [for recent reviews, see Stillman (1989) and Challberg and Kelly (1989)].

We have previously shown that the presence of numerous O^6-MeG residues throughout the SV40 origin of replication inhibits the binding of T antigen to both binding sites I and II. Futhermore, multiple substitution with the methylated base facilitates the helicase activity of T antigen (Bignami & Lane, 1990). The synthetic 109-mer oligonucleotide used in those experiments contained a very high density of O^6-MeG residues. In comparison, the D_{37} value for CHO cells is associated with approximately 1 O^6-MeG residue per 10^5 nucleotides (Aquilina et al., 1990). Our aim is to understand, in molecular terms, the effects of biologically relevant levels of O^6-MeG on mammalian DNA replication. We report here that single O^6-MeG bases may have profound consequences for both binding and unwinding by purified T antigen and that the effects exhibit considerable context dependence.

Materials and Methods

Oligodeoxynucleotides. Oligonucleotides were synthesized by 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) polyacrylamide gels [acrylamide/bis(acrylamide), 19:1 w/v] containing 7 M urea in TBE buffer (89 mM Tris-borate, pH 8.2, and 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.

Construction of 5'-End-Labeled Substrates. Single-stranded oligonucleotides (1 μg) were labeled at the 5' terminus with T4 polynucleotide kinase (10 units/20 μCi of [γ-^{32}P]ATP in 10 μL of T4 polynucleotide kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 5 mM dithiothreitol). The polynucleotide kinase was inactivated by heating at 65 °C for 15 min, and complementary unlabeled 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 (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/protein A/Sepharose column (PAb 419). 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, 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 (1988). Reaction mixtures (50 μL) containing 40 mM creatine phosphate (di-Tris salt, pH 7.8), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mg/mL bovine serum albumin, 0.3 μg of poly(dI-dC), 2.5 ng of 5'-^{32}P-labeled oligonucleotide, 4 mM ATP, and T antigen (0–150 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 7.0, and 5 mM $MgCl_2$). The filter was washed 3 times with 1-mL portions of NC buffer and dried, and the radioactivity was determined by liquid scintillation counting.

Delayed Oligonucleotide Migration 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 was added 5 μL of loading buffer (50% glycerol, 50 mM EDTA, 0.2% bromophenol blue, and 0.2% xylene cyanol), and a 25-μL aliquot of the reaction mixture was immediately loaded into a 1.5-mm-thick nondenaturing 5% polyacrylamide gel in TBE buffer. Electrophoresis was carried out at room temperature at 10 V/cm for 3.5 h with continuous circulation of the buffer. The gels were dried, and the DNA was 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. (1988). Partial duplex substrates were constructed by annealing end-labeled unsubstituted 41-mer oligonucleotides, complementary to a region which includes binding site I, to a 109-mer containing the origin of replication. Annealing was carried out as described above with the exception that the unlabeled 109-mer was added in a 10-fold molar excess. The standard reaction mixture (30 μL) containing 40 fmol of ^{32}P-labeled 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 of *Escherichia coli* single-strand binding protein (ssb) when indicated, and T antigen (0–150 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

