Differential Induction of Structural Changes in the Simian Virus 40 Origin of Replication by T Antigen

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The ATP-dependent binding of the simian virus 40 (SV40) large tumor antigen (T antigen) to the SV40 origin of replication (*ori*) results in the structural distortion of two critical elements within flanking regions of *ori* and the untwisting of the DNA helix. We examined the effect of changes in temperature, ATP concentration, and other reaction parameters on the generation of these DNA structural changes. We found that induction of the two localized structural transitions were highly and differentially sensitive to reaction conditions. Significant distortion of the early palindrome element, shown previously to result from DNA melting, required low levels of ATP (10 to 30 μ M) but temperatures above 25°C. Distortion of the AT tract occurred at low temperatures (5°C) but required relatively high concentrations of ATP (>300 μ M). Thus, T antigen can induce structural changes within one critical element of *ori* without generating significant structural distortion within the second element. The response of *ori* untwisting to reaction conditions generally increased in parallel with or fell intermediate between the inductions of localized structural transitions. We suggest that *ori* untwisting and localized structural distortions are interdependent consequences of T-antigen binding to *ori*. These results suggest a model for the structural events occurring during the initial steps of SV40 DNA replication.

The initiation of DNA replication requires gross changes in the duplex DNA structure comprising the origin of replication. The DNA must be denatured to allow the enzymatic machinery access to the bases in order to synthesize the complementary strand. The nature of the protein-DNA interactions that can generate sufficient force to melt DNA sequences within an origin remains poorly understood. We are studying the initiation of DNA replication using simian virus 40 (SV40) with the goal of understanding the molecular events required to achieve large-scale denaturation of an origin of replication.

SV40 is widely used as a eukaryotic model system *in vitro* to examine the mechanistic steps constituting the initiation of DNA replication (1, 6, 23). The SV40 core origin of replication (*ori*) is contained within a fairly small region (approximately 65 bp), and regions critical for DNA replication have been defined by detailed genetic investigation (10, 12). The initiation of DNA replication requires sequence-specific binding to *ori* of the SV40 large tumor antigen (T antigen), the only virus-encoded protein required for replication of the SV40 chromosome. Efficient binding of T antigen to *ori* at elevated temperatures (e.g., 37°C) requires ATP and results in a complex that protects the complete core *ori* sequence from DNase I digestion (2, 9, 11). This ATP-dependent complex contains up to 12 monomers of T antigen arranged in a structure with a distinct bilobed appearance (9, 17). The addition of single-stranded DNA-binding protein (SSB; e.g., human SSB or *Escherichia coli* SSB) to the complex induces stable denaturation of *ori* in an ill-defined process and results in bidirectional unwinding of DNA outward from *ori* because of the DNA helicase activity of T antigen (8, 14, 15, 26).

Chemical probing of the ATP-dependent complex has

shown that the binding of T antigen induces structural alterations of two DNA elements in the core *ori* (Fig. 1) (3, 21). In the early-gene side of *ori*, T antigen melts approximately 8 bp of DNA partially contained within one arm of a 15-bp imperfect inverted repeat (the early-palindrome element). The second significant change is found within a 17-bp AT tract that becomes structurally distorted, although the DNA remains essentially double stranded in character. These two elements flank a central region containing four conserved GAGGC repeats that are thought to bind and properly position T antigen for induction of the structural changes and for the unwinding of *ori* (3, 21). Each of these three regions is essential for events occurring during the initiation of replication; mutation of any element is deleterious both to SV40 DNA replication and to unwinding of DNA containing *ori* (7, 12).

Topological assays measuring other aspects of DNA structure, namely untwisting of the DNA helix mediated by T antigen, have also been developed (9a, 22). These studies show that T antigen significantly untwists the DNA helix (increases the number of base pairs per helical turn) concurrently with the formation of the ATP-dependent complex. While certain of the structural changes found by chemical probing would be expected to untwist the DNA helix (e.g., DNA melting), the relationship between DNA structural changes detected chemically and those detected by alterations in DNA topology has not been determined.

