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Localized melting and structural changes in the SV40 origin of replication induced by T-antigen

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Replication of simian virus 40 (SV40) DNA is dependent upon the binding of the viral T-antigen to the SV40 origin of replication. Structural changes in the origin of replication induced by binding of T-antigen were probed by chemical modifications of the DNA. In the presence of ATP, T-antigen rendered two of three domains in the SV40 core origin hypersensitive to attack by either dimethyl sulfate or potassium permanganate ($KMnO_4$). One of these domains, the early palindrome, was shown to contain an 8-bp region of melted DNA as determined from methylation of cytosine residues and by nuclease S1 cleavage of methylated DNA. DNA melting was not dependent upon either the hydrolysis of ATP or the binding of T-antigen to an adjacent site (site I). A second domain, the A/T element, was extensively modified by $KMnO_4$ but no significant melting was detected. Rather, the pattern of modification indicates that T-antigen caused a conformational change of the double-stranded DNA in this region. These results suggest that T-antigen, in the presence of ATP, destabilizes the SV40 origin by melting and structurally deforming two flanking regions within the core origin sequence. These DNA structural changes may provide access to other replication factors, allowing complete denaturation of the SV40 origin and the initiation of SV40 DNA replication.

Key words: DNA conformation changes/DNA melting/methylation protection/potassium permanganate oxidation/nucleotide effects

Introduction

Simian virus 40 (SV40) is a circular double-stranded DNA virus of 5243 bp which lytically infects cell lines permissive for viral replication. Approximately 12 h after infection, DNA replication is initiated from the SV40 origin of replication (*ori*) and proceeds bidirectionally. SV40 DNA replication requires the viral-encoded large tumor antigen (T-antigen), an 82-kd phosphoprotein. With the exception of T-antigen all proteins essential for replication are supplied by the host.

The requirement for T-antigen entails a direct interaction between the protein and *ori* DNA sequences, in which there are two major binding sites for T-antigen (I and II). Deletion studies of *ori* sequences have shown that only site II is essential for DNA replication *in vivo* and *in vitro*; site I, though not critical for DNA replication, augments replication ~2- to 10-fold *in vivo* and 2- to 3-fold *in vitro* (Stillman *et al.*, 1985; DeLucia *et al.*, 1986; Li *et al.*, 1986). Similar deletion analyses have defined the precise boundaries of essential *ori* sequences containing binding site II. This 'core origin' sequence is contained within a 64-bp region (Deb *et al.*, 1986a). Extensive mutagenesis of the *ori* core has revealed three critical elements that include a 17-bp A/T tract, a central 23-bp perfect inverted repeat containing four GAGGC boxes, and a 10-bp segment containing the distal arm of an imperfect inverted repeat, termed the early palindrome (Deb *et al.*, 1987). The central pentanucleotide boxes appear to constitute recognition sequences for T-antigen (Tegtmeyer *et al.*, 1983). The requirement for the two flanking domains is not understood, although the A/T element is known to exist in a structure other than normal 'B-DNA' (Deb *et al.*, 1986b) and enhances the binding of T-antigen to the central pentanucleotide region (Müller *et al.*, 1987). Base substitution in any one of the three elements severely inhibits SV40 DNA replication *in vivo* (Deb *et al.*, 1987), replication *in vitro* with either crude cytoplasmic extracts or purified proteins, as well as inhibiting the *ori*-dependent DNA unwinding reaction catalyzed by T-antigen (Dean *et al.*, 1987b).

Recently, the role of T-antigen in SV40 DNA replication has become better defined. Efficient binding of T-antigen to site II under conditions that support SV40 DNA replication (e.g. incubation at 37°C) requires the presence of ATP, although dATP, ADP, or the nonhydrolyzable analog of ATP, adenosine 5'-[β,γ-imido]triphosphate (p[NH]ppA), can substitute for ATP (Dean *et al.*, 1987c; Deb and Tegtmeyer, 1987; Borowiec and Hurwitz, 1988). Electron microscopy has revealed that the complex formed in the presence of ATP has a bilobed structure and contains a large number of T-antigen monomers (Dean *et al.*, 1987c). This complex has been examined by DNase I protection assays and was found to protect the complete core origin region (Deb and Tegtmeyer, 1987; Borowiec and Hurwitz, 1988). In addition to its site-specific DNA-binding function, T-antigen also possesses an ATP-dependent DNA helicase activity (Stahl *et al.*, 1986). Dean *et al.* (1987a) have recently shown that T-antigen can unwind circular DNA molecules containing the SV40 *ori*. This reaction proceeds bidirectionally from *ori* and requires a single-strand DNA binding protein (SSB) and ATP (Dodson *et al.*, 1987; Dean *et al.*, 1987a,b). This unwinding reaction is thought to reflect a required step in the pathway of SV40 DNA replication since *ori* mutations similarly affect DNA unwinding and replication (Dean *et al.*, 1987b). Thus, the initiation of SV40 DNA replication appears to proceed through a pathway in which T-antigen first forms an ATP-dependent complex at *ori* that then melts the DNA at *ori* allowing the entry of other essential replication proteins such as DNA polymerase α/primase complex and HeLa SSB (Wobbe *et al.*, 1987).

In this report, we have examined the changes in *ori* structure induced by the ATP-dependent binding of T-antigen using the techniques of methylation protection and potassium permanganate ($KMnO_4$) oxidation. These experiments show

that the ATP-dependent T-antigen complex caused significant alterations in chemical modification in each of the three critical domains of *ori*. We find that the T-antigen complex contains a central region of numerous T-antigen–*ori* contacts flanked on either side by melted or helically distorted DNA. These results indicate possible roles for the two outer *ori* domains and lead to a general model for the initiation of SV40 DNA replication.

Results

T-antigen binding to the SV40 *ori* was recently examined under conditions that support *ori* DNA replication *in vitro* (Dean *et al.*, 1987b; Deb and Tegtmeyer, 1987; Borowiec and Hurwitz, 1988). Under these conditions, incubations at 37°C in the presence of ATP, the binding of T-antigen to the *ori* core (binding site II) was stimulated ~10-fold compared with the binding observed in the absence of ATP. DNase I protection analysis showed that the entire 64-bp core origin was protected in this ATP-dependent complex (Deb and Tegtmeyer, 1987; Borowiec and Hurwitz, 1988). In the absence of ATP, T-antigen weakly protected only the central 20–25 bp of the core origin containing the four GAGGC elements. In this paper, we have examined the effects of the ATP-dependent complex on the DNA structure at *ori* using two different *ori*-containing DNA molecules (Figure 1). The first DNA, termed *ori* wt, contained the wild-type *ori* and auxillary sequences including T-antigen binding site I. The second DNA, *ori* core, contained only the SV40 core *ori* (nt 5211 to 31) (Deb *et al.*, 1986a) flanked by plasmid DNA sequences. For ease of identification, the locations of the three critical domains in *ori* are defined as shown in Figure 1.