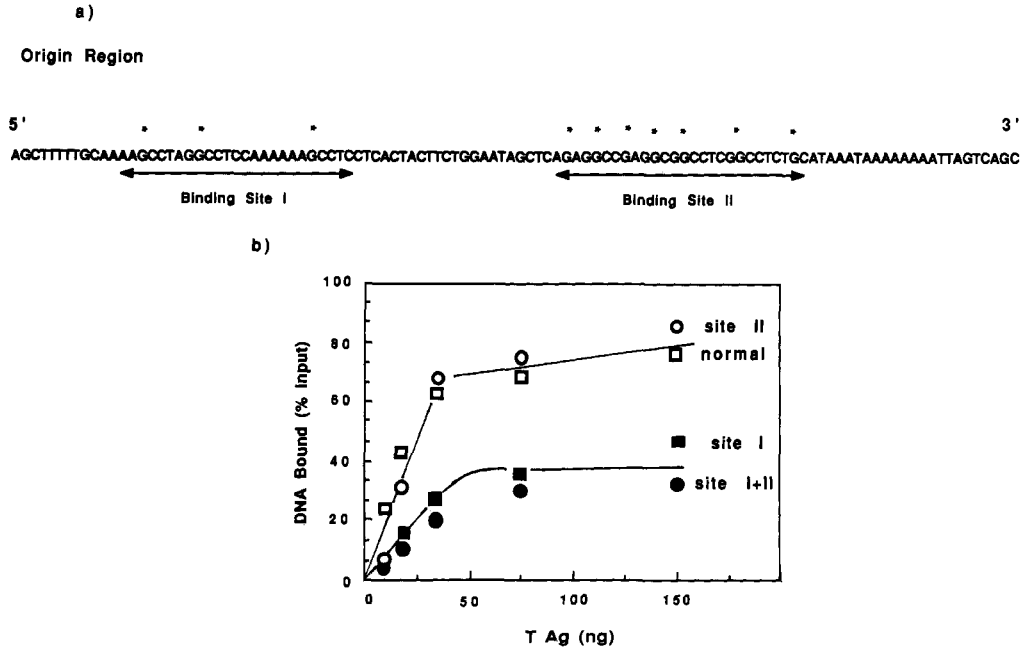

FIGURE 1: (a) Oligonucleotide sequence of the region of the SV40 origin of replication contained in the *Hin*dIII–*Nco*I fragment. The positions which were substituted by O^6-MeG are represented by asterisks. (b) Binding of T antigen to the oligonucleotides containing the SV40 origin. Increasing concentrations of purified T antigen were incubated with 5′-labeled oligonucleotides annealed to the complementary strand. Unsubstituted substrate (normal, □); substrate containing O^6-MeG in binding site II (site II, ○); substrate containing O^6-MeG in binding site I (site I, ■); substrate containing O^6-MeG in binding sites I and II (site I+II, ●). After 15-min incubation, the reaction mixtures were adsorbed onto nitrocellulose filters, and the bound radioactivity was determined. Each curve represents the mean of three independent experiments.

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 oligomers. Helicase activity was determined on the basis of densitometric tracing of autoradiograms with an 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

Inhibition of T-Antigen Binding by Multiple O^6-MeG. The oligonucleotides used in this study are synthetic 109-mers corresponding to the *Hin*dIII–*Nco*I fragment of the SV40 genome (nucleotides 5172–37) and comprise T-antigen binding site I (nucleotides 5184–5211), the early palindrome (nucleotides 5211–5232), binding site II (nucleotides 5232–12), and the 3′-AT-rich domain (nucleotides 15–31) (Deb et al., 1986; Borowiec & Hurwitz, 1988). Figure 1a shows the sequence of the top strand of this origin region and indicates all the positions of substitution by O^6-MeG within binding sites I and II. Three multiple-substituted substrates were synthesized: the first contained all methylated bases in binding sites I and II; the second, three in binding site I alone; and the third, seven in binding site II alone. The unmodified (normal) or modified top strand was then annealed to the complementary bottom strand which had been previously 5′-end-labeled with ^{32}P. The resulting double-stranded oligonucleotides were incubated with increasing concentrations (0–150 ng) of purified T antigen at 37 °C for 15 min in the presence of ATP, and binding of T antigen to DNA was measured by a nitrocellulose filter binding assay (Borowiec & Hurwitz, 1988). In the binding conditions we used, T antigen protects binding sites I and II with little cooperation between the two sites (Deb & Tegtmeyer, 1987; Mastrangelo et al., 1989). We have previously shown by this method that the presence of 14 O^6-MeG bases in this sequence reduced T-antigen binding by more than 60% and pretreatment of the substrate with purified *E. coli* MT substantially restored this activity (Bignami & Lane, 1990). A similar inhibition of binding was observed with the substrate containing 10 O^6-MeG confined within binding sites I and II (Figure 1b). The presence of three O^6-MeG in binding site I was sufficient to reduce T-antigen binding to a similar extent. In contrast, the substrate which contained seven methylated guanine bases within binding site II bound T antigen with an efficiency closely similar to that of the normal substrate (Figure 1b).