We have examined the influence of ATP levels, temperature, and other factors on the structural distortion of *ori* mediated by T antigen. We show that the ATP-dependent complex of T antigen bound to *ori* can differentially induce localized structural distortions within two critical regions of *ori*. We also show that changes in DNA topology generally parallel DNA structural alterations that are detectable by chemical probing. These results suggest that changes in DNA topology and structural alterations in the early palindrome and AT-tract regions are related events arising from the binding of T antigen.

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FIG. 1. Schematic of the SV40 origin of replication. The SV40 core *ori* extends from SV40 positions 5211 to 31 (10) of the 5,243-bp SV40 genome. The core *ori* is considered here to be identical to T-antigen-binding site II. The three regions within the *ori* critical for DNA replication are the early palindrome element (EP), a central palindrome containing four GAGGC repeats (GAGGC), and a 17-bp AT tract (AT). Located outside the core *ori* is T-antigen-binding site I. The approximate location of each region is indicated by the SV40 sequence positions above the diagram. The late-gene side of *ori* is to the right.

MATERIALS AND METHODS

Preparation of T antigen. T antigen was purified from Sf9 insect cells infected with recombinant baculovirus strain vEV55SVT (20). Forty-eight hours postinfection, the Sf9 cells were collected and pelleted. The cells were suspended in 5 ml of lysis buffer (20 mM Tris-HCl [pH 9.0], 0.3 M NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.2 μ g of aprotinin per ml, 0.2 μ g of leupeptin per ml, 0.1 μ g of antipain per ml, 50 μ M EGTA) per ml of pellet volume. After incubation at 0°C for 15 min, the suspension was centrifuged at $40,000 \times g$ for 30 min (4°C). The supernatant was neutralized with a one-half volume of neutralization buffer (lysis buffer containing 100 mM Tris-HCl [pH 6.8]), and the lysate was then stored at -80°C. T antigen was immunoaffinity purified from this lysate according to the method of Mastrangelo et al. (17).

KMnO₄ footprinting of DNA. Standard reaction mixtures (30 μ l) contained 40 mM creatine phosphate (di-Tris salt, pH 7.8), 7 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 μ g of pSV01 Δ EP DNA (25), 4 mM ATP, 23.3 μ g of creatine phosphokinase per ml, and 1.0 μ g of T antigen. Reactions were incubated at 37°C for 60 min prior to modification with KMnO₄. KMnO₄ was added from a freshly prepared stock solution (60 or 200 mM) to give a final concentration of 6 mM, and the reaction was incubated an additional 4 min. The primer extension and gel electrophoresis conditions were essentially as described by Borowiec and Hurwitz (3). For kinetic examination of changes in DNA structure induced by T antigen, the concentration of KMnO₄ used to modify the DNA was increased to 20 mM (final), and the incubation time with this chemical probe was shortened to 30 s.

The level of KMnO₄ oxidation in each region was quantitated by densitometric scanning of autoradiographs with a Helena Quick Scan densitometer. Autoradiographs with various exposure times were used to ensure that the band intensity was within the linear range of film sensitivity. The extent of KMnO₄ oxidation of the early palindrome was determined by quantitating the intensity of the region corresponding to top-strand thymine residues 5210, 5214, 5217, and 5218. For the AT tract, bands corresponding to thymine residues 16, 20, 29, and 30 of the top strand were used. The overall intensity of each region was separately normalized by comparison with the intensity of two control thymine residues (SV40 positions 5159 and 5163) located outside both the SV40 core *ori* and T-antigen-binding site I (adjacent to the core *ori*). The maximum normalized value for the early palindrome and the AT tract for each titration was set at 1.0. The fraction of maximum oxidation was then calculated for each point. When a kinetic analysis was performed, regions of the AT tract proximal and distal to the central GAGGC element were examined separately. The proximal region was

considered to minimally contain the region encompassed by thymine residues located at SV40 positions 16 and 20; the distal region contained thymines at positions 29 and 30.