Methylation protection analysis of T-antigen bound to ori wt

T-antigen binding to *ori* was first probed by methylation protection to determine whether the presence of ATP resulted in new or modified contacts between T-antigen and *ori* DNA. T-antigen was incubated with the *ori* wt DNA fragment and the DNA then alkylated with the methylating agent dimethyl sulfate (DMS). The modified DNA was cleaved using standard Maxam and Gilbert (1980) 'G-only' sequencing conditions that break the DNA at methylated guanosines and, to a lesser extent, at methylated adenosine residues. T-antigen-dependent changes in the *ori* wt methylation pattern were first examined with the fragment 5′-labeled on the top strand (Figure 2A). The addition of T-antigen alone altered the methylation pattern in two regions of *ori* wt (lane 2). A number of protected guanosine residues were detected in T-antigen binding site II, all of which occurred in the central region containing the four conserved GAGGC elements. These weak-to-modest protections (summarized in Figure 7) corresponded to those noted previously in binding experiments carried out at low temperatures (Tjian, 1979; DeLucia *et al.*, 1983). These data are also consistent with recent DNase I protection studies of T-antigen binding in the absence of ATP under conditions similar to those employed here. In those studies, T-antigen weakly protected a 20–25-bp region containing the central pentanucleotide sequences (Deb and Tegtmeyer, 1987; Borowiec and Hurwitz, 1988). Methylation changes were also observed at T-antigen binding site I. The three guanosine residues protected and two enhanced adenosine cleavages are listed in the legend to Figure 2. These findings are consistent with

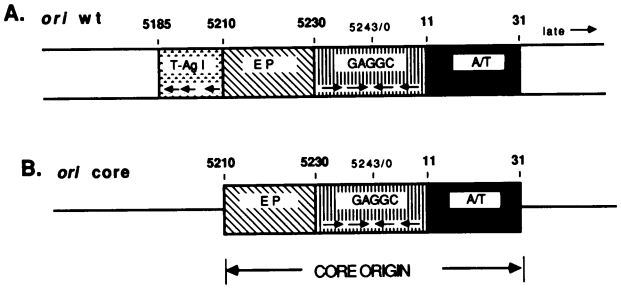

Fig. 1. DNA templates used to examine the effects of T-antigen and ATP on *ori* structure. Two DNA fragments, *ori* wt (**A**) and *ori* core (**B**), were used. The *ori* wt DNA contains SV40 sequence positions nt 5094 to 161. This region includes the core *ori* (nt 5211 to 31) and T-antigen binding site I (nt 5185 to 5212). T-antigen binding site II is used interchangably with the *ori* core. For $KMnO_4$ modification experiments, *ori* wt was contained in plasmid pSV01ΔEP (Wobbe *et al.*, 1985). The *ori* core DNA fragment contains SV40 sequence positions nt 5171–5177 and 5209 to 43, surrounded by vector sequences. This fragment was prepared from plasmid pOR1 (DeLucia *et al.*, 1986). SV40 sequence positions are indicated by boxes, while vector sequences are shown by a single line. For ease of identification, the *ori* core domains are indicated as follows: A/T region, nt 12–31; central pentanucleotide region (GAGGC), nt 5230 to 11; early palindrome region (EP), nt 5210–5229; and T-antigen binding site I (T-Ag I), as above. The arrows in T-antigen binding site I and the GAGGC element indicate the positions and orientation of the GAGGC sequences. The actual 15-bp early palindrome sequence is located at nt 5213–5227. The late gene side of SV40 is indicated as in (**A**).

previous studies (Tjian, 1979; DeLucia *et al.*, 1983) with the exception that we did not observe protection of the guanosine residue at position 5186 and did note a novel enhanced cleavage of an adenosine residue at position 5202. We note that while a few of the weakest protections or enhancements did not reproduce well in the reduced figure, each was clearly observable in the original autoradiographs.

The addition of ATP to the T-antigen binding reaction significantly altered the DNA methylation pattern at binding site II (Figure 2A, lane 3). In addition to increasing the protection of guanosine residues in the central pentanucleotide element, enhanced cleavages were also observed at top-strand positions 5211 (strong), 5213 (weak) and 5216 (medium, marked with arrowheads). Surprisingly, each of these strand scissions occurred at cytidine residues in the distal arm of the early palindrome region. ADP (lane 4) and the nonhydrolyzable analog of ATP, p[NH]ppA (lane 5) also induced enhanced cleavage of these cytidine residues. When p[NH]ppA replaced ATP, the extent of cytidine cleavage at nucleotide 5211 was somewhat reduced. Cleavage at these positions was not observed with ATP in the absence of T-antigen (data not shown). The presence of ATP, ADP or p[NH]ppA did not cause any significant changes in the methylation pattern of T-antigen binding site I (Figure 2A, lanes 3–5).

The only position on cytosine with appreciable reactivity towards DMS is N-3 (Lawley, 1966). Normally, this position is not susceptible to DMS attack in native DNA since the N-3 is hydrogen bonded to the complementary strand. Kirkegaard *et al.* (1983) have shown previously that methylation of cytidine residues located in single-stranded regions can lead to strand scission using DMS guanosine sequencing chemistry. However, cleavage was also dependent upon treatment of the methylated DNA sample with hydrazine prior to the standard piperidine cleavage step.

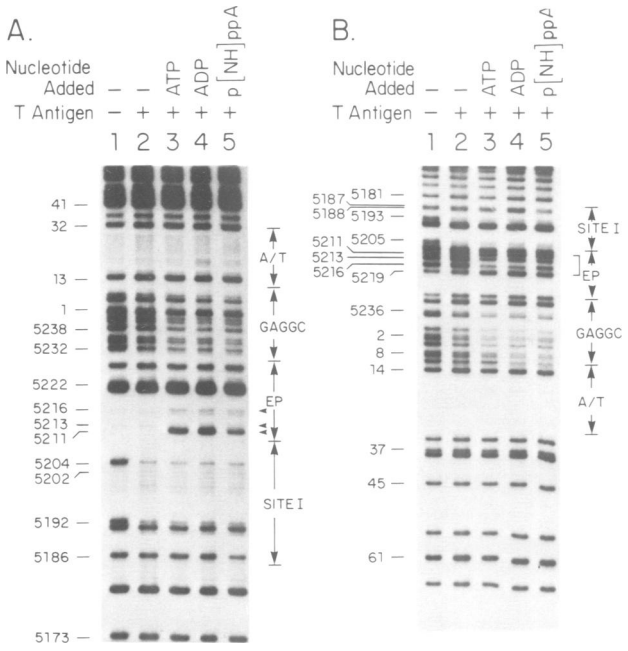

Fig. 2. Methylation protection analysis of T-antigen binding to *ori* wt. T-antigen (1.2 μg) was incubated, as indicated, with the *ori* wt fragment end-labeled on either the top strand (**A**) or bottom strand (**B**). The sequence of the top strand is shown as in Figure 7. The binding reactions also contained 4 mM ATP, ADP or p[NH]ppA as indicated. The binding was probed with DMS and the methylated DNA sample cleaved with Maxam and Gilbert (1980) 'G-only' sequencing chemistry. The cleaved samples were then separated on a 6% polyacrylamide sequencing gel and the gel autoradiographed. SV40 DNA sequence positions are indicated on the left-hand side of the figure. Enhanced cytidine residues are shown by arrowheads on the right-hand side of the figure. Changes in guanosine methylation dependent upon the presence of T-antigen and ATP are indicated by a bracket on the right side. Also shown on the right are regions of the *ori* sequence corresponding to the early palindrome (EP), central pentanucleotide (GAGGC) and A/T (A/T) elements. Changes in guanosine (G) and adenosine (A) methylation observed with T-antigen binding to site I were as follows: (P), protection; (E) enhancement; sequence positions as indicated; site I top strand, G5191 (P), G5192 (P), A5199 (E), A5202 (E) and G5204 (P); bottom strand, G5193 (P), G5194 (P), G5196 (P), G5205 (P), G5206 (P) and G5208 (P). Certain weak protections or enhancements, while poorly visible in the reduced figure, were clearly seen in the original autoradiographs. The boundaries of T-antigen binding sites I and II were determined from DNase I protection analysis (Borowiec and Hurwitz, 1988).