Binding of T antigen to these origin-containing substrates can also be assayed by delayed migration of the resulting complexes through agarose or acrylamide gels (Dean et al., 1987; Bignami & Lane, 1990). When the complex formed with the normal substrate under the same conditions as for the nitrocellulose binding assay was fixed with glutaraldehyde and electrophoresed through a 5% polyacrylamide gel, two major and several minor delayed-migrating bands were observed only in the presence of T antigen (Figure 2). The intensity of both major bands was similar at 75 and 150 ng of T antigen in accordance with the saturation of T-antigen binding observed in the filter binding assay (see Figure 1b). The presence of modifications in binding site II alone did not significantly alter the level of complex formation. In contrast, neither major delayed-migrating species was formed from the substrate containing modifications in binding site I alone, and only the minor bands, possibly representing T-antigen–oligonucleotide complexes with different conformations, were observed.

In conclusion, both the nitrocellulose filter binding assay and delayed migration analysis indicate that T-antigen binding is unaffected by multiple modifications in binding site II and all of the previously observed reduction of T-antigen binding can be ascribed to the presence of O^6-MeG in binding site I.

Site-Dependent Inhibition of T-Antigen Binding by Single O^6-MeG. Since T-antigen binding is strongly affected by modification of only three positions within binding site I, each

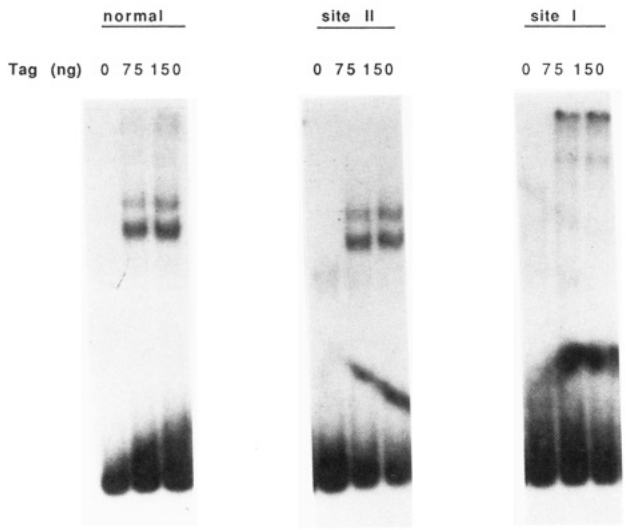

FIGURE 2: Polyacrylamide gel electrophoresis of T-antigen–oligonucleotide complexes. T antigen was incubated with the unsubstituted substrate (normal), a substrate containing O^6-MeG in binding site II (site II), or a substrate containing O^6-MeG in binding site I (site I) as for the nitrocellulose binding assays. Reactions were carried out at 37 °C for 15 min 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 analyzed by autoradiography.