Topoisomer shift assay (DNA untwisting). Standard reaction mixtures (30 μ l) containing 40 mM creatine phosphate (di-Tris salt, pH 7.7), 7 mM MgCl₂, 1 mM dithiothreitol, 4 mM ATP, 10 U of HeLa topoisomerase I, 0.9 μ g of bovine serum albumin, 0.3 μ g of relaxed pSV01 Δ EP DNA, and 0.75 μ g of T antigen were incubated for 45 min at 37°C. Reactions were terminated by the addition of a solution (5 μ l) containing 2% sodium dodecyl sulfate (SDS), 0.1 mM EDTA, 1 μ g of glycogen, and 5 μ g of proteinase K followed by an additional incubation for 30 min at 37°C. Following ethanol precipitation, the products were subjected to electrophoresis through agarose gels (1.8%) containing chloroquine phosphate (2 to 4 μ g/ml). The ethidium bromide-stained gels were photographed, and the photographic negatives were scanned with an LKB 2202 Ultrascan laser densitometer. The relative amount of DNA contained in each topoisomer band was determined, and the (relative) average linking number was calculated. The change in the average linking number was then calculated and normalized with respect to the maximum linking number difference determined for each experiment.

RESULTS

We examined the effects of various reaction conditions on the ability of T antigen to distort the structure of DNA. Conformational changes in *ori* were examined by two complementary methods. Local structural transitions were probed by chemical modification with potassium permanganate (KMnO₄), while the amount of DNA untwisting generated by T antigen was examined by quantitating changes in the distribution of plasmid topoisomers.

DNA structural changes as a function of ATP concentration. The effect of ATP concentration on T-antigen-mediated DNA structural changes was examined (Fig. 2). Localized structural distortions in the early-palindrome element and the AT tract can be detected by potassium permanganate (KMnO₄) oxidation of T-antigen-*ori* complexes (3). While KMnO₄ reacts poorly with DNA in a typical B-DNA conformation, significant changes in the structure of the DNA duplex (e.g., DNA melting or pronounced DNA bending) lead to hypersensitivity to KMnO₄ oxidation, predominantly at thymine residues (4).

T-antigen-*ori* complexes were formed in the presence of increasing amounts of ATP, and the complexes were then modified with KMnO₄. The reactive sites within *ori* were determined by the extension of a ³²P-end-labeled DNA primer through the *ori* region with the Klenow fragment of *E. coli* DNA polymerase I, which stalls at oxidized thymines. The sites of modification, corresponding to regions of DNA

with significant distortion, were visualized by autoradiography of the primer extension products separated on a DNA sequencing gel. While modification of either strand can be examined by using an appropriate oligonucleotide primer, we have found that a primer complementary to the top strand (the adenine-rich strand in the AT tract) is most informative for examination of *ori* DNA conformation.

ori-containing DNA incubated with T antigen in the absence of ATP was poorly oxidized by KMnO_4 , consistent with past probing studies (Fig. 2A, lane 1) (3). As the level of ATP in the reaction was increased from 0 to 3 mM, the early-palindrome and AT-tract elements were found to respond at different nucleotide concentrations. The early-palindrome element was reactive to the chemical probe at lower concentrations of ATP, with significant reactivity observed at 10 μ M ATP. Significant modification of the AT tract was not observed at concentrations of ATP below 100 μ M. The early-palindrome element thus requires lower ATP levels than the AT tract to undergo structural transitions detectable by this method. In addition, while the overall sensitivity of each *ori* region to chemical attack was strongly influenced by the ATP concentration, the relative intensity