Since our reaction conditions did not include hydrazine treatment, DNA strand breakage at the cytidine residues observed in Figure 2A was unexpected. Control experiments showed that the observed cleavage required both DMS methylation and piperidine cleavage steps (data not shown). These experiments also demonstrated that cytidine cleavage was only stimulated by hydrazine, indicating that this reaction was not a prerequisite step but rather enhanced the cleavage reaction. Thus, we suggest that T-antigen in the presence of ATP (as well as ADP and p[NH]ppA) can induce localized melting of the DNA in the early palindrome region. This DNA melting allows methylation of the cytosine N-3 and subsequent strand breakage by piperidine. Below, we present further evidence of melting at the *ori* dependent upon T-antigen and ATP.

The effect of T-antigen on the methylation pattern of the *ori* wt fragment labeled on the bottom strand was examined (Figure 2B). T-antigen in the absence of ATP protected nine guanosine residues in binding site II (Lane 2), consistent with previous studies of T-antigen binding of *ori* wt at low temperature (Tjian, 1979; DeLucia *et al.*, 1983) (see Figure 7). Unlike these previous studies, we did not detect the protection of guanosine residues located at positions 5187 and 5188.

The addition of ATP to the T-antigen binding reaction caused further changes in the methylation protection pattern within binding site II localized to the early palindrome region (Figure 2B, lane 3). T-antigen and ATP induced two novel guanosine enhancements at positions 5211 and 5219, and protected two guanosines at positions 5213 and 5216 (marked with brackets). Note that guanosines at positions 5211, 5213 and 5216 are located complementary to the top-strand cytidine residues that, in the presence of T-antigen and ATP, showed enhanced alkylation. The presence of ATP also significantly strengthened protection of guanosines contacted by T-antigen in the central pentanucleotide region (lane 3). The substitution of ADP (lane 4) or p[NH]ppA (lane 5) for ATP resulted in similar changes in the methylation pattern, although weaker interactions in the early palindrome region were again found in the presence of p[NH]ppA. The presence of ATP, ADP or p[NH]ppA did not cause any methylation changes in T-antigen binding site I.

Methylation protection analysis of T-antigen bound to ori core

The DMS footprinting reactions using *ori* wt DNA indicated that, in the presence of ATP, T-antigen formed new contacts and induced DNA melting within the early palindrome region of site II. This region is situated between the consensus pentanucleotide sequences of T-antigen binding site I and II. Thus, the possibility existed that the interaction between T-antigen and site I could influence the induction of the protection and enhancement observed in the early palindrome region. To explore this possibility, the DMS footprinting experiments were repeated on the *ori* core DNA fragment lacking site I.

T-antigen binding to the *ori* core DNA end-labeled on the top strand is shown in Figure 3A. T-antigen in the absence of ATP weakly protected 10 guanosine nucleotides in the central pentanucleotide region of site II (Figure 3A, lane 2). In the presence of ATP, T-antigen again resulted in enhanced cleavage of cytidine residues at positions 5211, 5213 and 5216 (arrowheads), and increased the protection of the pentanucleotide elements (lane 3). The replacement of ATP with ADP or p[NH]ppA gave similar results (lanes 4 and 5 respectively). No changes in methylation were observed at sequences outside the region protected from DNase I cleavage by T-antigen bound at site II (Borowiec and Hurwitz, 1988). T-antigen binding to the *ori* core DNA fragment labeled on the bottom strand is shown in Figure 3B. In the absence of ATP, T-antigen shielded identical guanosines in the consensus pentanucleotide sequences as were noted with *ori* wt DNA (Figure 3B, lane 2). With the addition of ATP (lane 3), T-antigen also weakly protected two guanosine residues (nt 5213 and 5216) and enhanced the methylation of two guanosine residues at nucleotides 5211 and 5219 (marked with bracket). The substitution of ADP or p[NH]ppA for ATP caused similar changes in the methylation pattern (lane 4 and 5).

Comparison of the T-antigen, ATP-dependent methylation changes on *ori* core and *ori* wt DNA fragments indicated that similar alterations occurred in these molecules. We conclude that the ATP-dependent complex formed between

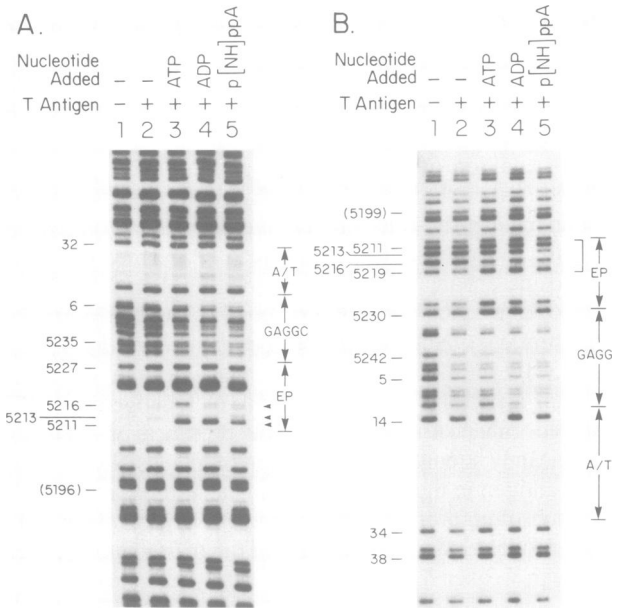

Fig. 3. Methylation protection analysis of T-antigen binding to *ori* core. T-antigen, as indicated, was incubated with the *ori* core DNA fragment end-labeled on either the top strand (**A**) or bottom strand (**B**). Binding reactions also contained 4 mM ATP, ADP or p[NH]ppA as indicated. Samples were prepared as described in Materials and methods. SV40 sequence positions are indicated on the left side of the figure; positions in parentheses indicate vector sequences with numbering continued from the SV40 DNA. The location of *ori* regions and changes in methylation are indicated as in Figure 2.

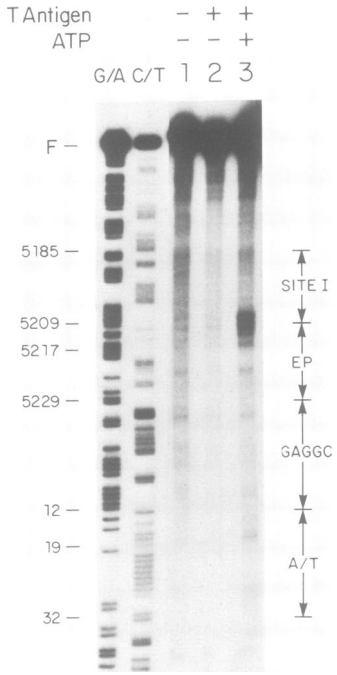

Fig. 4. Nuclease S1 sensitivity of DNA methylated in the presence of T-antigen and ATP. T-antigen (2.0 μg; **lanes 2** and **3**) was incubated with *ori* wt DNA fragment 5′ end-labeled on the bottom strand in the absence (**lanes 1** and **2**) or presence (**lane 3**) of ATP. After DMS treatment followed by deproteinization, the DNA samples were treated with nuclease S1 to nick the DNA fragment at those nucleotides methylated at hydrogen-bonding positions (particularly the N-1 position of adenosine). The samples were then subjected to PAGE under denaturing conditions. **Lanes G/A** and **C/T** indicate purine and pyrimidine sequencing ladders respectively. SV40 sequence positions are indicated on the left-side of the figure and *ori* elements on the right.

T-antigen and the *ori* core is sufficient to induce helical melting as well as novel guanosine contacts and enhancements in the early palindrome region. T-antigen bound at site I has only minor effects on T-antigen bound to site II as determined by methylation protection/enhancement. This observation is consistent with past studies showing only weak cooperativity between T-antigen bound to these two sites (Tegtmeyer *et al.*, 1983).