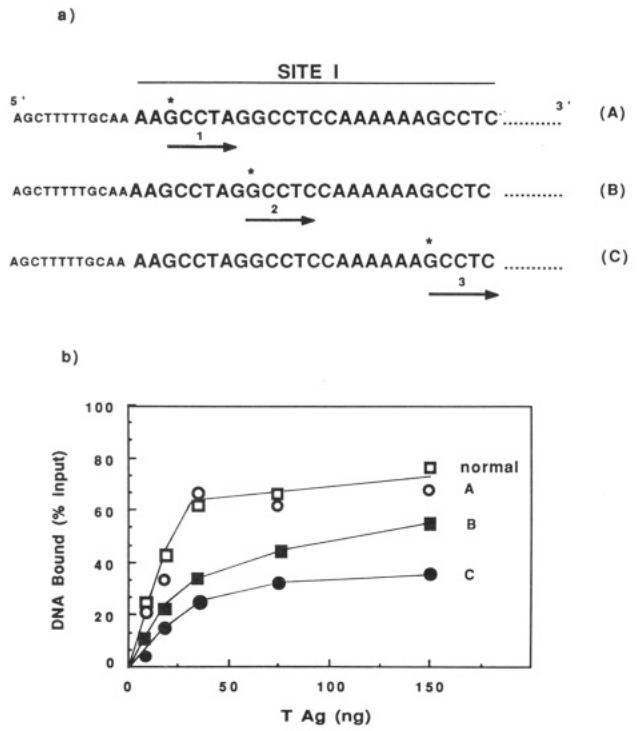

FIGURE 3: (a) Oligonucleotide sequence of a region of the top strand of the 109-mer indicating the positions in binding site I which were substituted by O^6-MeG (asterisks). The arrows indicate the 5′ to 3′ direction of the sequences necessary for T-antigen–DNA interaction (pentanucleotides 1, 2, and 3). (b) Binding of T antigen to the unsubstituted substrate (normal, □), substrate A (○), substrate B (■), and substrate C (●) containing O^6-MeG in the indicated position.

position was evaluated separately. Figure 3a shows the three singly modified substrates in which O^6-MeG is present in each of the imperfect repeats 5′-GCCTN-3′ (pentanucleotides 1, 2, and 3) which correspond to the T-antigen contact sites (De Lucia et al., 1983). When substrate A containing the modification in pentanucleotide 1 was used in a standard nitrocellulose filter binding assay, no alteration in T-antigen binding was observed (Figure 3b). In contrast, significant reduction in the level of T-antigen binding was observed with substrates

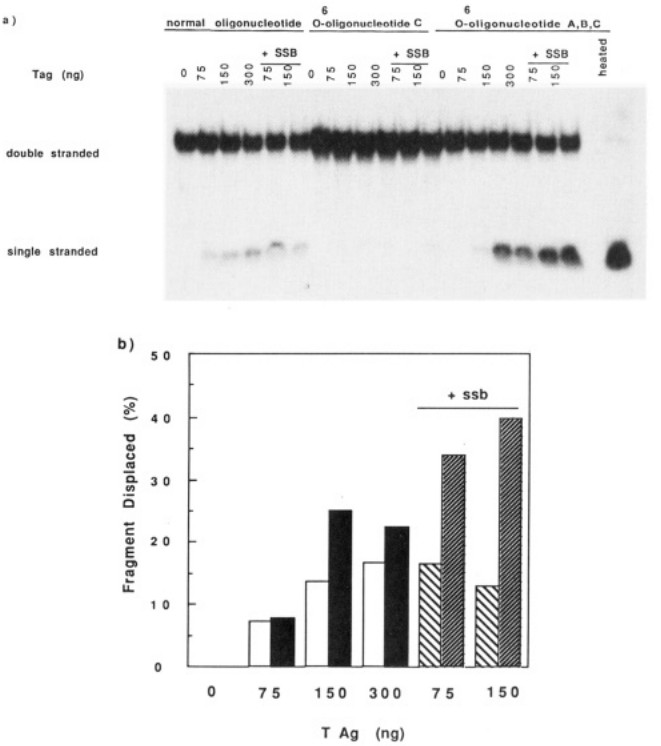

FIGURE 4: Helicase activity of T antigen on the normal and the O^6-MeG substrates. (a) Autoradiogram from a helicase assay using the normal and the O^6-MeG substrates. The substrates consist of the 109-mer oligonucleotide, either unsubstituted (normal), containing a single O^6-MeG (oligonucleotide C, Figure 3), or three O^6-MeG (site I, Figure 1) annealed to a 41-mer complementary to the 5′ end of the *Hin*dIII–*Nco*I fragment excluding the first three bases. Displacement of the single-stranded 41-mer by increasing concentrations of T antigen was measured in the presence or absence of *E. coli* ssb protein (ssb). (b) Quantitation of the displacement of the 41-mer from densitometric scanning of the autoradiograms. Normal substrate in the absence (□) or in the presence of ssb (▨); three O^6-MeG-containing substrate in the absence (■) or in the presence of ssb (▨).