FIG. 2. Response of T-antigen-dependent structural changes in *ori* to ATP concentration. (A) Representative autoradiograph showing the KMnO_4 oxidation of *ori* as a function of ATP concentration. Form I pSV01 Δ EP (containing the SV40 *ori*) (25) was incubated with 1.0 μ g of T antigen and various concentrations of ATP (as indicated) at 37°C for 1 h. The DNA was then modified with KMnO_4 , and the sites of modification were probed by primer extension as described in Materials and Methods. The locations of the three critical *ori* regions are shown on the right side of the autoradiograph and are designated as follows: EP, early palindrome; GAGGC, central element containing four GAGGC repeats; AT, 17-bp AT tract. Various SV40 sequence positions are shown on the left. (B) DNA untwisting mediated by T antigen. Form I pSV01 Δ EP (0.3 μ g) was incubated with 0.75 μ g of T antigen and various concentrations of ATP (as indicated). Topoisomerase I was also present in each reaction to relieve any DNA supercoils generated by the binding of T antigen. After incubation, the product DNA was subjected to agarose gel electrophoresis in the presence of 2 μ g of chloroquine phosphate per ml. The position of form II (nicked) DNA is indicated on the left. (C) Quantitation of *ori* structural changes. The levels of DNA untwisting and KMnO_4 oxidation in the early palindrome and AT tracts were determined and normalized as described in Materials and Methods. Symbols: ●, early palindrome oxidation; ■, AT-tract oxidation; Δ , DNA untwisting. Maximum DNA untwisting was found at 3 mM ATP, at which point 1.3 turns were removed.

of each thymine within a single element was not affected by nucleotide concentration. This suggests that the changes in ATP level do not cause distinct structural transitions, but rather affect either the fraction of *ori* molecules undergoing a structural transition or the degree of distortion within each *ori* molecule.

The extent of KMnO_4 modification in the early-palindrome and AT-tract regions was quantitated by densitometric scanning of the autoradiographs. The levels of modification were separately normalized by comparison with control bands located outside the *ori* region. The fraction of maximum KMnO_4 oxidation in each region was plotted as a function of ATP concentration (Fig 2C). The structural distortions within each element had a sigmoidal dependence on ATP levels and were found to plateau at high concentrations of nucleotide. Comparison of the two curves confirmed the differential response of each region to ATP. The concentration of ATP resulting in 50% maximal reactivity was found to be 35 μ M for the early palindrome, while fivefold-higher levels of ATP (175 μ M) were required to achieve 50% maximum reactivity in the AT tract.

The effect of ATP concentration on topological changes in DNA structure induced by T antigen was examined. *ori*-containing plasmid DNA was incubated with T antigen and various concentrations of ATP. HeLa topoisomerase I was

FIG. 3. Influence of temperature on T-antigen-dependent structural changes in *ori*. (A) Representative autoradiograph of the KMnO_4 oxidation pattern in *ori* as a function of incubation temperature. Form I pSV01 Δ EP (0.5 μg) was incubated with 1.0 μg of T antigen and 4 mM ATP for 1 h at various temperatures (as indicated). The DNA was then modified with KMnO_4 , and the sites of modification were probed by primer extension as described in Materials and Methods. SV40 sequence positions are indicated as described in the legend to Fig. 2. (B) DNA untwisting mediated by T antigen. Form I pSV01 Δ EP (0.3 μg) was incubated with 0.75 μg of T antigen and 4 mM ATP for 60 min at various temperatures (as indicated). Topoisomerase I was present in all reactions except that of lane 1. After incubation, the product DNA was subjected to agarose gel electrophoresis in the presence of 2 μg of chloroquine phosphate per ml. The position of form II (nicked) DNA is indicated on the left. (C) Quantitation of *ori* structural changes. The levels of DNA untwisting and KMnO_4 oxidation in the early palindrome and AT tracts were determined and normalized as described in Materials and Methods. Symbols: ●, early palindrome oxidation; ■, AT-tract oxidation; △, DNA untwisting. Maximum DNA untwisting was found at 30°C, at which point 0.9 turn was removed.

present in each reaction to relieve any DNA supercoiling generated by T-antigen binding to the *ori*. T antigen does not catalyze gross DNA unwinding of the plasmid DNA molecules in these reactions, since an SSB was not added. After incubation, the reaction mixtures were deproteinized and subjected to agarose gel electrophoresis in the presence of chloroquine to allow visualization of the entire plasmid topoisomer distribution (Fig. 2B).

Increases in the concentration of ATP from 0 to 3 mM caused the distribution of plasmid DNA topoisomers to broaden and shift upwards by one to two bands (Fig. 2B, cf. lane 9 and 10). Since the origin DNA does not wrap around T antigen in a nucleosomelike configuration (9), the upward shift in topoisomer distribution most likely results from T antigen untwisting the DNA helix. We will refer to such topological changes as DNA untwisting, defined as an increase in the number of base pairs per helical turn (as

opposed to changes in the direction of the helical axis, such as the bending of DNA).