Nuclease S1 cleavage of methylated ori wt fragment

The observed ATP-dependent cytidine methylation suggested that T-antigen can induce localized melting of the DNA duplex in the early palindrome region. The location of the initial melting site of DNA within the *ori* core has implications on the mechanism of the melting reaction as well as on the location of initiation sites of nascent DNA strand synthesis (e.g. see Hay and DePamphilis, 1982). For these reasons, we wished to verify the existence of single-stranded regions in the T-antigen/*ori* complex and determine their location. The method of Siebenlist (1979) was used to locate single-stranded regions on an end-labeled duplex DNA fragment. Briefly, localized DNA melting by a protein allows methylation at the N-1 position of adenine. The N-3 position of cytosine can also be alkylated (see Figures 2 and 3), but this site has a reactivity roughly one-third that of the N-1 of adenine (Lawley, 1966). After deproteinization of the DNA sample, methylation at these sites prevents complete renaturation of the DNA fragment. Treatment of the end-labeled fragment with the single-strand specific nuclease S1 results in nicking at the unpaired regions. Nuclease S1 cleavage of methylated DNA has been shown to occur primarily at AT base pairs, while methylated GC base pairs are poorly cleaved (Siebenlist, 1979). These cleavage sites can be localized after electrophoresis of the DNA on a polyacrylamide sequencing gel.

T-antigen binding to *ori* wt DNA labeled on the bottom strand was probed using the DMS/nuclease S1 procedure (Figure 4). Treatment of the DNA fragment, methylated in the absence of T-antigen, with nuclease S1 resulted in no discrete DNA cleavage products (lane 1). Similar treatment of the *ori* wt DNA fragment incubated with T-antigen in the absence of ATP also did not result in cleavage at internal positions (lane 2) indicating that T-antigen bound to *ori* wt DNA in the absence of ATP did not detectably melt this fragment. In the presence of T-antigen and ATP, a single discrete region of the *ori* wt was hypersensitive to nuclease S1 nicking following methylation (lane 3). The S1-sensitive site was localized to the early palindrome region at positions 5210−5217, after accounting for the slower electrophoretic migration of the nuclease-S1-generated products relative to the sequence ladder because of the lack of 3′-phosphate ends on nuclease S1 products. This cleavage site completely overlapped the positions of enhanced cytidine cleavage observed with T-antigen binding to the *ori* wt and *ori* core fragments (Figures 2A and 3A, lanes 3−5). The A/T region showed only a weak nuclease S1 sensitive site at nucleotide 19 which amounted to <5% of the signal observed within the early palindrome region. We conclude that only sequences within the *ori* significantly melted by T-antigen in the presence of *ori* are located in the early palindrome region at positions 5210−5217. Similar results were obtained

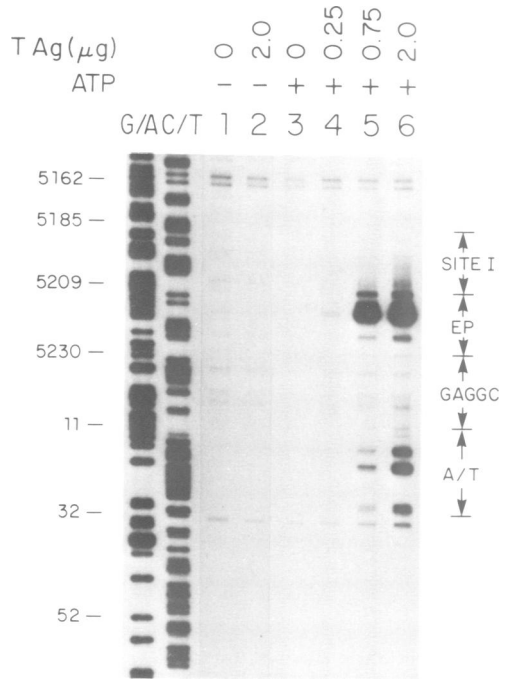

Fig. 5. Effects of T-antigen concentration and ATP on the $KMnO_4$ oxidation pattern of *ori* wt. T-antigen (as indicated) was incubated (37°C) with RFI pSV01ΔEP (Wobbe *et al.*, 1985) in the absence (**lanes 1** and **2**) or presence (**lanes 3–6**) of 4 mM ATP. After 60 min, $KMnO_4$ was added to 6 mM and the reaction incubated for an additional 4 min. The reaction was then quenched and the DNA purified as described in Materials and methods. Aliquots of the DNA sample were removed and subjected to primer extension using a 5′-^{32}P-labeled primer complementary to top strand sequences and Klenow DNA polymerase. The extension products were then separated by PAGE using sequencing conditions and the gel autoradiographed. G/A and C/T indicate dideoxy sequencing ladders. SV40 DNA sequence positions are indicated on the left-hand side of the figure. Regions of the *ori* sequence are as indicated in Figure 2.

using an *ori* wt DNA fragment end-labeled on the top strand (data not shown).

ori is rendered hypersensitive to $KMnO_4$ attack by T-antigen and ATP

We used $KMnO_4$ to probe for changes in DNA structure induced by T-antigen binding to *ori*. Gralla and coworkers have recently shown that $KMnO_4$ can recognize thymidine residues in regions of distorted DNA structure (Borowiec *et al.*, 1987). Specific sites in DNA become hyper-reactive to $KMnO_4$ oxidation when the DNA is sharply bent by a DNA-binding protein (Borowiec *et al.*, 1987) or is melted (S.Sasse-Dwight and J.Gralla, personal communication). The primary target in DNA sensitive to $KMnO_4$ attack is the thymine ring that is oxidized at the 5,6 double-bond (Rouet and Essigman, 1985). Oxidation at this position strongly inhibits primer extension by the Klenow fragment of DNA polymerase I, resulting in an elongation product terminating one nucleotide upstream of the modified thymidine residue. Thus, the sites at which a DNA-binding protein distorts the DNA helix can be localized by first treating the protein–DNA complex with $KMnO_4$, followed by extension of a ^{32}P-labeled primer through the region with the Klenow DNA polymerase. The extension products are then separated on a DNA sequencing gel to determine the sites of modification.

The ability of T-antigen to distort DNA structure in the *ori* region was examined. T-antigen was incubated with RFI pSV01ΔEP containing the *ori* wt sequence. The binding reaction mixture was then treated with $KMnO_4$ and the DNA probed by primer extension using a primer complementary to the top strand sequence (Figure 5). Treatment of the *ori* wt DNA with $KMnO_4$, in the absence of T-antigen and ATP, resulted in a minimal level of modification throughout the entire region (lane 1). The addition of 2.0 μg of T-antigen in the absence of ATP did not alter the pattern of modification (lane 2). These results showed that T-antigen alone did not induce changes in DNA structure sensitive to $KMnO_4$ modification, even though at this concentration of T-antigen, binding is completely saturated at site I and partially at site II (Figure 2) (Borowiec and Hurwitz, 1988).

We next examined the effect of ATP on the $KMnO_4$ oxidation pattern of T-antigen bound to *ori* wt. ATP in the absence of T-antigen (lane 3) or with 0.25 μg of T-antigen (lane 4) did not induce significant modification. When 0.75 μg of T-antigen was used, a striking enhancement of $KMnO_4$ modification was observed in the early palindrome region with a minor increased oxidation in the A/T region (lane 5). The addition of 2.0 μg of T-antigen resulted in a slightly greater $KMnO_4$ modification of the early palindrome region while inducing a marked enhancement at the A/T region (lane 6). Similar results were obtained using a primer complementary to bottom strand sequences (data not shown). Thus, T-antigen in the presence of ATP can induce significant conformational changes in two of the three regions of the *ori* essential for the initiation of SV40 DNA replication. The early palindrome and A/T regions are both extensively modified by $KMnO_4$, while no changes were detected in the central pentanucleotide element. The extent of modification of the two $KMnO_4$-sensitive regions was dependent on the level of T-antigen used; the early palindrome required less T-antigen to distort the DNA helix than the A/T region.