B or C containing modifications in either pentanucleotide 2 or 3. Inhibition of T-antigen binding with substrate C was more pronounced than within substrate B and was similar in extent to that observed with the substrate containing the three modifications in binding site I (Figure 1b). Pretreatment of substrate C with purified *E. coli* Ada MT, which demethylates O^6-MeG in situ, increased T-antigen binding by approximately 35% at each T-antigen concentration. By way of comparison, a substrate in which the O^6-MeG·C base pair in pentanucleotide 3 was replaced by an A·C mismatch exhibited an even more pronounced reduction in T-antigen binding, and the maximum extent of binding to this substrate was 5% (data not shown).

Thus, a single O^6-MeG residue is sufficient to produce a pronounced inhibition in T-antigen binding to the SV40 origin, and a strong position dependence is also evident.

Effect of O^6-MeG on DNA Helicase Activity of T Antigen. We determined the ability of purified T antigen to unwind partially duplex substrates substituted in binding site I by O^6-MeG. Substrates were prepared by annealing an unsubstituted radioactively-labeled 41-mer corresponding to the bottom strand of binding site I (Bignami & Lane, 1990) to the 109-mer top strand containing O^6-MeG at various positions. The resulting partially double-stranded substrate comprises a 41 bp duplex region with a 3-base overhanging region at the 5′ end and a 3′ overhang of 65 bases. When the unsubstituted substrate was incubated with T antigen in the presence of ATP and an ATP-regenerating system, a T-antigen concentration-dependent displacement of the 41-mer was ob-

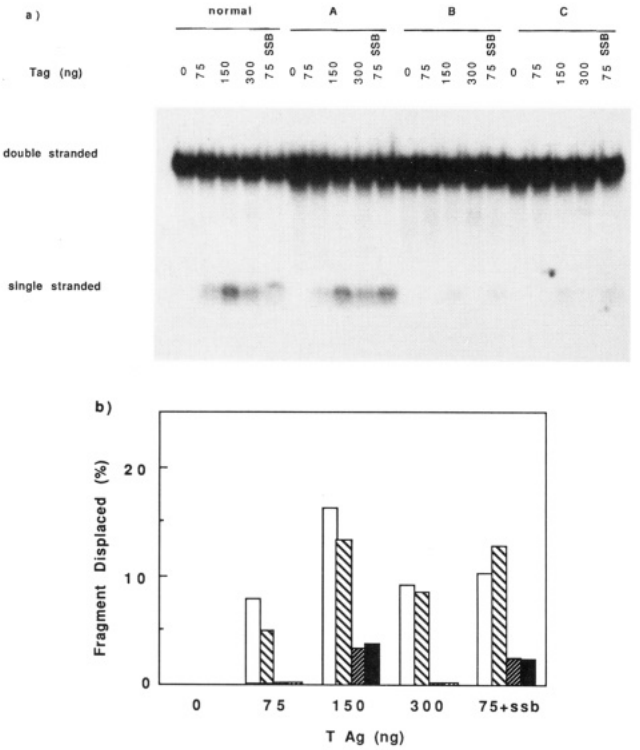

FIGURE 5: Helicase activity of T antigen on binding site I substituted with single O^6-MeG. (a) Autoradiogram from a helicase assay using the normal or O^6-MeG containing substrates A, B, and C (see Figure 3). (b) Quantitation of the displacement of the 41-mer from densitometric scanning of the autoradiograms. Unsubstituted substrate (▧); substrate A (▨); substrate B (□); substrate C (■) in the presence or absence of *E. coli* ssb.

served (normal oligonucleotide, Figure 4). As we previously reported (Bignami & Lane, 1990), concentrations of T antigen between 75 and 150 ng catalyzed maximal displacement of the single-strand fragment. In the presence of purified *E. coli* ssb, maximal displacement remained unchanged but was achieved at lower T-antigen concentrations.

When all three pentanucleotide repeats of binding site I were substituted with the methylated base (O^6-oligonucleotide A,B,C; Figure 4), the single-stranded fragment was released to a greater extent than from the normal substrate, both in the presence and in the absence of ssb. In all cases, no displacement occurred in the absence of ATP, indicating that the helicase activity of T antigen was being observed (data not shown).