The amount of DNA untwisting was calculated by densitometric scanning of the gel and quantitation of the difference between the center of each topological distribution. The fraction of maximal DNA untwisting was plotted as a function of ATP concentration (Fig. 2C). The untwisting of *ori* by T antigen was found to have a sigmoidal dependence on ATP concentration. DNA untwisting approached a plateau at the highest levels of ATP, at which point 1.3 helical turns were removed. The level of ATP giving rise to 50% maximal untwisting was calculated to be 140 μM . The DNA untwisting curve appeared to parallel the KMnO_4 reactivity of the two elements, with a particularly close overlap between DNA untwisting and modification of the AT tract.

Sensitivity of the early palindrome and AT tract to temperature. The response of DNA structural changes to incubation temperature was examined. T-antigen-*ori* complexes were formed at various temperatures in the presence of 4 mM ATP and then chemically probed with KMnO_4 . A representative autoradiograph of the separated primer extension products is shown in Fig. 3A. The autoradiograph indicated that the early palindrome and AT-tract regions were differentially sensitive to temperature. Surprisingly, the AT tract was modified at lower temperatures than the early palindrome element. This response was opposite to that observed with ATP, when the early palindrome was the more sensitive of the two elements at low concentrations of nucleotide.

The level of KMnO_4 attack in each of the two regions was quantitated, and the normalized oxidation of each region was determined as a function of temperature (Fig. 3C). The level of KMnO_4 oxidation in the AT tract increased sharply from 0 to 10°C and then decreased gradually from these peak levels. The behavior of the early palindrome element with respect to temperature was quite different, verifying the differential sensitivity of the two *ori* elements to temperature. The early palindrome was poorly reactive to KMnO_4 below 20°C but became increasingly reactive to the chemical probe up to the highest temperatures tested. The temperatures that resulted in 50% maximum KMnO_4 oxidation were determined for each of the two regions. The thermal midpoints were found to differ by over 25°C, with the midpoints calculated to be 2 and 28°C for the AT-tract and early palindrome elements, respectively.

Parallel experiments were performed to determine the response of DNA untwisting to temperature. T antigen was incubated at various temperatures with *ori*-containing DNA, 4 mM ATP, and topoisomerase I. The DNAs were then subjected to agarose gel electrophoresis to visualize their topoisomer distribution (Fig. 3B). Since the helical twist of DNA is temperature dependent (13), control reactions lacking T antigen were performed at each temperature. Note that the HeLa topoisomerase I was active at the lowest temperatures tested (i.e., 0°C; Fig. 3B, cf. lanes 1 and 2). The amount of T-antigen-dependent DNA untwisting at each temperature was calculated and plotted (Fig. 3C). The T-antigen-mediated DNA untwisting was strongly dependent on temperature, rising sharply from the minimal levels observed in the low-temperature range ($\leq 10^\circ\text{C}$). DNA untwisting plateaued at temperatures of 25 to 30°C and then declined slightly at 37°C. The thermal midpoint for DNA untwisting in the increasing portion of the curve was calculated to be 17°C. Comparison of DNA untwisting with KMnO_4 oxidation indicated that untwisting, in general, paralleled the response of the early palindrome element but had a thermal midpoint that was intermediate between those calculated for the early palindrome and AT-tract elements.

Titration of T antigen. The response of DNA structural changes to the amount of added T antigen was examined. Since *ori*-dependent DNA untwisting has been examined in parallel studies (9a), we probed structural changes in *ori* by KMnO_4 modification. *ori* DNA was incubated with 4 mM ATP and various amounts of T antigen at 37°C, and the DNA was then modified with KMnO_4 . Autoradiography showed that structural changes induced in *ori* were highly dependent on the amount of T antigen added, a result consistent with those of preliminary studies (Fig. 4A) (3). The level of oxidation in each region was quantified and separately normalized, and these levels were plotted as a function of the amount of T antigen added (Fig. 4B). DNA untwisting as a function of T antigen is also presented (9a).