ATP analogs differentially alter the $KMnO_4$ modification pattern

A variety of nucleotides, including ADP and p[NH]ppA, replaced ATP in supporting T-antigen complex formation at site II (Dean *et al.*, 1978c; Borowiec and Hurwitz, 1988) and induced DNA melting in the early palindrome region (Figures 2 and 3). The ability of these nucleotides to induce changes in DNA structure detected by $KMnO_4$ modification was examined using a primer complementary to the top strand (Figure 6A). In the absence of T-antigen (lane 1), or in the presence of T-antigen without added nucleotide (lane 2), only a basal level of $KMnO_4$ modification was seen. Upon addition of T-antigen and ATP, significant $KMnO_4$ oxidation of both the early palindrome and the A/T regions was observed (Figure 6A, lane 3). The substitution of ADP for ATP induced significant changes in DNA structure, though the pattern differed substantially from that detected with ATP (lane 4). While the presence of ADP rendered the early palindrome region hypersensitive to $KMnO_4$, the A/T region was modified only slightly above background. This pattern was unchanged even at the highest levels of T-antigen tested (2.0 μg, data not shown). When p[NH]ppA was tested, a third oxidation pattern emerged (lane 5). The early palindrome was strongly modified by $KMnO_4$, but the length of the region attacked was shortened with the thymidine residue at position 5225 no longer hypersensitive

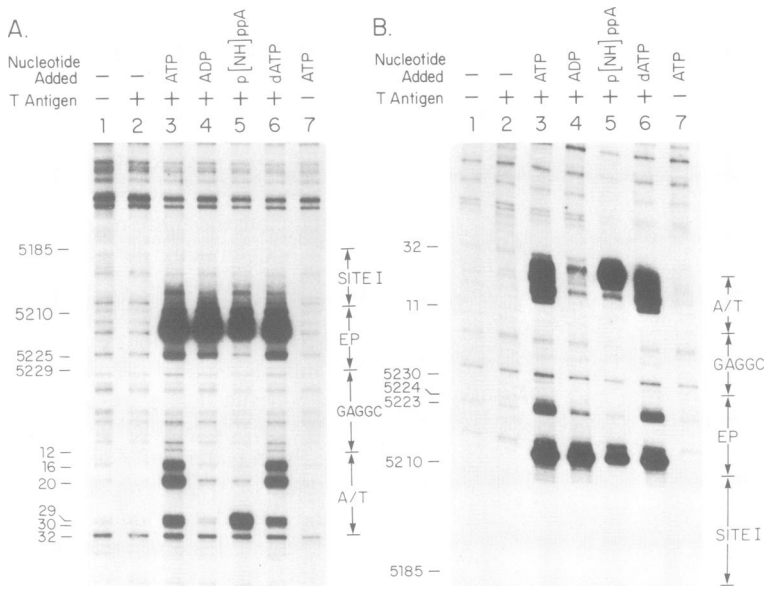

Fig. 6. $KMnO_4$ modification pattern of *ori* wt DNA in the presence of T-antigen and various nucleotide effectors. T-antigen (1.5 μg; as indicated) was incubated at 37°C with *ori* wt plasmid in the absence of any nucleotide (**lanes 1** and **2**) or with 4 mM ATP (**lane 3**), ADP (**lane 4**), p[NH]ppA (**lane 5**) or dATP (**lane 6**). After 60 min, samples were treated with $KMnO_4$ and the DNA then probed by primer extension footprinting using a ^{32}P end-labeled primer complementary to top strand (**A**) or bottom strand (**B**) sequences. The extension products were then separated by PAGE using sequencing conditions and the gel autoradiographed. SV40 sequence positions are indicated on the left-hand side of the figure. Regions of the *ori* sequence corresponding to the early palindrome, central pentanucleotide and the A/T-rich elements are shown on the right side of the figure.

to $KMnO_4$ attack. Similar changes occurred in the A/T tract; oxidation of thymidine residues 29 and 30 of the top strand were strongly enhanced, yet the thymidine sites proximal to the central pentanucleotide element (nt 16 and 20) were no longer significantly modified above background. dATP, shown to be 30-fold more efficient than ATP in supporting formation of the T-antigen complex at *ori* core (Borowiec and Hurwitz, 1988), produced an identical modification pattern to that found with ATP (Figure 6A; cf. lanes 3 and 6).

The bottom strand sequences were similarly examined by primer extension of modified DNA using a probe complementary for that strand (Figure 6B). T-antigen in the presence of ATP resulted in strong modification of *ori* wt in both the early palindrome and A/T regions (Figure 6B, lane 3). To determine thymidine residues in the A/T region which were rendered hypersensitive to $KMnO_4$ oxidation, primer extension products were subjected to gel electrophoresis for significantly greater distances (data not shown). Careful examination of these autoradiographs indicated that the proximal 12 thymidines on the bottom strand of the A/T region became hypersensitive to $KMnO_4$ modification by T-antigen in the presence of ATP (see Figure 7). The addition of ADP *in lieu* of ATP again poorly induced $KMnO_4$ oxidation of the A/T sequence, while allowing strong modification of the early palindrome (lane 4). It was noted that thymidine residues 5223 and 5224 were oxidized less effectively in the presence of ADP than in the presence of ATP. The addition of p[NH]ppA again resulted in a pattern which differed from that seen with either ATP or ADP. Both the A/T and early palindrome regions were attacked less effectively at sites proximal to the central pentanucleotide sequence (lane 5). The presence of dATP induced a $KMnO_4$ oxidation pattern virtually indistinguishable from that seen with ATP (Figure 6B, lane 6). The regions modified were not significantly altered when linear *ori* wt plasmid DNA was used in place of RFI plasmid (data not shown).

Discussion

The initiation of SV40 DNA replication requires the action of the multifunctional viral T-antigen at *ori*. The recent discovery and characterization of T-antigen helicase and *ori*-dependent DNA-unwinding activities (Stahl *et al.*, 1986; Dean *et al.*, 1987a; Dodson *et al.*, 1987) as well as observations of an ATP-dependent complex of T-antigen with *ori* core (Dean *et al.*, 1987b; Deb and Tegtmeyer, 1987; Borowiec and Hurwitz, 1988) suggest that T-antigen acts to nucleate DNA melting at *ori* and then propagates the melted region outwards. For these reasons, we examined the structure of the *ori* region complexed to T-antigen in the presence of ATP. Under conditions which support SV40 DNA replication, T-antigen induced striking changes at *ori*, including localized melting of core sequences and the formation of double-stranded DNA with distorted structure.