The effects of substitutions with single O^6-MeG were measured in a similar fashion. Since a single substitution in pentanucleotide 3 drastically decreased T-antigen binding to the origin fragments, we first tested the ability of this single O^6-MeG to affect DNA unwinding. Figure 4 shows that whereas three modified bases enhanced helicase activity, the single O^6-MeG present in substrate C was severely inhibitory.

A more complete analysis is presented in Figure 5 in which comparison is made of the effects on helicase activity of single substitutions within each of the pentanucleotide repeats (substrates A, B, or C). Unwinding of substrate A was quantitatively similar to that of the normal substrate both in the presence and in the absence of ssb (Figure 5). In contrast, the single O^6-MeG in either substrate B or substrate C effectively abolished the helicase activity of T antigen.

Thus, while the presence of three O^6-MeG in the 41-mer base pair duplex region increases T-antigen-catalyzed unwinding, a single methylated base can have severe inhibitory effects depending on its position in the duplex.

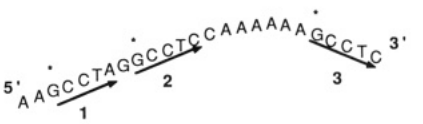

FIGURE 6: Sequence of binding site I showing the potential bent structure. The positions which were substituted by O^6-MeG are represented by asterisks; the sequence underlined represents pentanucleotides 1, 2, and 3.

Discussion

The SV40 origin of replication contains numerous repeated GC-rich sequence motifs that have been shown by site-directed mutagenesis to affect the efficiency of viral DNA replication. The T-antigen binding site II is essential for SV40 replication, and mutations within this region abolish replication both in vivo and in vitro (DeLucia et al., 1986; Deb et al., 1987; Parsons et al., 1990). However, multiple substitutions by O^6-MeG (7 in the 27 bp palindrome) in this region were without effect on T-antigen binding, indicating that the presence of the methylated base is not, in itself, sufficient to measurably reduce T-antigen–origin interactions.

In contrast to the insensitivity of binding site II to the presence of several methylated bases, a single O^6-MeG residue in binding site I can be sufficient to significantly reduce T-antigen binding. Each of the three positions of single O^6-MeG substitution in binding site I lies in a similar pentanucleotide (GCCTN) comprising a T-antigen contact locus (De Lucia et al., 1983). Despite the sequence homology of these loci, the observed inhibition of T-antigen binding was profoundly dependent on the position of the methylated base within binding site I. Our observation that methylation of pentanucleotide 1 was without effect whereas a substitution in pentanucleotide 2 or 3 caused substantial reduction in T-antigen binding indicates that pentanucleotides 2 and 3 are important in T-antigen–origin interactions and is in agreement with deletion and base substitution analysis. Further, ethylation interference analysis indicates that the sites protected by T antigen are directly adjacent to the 5′ ends of pentanucleotides 2 and 3 (Ryder et al., 1985). O^6-MeG in pentanucleotide 2 or 3 also prevented formation of a delayed-migrating T-antigen–oligonucleotide complex, indicating that the presence of O^6-MeG in these sequences affects the oligomerization of T antigen on the origin. Pentanucleotide 2 and 3 are approximately one helical turn apart and are separated by the 7 bp spacer sequence 5′-CAAAAA-3′. It has been suggested that the curvature induced by this sequence might be important for T-antigen oligomerization by allowing contact between T-antigen monomers bound to the adjacent pentanucleotides (Ryder et al., 1986) (see Figure 6). Bending of DNA induced by short A tracts results in a compression of the minor groove of the helix (Crothers et al., 1990). NMR studies indicate that the O^6-MeG·C base pair does not exhibit a normal Watson–Crick alignment and the methyl group is accommodated in the major groove by altering the position of the base pair such that the cytosine is forced into the minor groove (Patel et al., 1986; Li et al., 1988). The inhibition of T-antigen binding by a single O^6-MeG in either pentanucleotide 2 or pentanucleotide 3 suggests that the effects of O^6-MeG on DNA–protein interactions may be maximal in bent DNA regions. Alternatively, the presence of O^6-MeG adjacent to such regions of secondary structure may itself alter the degree of DNA curvature. In support of this second possibility, Patel et al. (1986) have shown that O^6-MeG·C base pairs exhibit altered O–P backbone angles.