A summary of T-antigen interactions with *ori* in the absence and presence of ATP is shown in Figure 7A and B. T-antigen was shown previously to bind poorly to *ori* core in the absence of ATP at elevated temperatures (Dean *et al.*, 1987c; Deb and Tegtmeyer, 1987; Borowiec and Hurwitz, 1988). This weak protection from DNase I cleavage was localized to the central pentanucleotide sequences (Figure 7A) that comprise the essential recognition elements for T-antigen binding to *ori* core (Tjian, 1979; Tegtmeyer *et al.*, 1983). The DMS methylation data confirm these observations, since T-antigen supported partial protection of the guanosine residues in this region. We noted differences

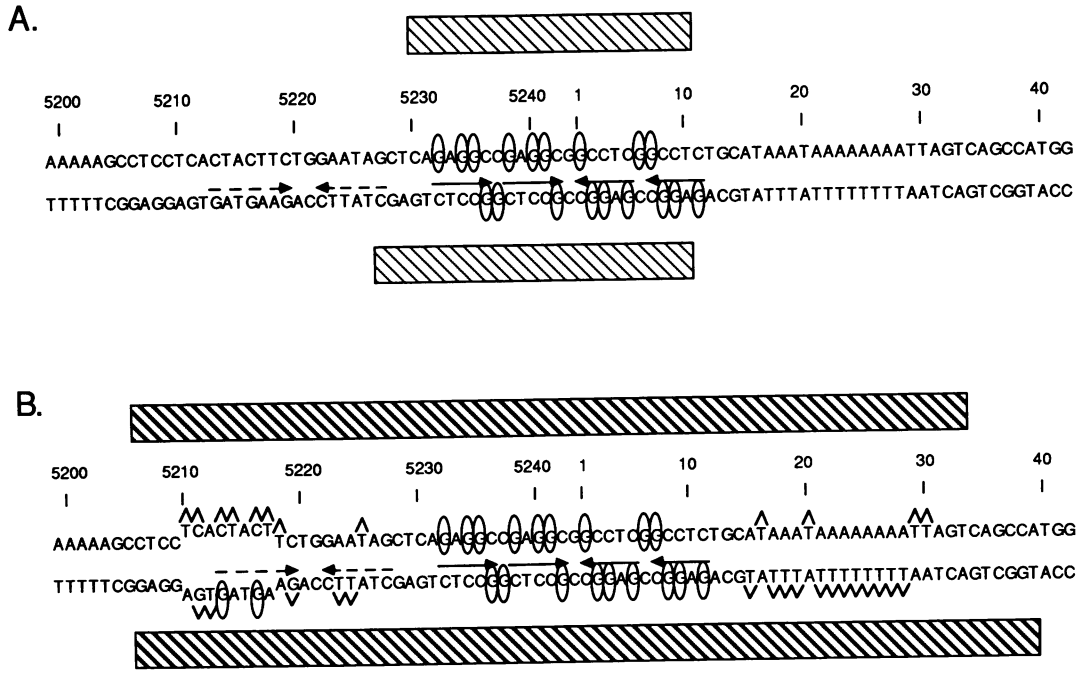

Fig. 7. Interaction of T-antigen with *ori* wt. This figure summarizes the results of DNase I footprinting (Borowiec and Hurwitz, 1988), DMS protection/enhancement, methylation/S1 cleavage and $KMnO_4$ oxidation experiment showing the effects of T-antigen binding to *ori* in the absence (**A**) or presence of ATP (**B**). DNase I protection of T-antigen binding to *ori* core ^{32}P labeled on the top or bottom strand is shown by cross-hatched boxes above and below the *ori* sequence respectively. The lighter cross-hatched box in (**A**) is indicative of weaker DNase I protection to *ori* core in the absence of ATP. Guanine residues protected from DMS methylation by T-antigen are indicated by the circled nucleotides; guanine or cytosine residues with enhanced methylation (DMS) and thymines with enhanced oxidation ($KMnO_4$) are shown by carets. Single-stranded regions at *ori* (**B** only), determined from cytosine methylation and methylation/S1 cleavage experiments, are indicated by increased separation between the top and bottom strands. SV40 sequence positions are indicated above the sequence. Dotted arrows at positions 5213–5227 indicate the 15-bp early palindrome sequence. Solid arrows in the central region correspond to conserved GAGGC sequences with P1 found at nt 5232–5236, P2 at 5238–5242, P3 at 1–5 and P4 at 7–11 (nomenclature from DeLucia *et al.*, 1983). Note that P3 and P4 are in an orientation opposite that of P1 and P2.

in the degree of protection within each GAGGC sequence; pentanucleotides P1 and P3 (see Figure 7) were protected more than P2 and P4. Similar quantitative differences in the binding of T-antigen to site II were previously observed in binding reactions performed at lower temperatures with T-antigen protecting P1 most effectively and P4 least effectively (DeLucia *et al.*, 1983; Jones and Tjian, 1984). In the absence of ATP, no methylation of $KMnO_4$ oxidation changes were observed in either the early palindrome or A/T regions, indicating that T-antigen alone does not induce significant DNA structural changes in these flanking sequences.

The regions of *ori* affected by T-antigen in the presence of ATP are summarized in Figure 7B. As previously shown, the ATP-dependent T-antigen complex protected roughly a 3-fold greater length of DNA from DNase I attack than did T-antigen without ATP (Borowiec and Hurwitz, 1988). Although the increase in the amount of DNA protected by T-antigen from DNase I presumably increases the number of T-antigen–*ori* contacts, only two novel contacts were observed (nt 5213 and 5216). The main changes induced by T-antigen in the presence of ATP were the increased reactivity of *ori* core sequences to DMS or $KMnO_4$ attack. In general, the modification pattern of T-antigen at *ori* is symmetrical. A region of extensive T-antigen–guanosine contacts is encompassed by two broad regions that are hypersensitive to either DMS or $KMnO_4$ attack. All ATP-dependent modifications were located in sequences shielded by T-antigen from DNase I cleavage indicating that no significant changes in DNA structure occurred outside regions closely bound by T-antigen. While gross symmetry exists, however, a closer examination of the ATP-dependent pattern indicates that $KMnO_4$ oxidation results from two distinct changes in the DNA structure: DNA melting in the early palindrome region and conformational changes of double-stranded DNA in the A/T region.

Melting of the early palindrome region

The early palindrome region contains approximately eight nucleotides of melted DNA (nt 5210–5217), detected by both cytidine cleavage and DMS/nuclease S1 reactions. Although this sequence is located at the border between binding sites I and II, melting is only dependent upon T-antigen bound to site II. The hypersensitivity to $KMnO_4$ in the 8-nt region presumably arises from oxidation of melted DNA, consistent with prior observations of $KMnO_4$ modifications of ssDNA (Rouet and Essigman, 1985; S.Sasse-Dwight and J.Gralla, personal communication).

How does the early palindrome region become melted? One possibility is that T-antigen induces the conversion of the early palindrome sequence (nt 5213–5227) to a cruciform structure. We do not believe this to be the case for a number of reasons. First, formation of a cruciform would leave the central two nucleotides of the palindrome unpaired (nt 5220–5221; TG/AC). Neither 3-methylcytidine cleavage nor DMS/nuclease S1 nicking of this

sequence was observed, suggesting that this region is not melted. Second, $KMnO_4$ and DMS modification of each arm of the inverted repeat was asymmetric. Of the 15 bases that showed ATP- and T-antigen-dependent changes, only three are located in the rightward region of the palindrome. Cruciform formation would be expected to result in a more symmetric distribution of modified residues. Finally, mutagenesis of the early palindrome sequence indicated that changes in the left arm severely inhibited DNA replication and *ori*-dependent DNA unwinding, while mutations in the right arm had only modest effects (Deb *et al.*, 1986a; Dean *et al.*, 1987a). These results strongly argue against the formation of a cruciform structure, at least during the initial steps of SV40 replication. A more likely explanation for the cause of melting arises from the sequence of the melted region. The critical melting sequences are contained in a region in which nine of 11 nucleotides in the top strand are pyrimidines (i.e. nt 5210–5220; 5′-TCACTACTTCT-3′). If the flanking non-core origin sequences are also considered, 14 of 16 bp have this polypurine/polypyrimidine asymmetry (pPu/pPy). pPu/pPy sequences have been shown to be sensitive to nuclease S1 cleavage (in the absence of alkylation) both *in vitro* and *in vivo* where such tracts are often found in the 5′ control region of transcribed genes (Pulleyblank *et al.*, 1985; Evans and Efstratiadis, 1986; Hoffman-Liebermann *et al.*, 1986). These and similar results have led to the conclusion that the asymmetric sequences are predisposed to form unstable duplex structures which are more easily melted than non-pPu/pPy tracts. The ATP-dependent T-antigen complex completely protected the pPu/pPy tract in *ori* from DNase I digestion and formed specific contacts to the sequence in the melted region (Figure 7B). We suggest that T-antigen binding to the pPu/pPy tract can further destabilize this tract and shift the equilibrium of the duplex DNA toward melting, resulting in the formation of 8 bp of ssDNA.

Structural changes in the A/T element

The second region in the *ori* core hypersensitive to $KMnO_4$ oxidation is the A/T domain. The A/T region has been shown to exist in a non-B DNA structure in the absence of T-antigen. DNA fragments containing this region migrate anomolously during polyacrylamide gel electrophoresis (PAGE) (Deb *et al.*, 1986b), behavior characteristic of a static DNA bend found in molecules with consecutive adenosine tracts (Koo *et al.*, 1986). This bend is not sensitive to $KMnO_4$ oxidation, since the A/T region was not significantly modified in the absence of T-antigen and ATP. In contrast, the binding of the ATP-dependent complex at *ori* resulted in extensive modifications over a wide region with 16 thymidine residues attacked by $KMnO_4$ over a 16-bp stretch. $KMnO_4$ oxidation of this entire 16-bp region was dependent upon the hydrolysis of ATP, although the T-antigen complex formed with p[NH]ppA resulted in modification to the distal third of this sequence. This is in sharp contrast to the broad modification of the early palindrome region observed with ATP, p[NH]ppA and ADP. While $KMnO_4$ reactivity of the early palindrome can be attributed to DNA melting, nuclease S1 analysis failed to detect any significant melting in the A/T region which could account for the broad hypersensitivity of this latter region to $KMnO_4$ oxidation. Therefore, $KMnO_4$ modification of the A/T region must arise from changes in the structure of double-stranded DNA.

There are two general changes in the structure of non-melted DNA that have the potential to increase thymidine sensitivity to $KMnO_4$. The first possible change is a sharp increase in the natural bending of the DNA. Significant additional bending of the DNA would widen the major and minor grooves on the 'back' side of the DNA helix away from the direction of the bend. This opening of the groove would facilitate $KMnO_4$ attack at this back side resulting in the observed hypersensitivity. Conversely, this bending would close the grooves on the 'front' side and further prevent $KMnO_4$ oxidation. DNA bending leading to enhanced $KMnO_4$ modification has been implicated in the interaction of *Escherichia coli lac* repressor with supercoiled *lac* operator DNA (Borowiec *et al.*, 1987). A second possible cause of $KMnO_4$ hypersensitivity is untwisting of the DNA helix. In this case, the pitch of the DNA is altered so that the number of base pairs per helical turn is increased and the stacking between base pairs reduced. This helical change would thus increase the accessibility of $KMnO_4$ to the reactive 5,6 double-bond of thymine. Modification of the DNA helix in this case is not confined to a specific face of the DNA, but rather is only dependent upon the length of untwisted DNA and the inability of the T-antigen to protect the thymine ring. McClellan and Lilley (1987) have proposed a somewhat similar model to account for the intrinsic hyper-reactivity of alternating $(A-T)_n$ sequences to OsO_4 modification. Each of these proposed changes in the structure of DNA supports a specific prediction of the helical placement of reactive thymines. In the case of DNA bending, $KMnO_4$ would selectively modify a single face of the DNA, while the opposite side would react negligibly with $KMnO_4$. DNA untwisting, on the other hand, would lead to a general $KMnO_4$ modification of the DNA helix with no preferred reactive face.

To determine which pattern of $KMnO_4$ modification occurred in the A/T region, the oxidized sites were plotted on a helix map of this DNA sequence (Figure 8) (Siebenlist *et al.*, 1980). Reactive thymine sites are found in relatively equal numbers on both faces of the DNA helix (nine thymines on side A versus six thymines on side B and one at the borderline). This lack of a unique hypersensitive face does not depend on the relative placement of modified thymines on the map. The faces were chosen to allow the greatest difference between the number of hypersensitive residues on the two faces. We believe, therefore, that the primary cause of $KMnO_4$ reactivity at the A/T region is an untwisting of the DNA helix. From the length of the modified region, it appears that the complete A/T region is subject to untwisting, since all but the most distal thymidine was attacked. Full untwisting of the A/T element requires ATP hydrolysis, since $KMnO_4$ oxidation of this region was limited to the distal six thymidine residues (nt 25–30) in the presence of p[NH]ppA, and was virtually absent with ADP. Thus, two distinct changes can be attributed to the structure of the A/T element; a static DNA bend formed by the natural sequence of this region, and a dynamic untwisting dependent upon the binding of T-antigen and the hydrolysis of ATP.

Model of the initiation of SV40 DNA replication

The changes in DNA structure induced by the ATP-dependent complex combined with the previously described reactions of T-antigen suggest the following model for the initiation of SV40 replication. T-antigen, in the absence of

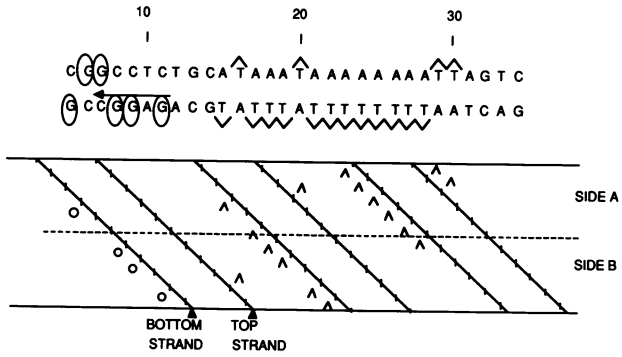

Fig. 8. Helix map of $KMnO_4$ reactive sites in the *ori* A/T region. The sequence of the A/T region of *ori* is shown in the top panel with modified residues indicated as in Figure 7. The helix map is shown below in register with the DNA sequence. The helix map is a representation of the three-dimensional location of chemically modified bases (Siebenlist *et al.*, 1980). The map is drawn as if the helix were cut along a single face, and the helix unfolded and flattened. Diagonal lines represent the sugar-phosphate backbone with tickmarks designating phosphate esters. The helical pitch is drawn with 10.5 bp/turn for A/T regions as suggested by Ulanovsky *et al.* (1986). Top and bottom strands are as indicated, with major and minor grooves represented by wide and narrow diagonal sections respectively. Thymine bases hyper-reactive to $KMnO_4$ are drawn in the major groove since $KMnO_4$ oxidation occurs across the thymine 5,6 double-bond. These reactive bases are indicated by carets. Sides A and B were arbitrarily chosen to give the largest difference between the number of hyper-reactive bases on each face. Also shown is a small portion of the central pentanucleotide element with guanine protection (from methylation) indicated by circles.

ATP, exists in a conformation that binds weakly to the central pentanucleotide element. With the addition of ATP, T-antigen undergoes a conformational change that allows the protein to bind efficiently to the central element and to flanking sequences within the core *ori*. T-antigen structurally deforms these flanking regions, melting sequences in the early palindrome region and untwisting the A/T region. At this point, the *ori* core DNA contains a central region with numerous contacts to T-antigen surrounded on either side by broad regions of melted or stressed DNA. In the presence of SSB, the ssDNA is complexed by the SSB, allowing T-antigen to melt additional sequences at *ori*. The presence of this extensive region of ssDNA serves as the entry site for the binding of additional replication factors (e.g. polymerase α/primase complex) to begin nascent strand synthesis.