While binding site I is required for the regulation of early transcription of the T-antigen gene (Rio et al., 1980; Rio &

Tjian, 1983), it is not essential for DNA replication (Stillman et al., 1985; Li et al., 1986; DeLucia et al., 1986). Recently, the stimulatory role of binding site I on replication has been attributed to a facilitating effect on T-antigen-dependent origin unwinding (Guo et al., 1989; Gutierrez et al., 1990). Multiple substitutions by O^6-MeG in either binding site I or binding site II also facilitated T-antigen-catalyzed unwinding (Bignami & Lane, 1990) even though the overall level of T-antigen binding remained unchanged (binding site II) or was reduced (binding site I). This facilitating effect on helicase activity is probably due to the helix-destabilizing property of O^6-MeG-containing base pairs (Gaffney & Jones, 1989). The effect is dependent on multiple substitution as a single O^6-MeG in pentanucleotide 1, which had no detectable effect on T-antigen binding, neither facilitated nor inhibited helicase activity. The reduction of T-antigen binding by a single O^6-MeG in either pentanucleotide 2 or 3 was clearly not compensated by decreased helix stability, and T antigen was unable to unwind these substrates. In agreement with these findings, a mutant lacking binding site I sequences up to the guanine of pentanucleotide 1 (where the methylated base had no effect) replicated almost normally in vitro whereas templates with deletions extending further into the region (pentanucleotides 2 and 3) replicated with reduced efficiencies (Stillman et al., 1985).

Damage to DNA alters normal protein–DNA interactions. DNA damaged by UV is also a poor substrate for T-antigen helicase activity (Gough & Wood, 1989), and UV photoproducts or certain chemical lesions block DNA polymerases in vitro (Moore et al., 1981). The presence of O^6-MeG inhibits recognition and/or cleavage of DNA by some restriction enzymes (Voigt & Topal, 1990). Recently, Tan and Li (1990) have shown that O^6-MeG·C base pairs influence the rate and extent of cytosine methylation at CpG sequences in hemimethylated duplexes in a way which is dependent on their position in the DNA sequence. We have used O^6-MeG in SV40 origin sequences to define single bases which are important in T-antigen–origin interactions, and our data complement those from base substitution and deletion analysis. More importantly, we have shown that the presence of O^6-MeG may not in itself be sufficient to alter protein–DNA interactions. The effects of the methylated base may be related more to its presence in regions of important secondary structure or to its ability to introduce structural distortions in particular sequence contexts. This sequence dependence may, in part, reconcile the inability of this methylated base to arrest DNA synthesis in vitro with its apparent cytotoxicity in vivo. There is a clear agreement between the positions in the pentanucleotides of binding site I whose alteration by deletion, base substitution, or guanine O^6-methylation affects T-antigen binding. Furthermore, an apparent correlation exists between the inhibition of T-antigen binding and DNA replication. We can now attempt to evaluate the effects of single O^6-MeG substitution on DNA replication in vitro by human cell extracts.

Acknowledgments

We thank R. Wood for comments on the manuscript, J. Gannon for the preparation of the T antigen, B. Sedgwick for the *E. coli* Ada MT, and I. Goldsmith for oligonucleotide synthesis.

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Characterization of Oligosaccharide Structures on a Chimeric Respiratory Syncytial Virus Protein Expressed in Insect Cell Line Sf9

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Received August 27, 1990; Revised Manuscript Received December 5, 1990

ABSTRACT: The oligosaccharide structures added to a chimeric protein (FG) composed of the extracellular domains of respiratory syncytial virus F and G proteins, expressed in the insect cell line Sf9, were investigated. Cells were labeled in vivo with [^{3}H]glucosamine and infected with a recombinant baculovirus containing the FG gene. The secreted chimeric protein was isolated by immunoprecipitation and subjected to oligosaccharide analysis. The FG protein contains two types of O-linked oligosaccharides: GalNAc and Galβ1-3GalNAc constituting 17 and 66% of the total number of structures, respectively. Only one type of N-linked oligosaccharide, constituting the remaining 17% of the structures on FG, was detected: a trimannosyl core structure with a fucose residue linked α1-6 to the asparagine-linked *N*-acetylglucosamine.