The suggested role of DNA structural changes in the initiation of SV40 DNA replication is consistent with past genetic studies of *ori* (Deb *et al.*, 1986a,b, 1987). There is a strong correlation between regions critical for replication and sites rendered hypersensitive to DMS or $KMnO_4$ modification by the ATP-dependent T-antigen complex. Single base pair changes at positions 5211–5219 were found to severely inhibit *ori*-dependent replication *in vivo* and *in vitro* as well as *ori*-dependent DNA unwinding (Deb *et al.*, 1987; Dean *et al.*, 1987b). Since the melted region was localized to nucleotides 5210–5217, this sequence overlaps that required for replication and suggests that the early palindrome may play a role to nucleate DNA unwinding. The A/T region is a second essential element for SV40 replication (Deb *et al.*, 1986b). Mutations across this region indicate that critical replication sequences were contained within nucleotides 17–31. Since we have shown that thymidine residues at nucleotides 15–30 were strongly modified by $KMnO_4$, there is again an intimate correlation between the observed DNA conformational changes and the function of this region in replication. Deb *et al.* (1986a) have shown that the placement and sequence of both the SV40 early palindrome and A/T regions are highly conserved with similar sequences in the replication origins of BK, JC, and SA12 papovaviruses. Thus, there is a strong probability that the changes observed in DNA structure at the SV40 *ori* may be representative of the events occurring in a number of viral origins.

The events observed at the SV40 *ori* are similar in many respects to the reactions found with two extensively studied prokaryotic origins, *E.coli oriC* and bacteriophage λ *ori*λ. In all three cases, multiple protomers of the specific origin binding protein [SV40 *ori*, T-antigen (Dean *et al.*, 1987c); *oriC*, dnaA protein (Fuller *et al.*, 1984); and *ori*λ, the λO protein (Dodson *et al.*, 1985)] accumulate at the *ori* and lead to changes in DNA structure. These changes allow the binding of additional proteins that can catalyze extensive unwinding of the DNA duplex from the *ori*. There are, however, differences in the structural features at each *ori*. A single-stranded region produced by the dnaA/ATP complex at *oriC* was found to be sensitive to cleavage by the single-strand specific enzyme, nuclease P1 (Sekimizu *et al.*, 1987). Similarly, binding of the λO protein to *ori*λ induced sensitivity to nuclease S1 or P1 cleavage at localized sites within this sequence (Schnos *et al.*, 1988). Although the ATP-dependent complex at the SV40 *ori* also yielded ssDNA, we were unable to detect nuclease P1 cleavage of *ori* sequences (data not shown). This may suggest that either the size of the melted region is smaller in the SV40 *ori* relative to those found in the prokaryotic origins or that the melted region within the dnaA/*oriC* or λO/*ori*λ complexes exist in conformations more easily accessible to enzymatic attack. A second major difference is that while T-antigen, dnaA, and λO proteins can all induce structural changes at their respective *ori*s, T-antigen alone contains an intrinsic DNA helicase activity that can catalyze extensive unwinding of the DNA duplex. The prokaryotic origins each require an additional protein factor, dnaB, for helicase activity (Baker *et al.*, 1986; Dodson *et al.*, 1986).

The elucidation of the conformational changes in the SV40 *ori* are essential for understanding the molecular events occurring during the initiation of DNA replication. The $KMnO_4$ oxidation procedure represents a sensitive assay to examine changes in DNA structure induced by T-antigen or other site-specific DNA binding proteins. The technique is simple and uses mild conditions, so that conformational changes can be probed quickly both *in vitro* and *in vivo*. It is likely that other pre-initiation replication complexes as well as transcription complexes locally alter the conformation of DNA at or near the sites of binding. The $KMnO_4$ oxidation procedure should prove useful for such studies.

Materials and methods

Preparation of T-antigen

T-antigen was isolated by immunoaffinity chromatography from COS-1 cells infected with cs1085 as described by Simanis and Lane (1985), as modified by Wobbe *et al.* (1985).

DMS footprinting

Reaction mixtures (20 μl) contained 40 mM creatine phosphate (di-Tris salt, pH 7.8), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.3 μg of pBR322ΔEP (ori$^-$) DNA, 15 ng of 5′-^{32}P-labeled *ori* core or *ori* wt fragment (1×10^6 c.p.m./pmol), 3 mM ATP, ADP or p[NH]ppA (as indicated), and 1.2 μg

of T-antigen. The *ori* wt and *ori* core DNA fragments were uniquely 5′-^{32}P-labeled as described previously (Borowiec and Hurwitz, 1988). Reactions were incubated for 60 min at 37°C, and then DMS added from a freshly diluted 200 mM stock solution to give a final concentration of 30 mM DMS. After 3 min at 37°C, the reaction was quenched with 46 μl of 3 M ammonium acetate, 1 M β-mercaptoethanol, 20 mM EDTA, and 100 μg/ml *E.coli* tRNA, and precipitated with ethanol. The methylated DNA was then cleaved using the Maxam and Gilbert (1980) 'G-only' sequencing reaction. Briefly, the DNA pellet was dissolved in 100 μl of 1.0 M piperidine, and the solution then transferred to a 1.5-ml screw-cap microcentrifuge tube (Sarstedt), and sealed tightly. The mixture was incubated at 90°C for 30 min, and then desalted and ethanol precipitated. Samples were electrophoresed on DNA sequencing gels (6% polyacrylamide) according to standard procedure (Maniatis *et al.*, 1982).

Nuclease S1 treatment of methylated DNA samples

The procedure used was essentially that of Siebenlist (1979). The end-labeled DNA fragment used was end-filled with the large fragment of *E.coli* polymerase I and dNTPs using standard conditions (Maniatis *et al.*, 1982) to reduce loss of 5′-^{32}P-labeled end. T-antigen binding and methylation conditions were the same as those used for DMS footprinting (above) through the initial ethanol precipitation. Pellets containing methylated DNA were dissolved in 50 μl of 30 mM sodium acetate (pH 4.3), 50 mM NaCl, 1 mM $ZnSO_4$ and 5% glycerol. Reaction mixtures were incubated at 37°C, and then 3 U nuclease S1 (Bethesda Research Laboratories) was added. Incubation was continued for 1 min, and the reaction quenched by the addition of 2.5 μl of 20% (w/v) SDS, 16.7 μl of 4 M ammonium acetate, and then precipitated with 3 vols ethanol. Samples were electrophoresed on DNA sequencing gels (6% polyacrylamide) according to standard procedures (Maniatis *et al.*, 1982).

$KMnO_4$ footprinting

Reaction mixtures (30 μl) contained 40 mM creatine phosphate (di-Tris salt, pH 7.8), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 μg of pSV01ΔEP (Wobbe *et al.*, 1985), 4 mM ATP, ADP or p[NH]ppA (as indicated), and 1 μg of T-antigen. After incubation for 60 min at 37°C, 3 μl of 60 mM $KMnO_4$ was added to give a final concentration of 6 mM $KMnO_4$. The reaction mixture was further incubated for 4 min at 37°C and then quenched by the addition of β-mercaptoethanol to 1.0 M. The mixture was diluted to 80 μl with water, and then desalted by centrifugation through a 1 ml Sephadex G-50-80 column pre-equilibrated in water. The sample was divided into two 35 μl portions in preparation for primer extension. To detect modifications on the top and bottom strands, *Eco*RI and *Hin*dIII pBR322 sequencing primers respectively, were used (Pharmacia PL). Primers were 5′ end-labeled with [γ-^{32}P]ATP and phage T4 polynucleotide kinase (Boehringer Mannheim). Primer extension and gel electrophoresis of samples were performed according to Borowiec *et al.* (1987). Sequence positions of modified residues were determined by comparison to dideoxy sequencing ladders (Sanger *et al.*, 1977).

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