The human respiratory syncytial virus (RSV)[1] genome codes for 10 proteins. Two of these, the F and G proteins, are expressed at the surface of the infected cell and the viral envelope and are important for cell fusion and viral attachment, respectively (Gruber & Levine, 1983; Levine et al., 1987). Both proteins are anchored to the membrane by hydrophobic domains that have been localized to the C-terminal end of the F protein (Collins et al., 1984) and the N-terminal end of the G protein (Wertz et al., 1985).

The F and G glycoproteins have been shown to be the principal targets of the host antibody response to RSV (Murphy et al., 1986). Vaccination with either of these glycoproteins will induce neutralizing antibodies and will protect animals from RSV challenge (Olmstead et al., 1986; Walsh et al., 1987). Since the F and G glycoproteins serve different viral functions (cell fusion and viral attachment, respectively), we reasoned that a vaccine inducing an immune response against both glycoproteins should be optimal. A chimeric, secreted protein (FG) composed of the signal and extracellular domains of the F protein linked to the extracellular domain of the G protein was constructed and expressed in the insect cell line Sf9 by using a baculovirus vector (Wathen et al., 1989). Animals vaccinated with this chimeric protein elicited an antibody response against both the F and G portions of the chimeric protein and were protected against RSV challenge (Brideau et al., 1989a). Generating a chimeric protein has the practical advantage of being able to produce and purify a single protein for a vaccine against RSV.

When synthesized by a mammalian cell, the F protein is a 68-kDa glycoprotein containing approximately 9 kDa N-linked oligosaccharides; no evidence for O-linked glycosylation has been found (Lambert, 1988). The mature G protein has a molecular mass of 90 kDa in mammalian cells and is heavily glycosylated. Approximately 12 kDa of the molecular mass on SDS–PAGE is contributed by N-linked oligosaccharides and as much as 45 kDa by O-linked oligosaccharides (Lambert, 1988; Wertz et al., 1989). The extensive O-glycosylation on the G protein is believed to be an important factor in the human immunoresponse to RSV (Wagner et al., 1989).

Several authors have reported that oligosaccharide structures synthesized by insect cells differ from those synthesized by mammalian cells [e.g., Butters and Hughes (1981), Hsieh and Robbins (1984), Wojchowski et al. (1987), Greenfield et al. (1988), Weber et al. (1986) and Kuroda et al. (1990)]. It has been demonstrated that *Aedes albopictus* cells do not synthesize complex-type oligosaccharides but they instead substitute truncated high-mannose structures on glycosylation sites that would contain complex-type structures if the protein was synthesized by a mammalian cell. Other authors have shown that insect cells appear to lack several of the glycosyltransferases involved in the addition of terminal sugars to N-linked oligosaccharides (Butters et al., 1981). O-Linked glycosylation also appears to differ, at least in Sf9 cells; thus, it has been demonstrated that these cells only to a limited extent substitute O-linked *N*-acetylgalactosamine with galactose and that, in analogy with N-linked oligosaccharides, no terminal sialic acid is added (Thomsen et al., 1990).

In this report we present a characterization of the oligosaccharide structures added to the FG protein when synthesized by the insect cell line Sf9.

Materials and Methods

D-[6-^{3}H]Glucosamine hydrochloride (32 Ci/mmol), D-[1-^{14}C]glucosamine hydrochloride (54 mCi/mmol), and sodium boro[^{3}H]hydride (407 mCi/mmol) were purchased from Amersham Corp. EN^{3}HANCE was from New England Nu-

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[1] Abbreviations: GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Gal, galactose; Man, mannose; Fuc, fucose; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; AcNPV, *Autographa californica* nuclear polyhedrosis virus; Sf9, *Spodoptera frugiperda* cell line 9; RSV, respiratory syncytial virus; MOI, multiplicity of infection; PFU, plaque-forming units; Con A, concanavalin A.

0006-2960/91/0430-2863$02.50/0