Structural changes induced in both the early palindrome and AT tract were found to have a roughly linear response to added T antigen. No obvious plateau was observed in either curve up to the highest levels of T antigen tested (2.0 μg). DNA untwisting showed a similar profile, though at the lowest T-antigen levels, DNA untwisting was detected without significant KMnO_4 modification. The levels of T antigen causing 50% maximal oxidation or untwisting were determined by using the data obtained with 2.0 μg as the maximum. The midpoints were found to be at 0.7 and 1.2 μg of T antigen for structural changes in the early palindrome and AT tracts, respectively, and at 0.6 μg of T antigen for DNA untwisting.

FIG. 4. Influence of T-antigen concentration on structural changes in *ori*. (A) Representative autoradiography of the KMnO_4 oxidation pattern in *ori* as a function of T-antigen levels. Form I pSV01 Δ EP (0.5 μg) was incubated with T antigen (as indicated) and 4 mM ATP for 1 h at 37°C. The DNA was then modified with KMnO_4 , and the sites of modification were probed by primer extension as described in Materials and Methods. SV40 sequence positions are indicated as described in the legend to Fig. 2. (B) Quantitation of *ori* structural changes. KMnO_4 oxidation in the early palindrome and AT tracts were determined and normalized as described in Materials and Methods. Symbols: ●, early palindrome oxidation; ■, AT-tract oxidation; ---, DNA untwisting. The DNA untwisting curve is from Dean and Hurwitz (9a). Maximum DNA untwisting was found at 2.0 μg of T antigen, at which point 1.9 helical turns were removed.

These responses were somewhat unanticipated because at the highest levels of protein (i.e., 2.0 μg of T antigen), the molar ratio of T antigen to DNA was greater than 80 to 1. Assuming that 3 molecules of T antigen can bind to site I (located outside the core *ori*) and up to 12 monomers can bind core *ori*-containing site II (17, 18), this level of T antigen nominally represents a fivefold excess over that needed to

completely occupy both sites. The linear response to the high T-antigen levels may reflect a low percentage of active molecules in the population. The DNA replication activity of low concentrations of similar T-antigen preparations showed a 2- to 4-fold stimulation when T antigen was treated with cellular protein phosphatase 2A and a 1.5-fold stimulation when T antigen was treated with human cdc2 kinase (cdc2 kinase generously provided by D. Beach) (9b, 19, 24). We note, though, that formation of ATP-dependent hexamers by T antigen in the absence of DNA occurs with more than 95% of the available T antigen in these and similar preparations of T antigen (9a, 17). Thus, a more likely hypothesis is that the core *ori* molecules are not saturated even at high levels of T antigen.

Kinetics of formation of structural changes within *ori*. The kinetics of induction of the *ori* structural changes was examined. To detect events occurring within the first few minutes of T-antigen binding to *ori*, we used higher concentrations of KMnO_4 and decreased the modification time relative to the conditions used above (see Materials and Methods). Individual reaction mixtures were also pre-warmed to 37°C in the absence of T antigen to reduce any effects of thermal equilibration during the initial period of T-antigen binding. T antigen was added to each reaction, and after various periods of incubation, the DNA was probed with KMnO_4 .

Autoradiography of the primer extension products revealed two distinct structural events within the AT tract distinguishable by their dissimilar kinetics of formation (Fig. 5A). The earliest structural transition occurred within the portion of the AT tract distal from the central GAGGC element minimally containing nucleotides located at SV40 positions 29 and 30. Strong KMnO_4 modification of this region was found in the first minute of binding, when only minor reactivity of the portion of the AT tract proximal to the GAGGC element (containing nucleotides 16 and 20) was detected. Structural changes in the proximal region of the AT tract as well as in the early palindrome element occurred at a slower rate, increasing gradually over the first 30 min of binding. Thus, it appears that separate conformational changes can occur in discrete regions of the AT tract during the first few minutes of T-antigen binding.

Levels of modification of the proximal and distal regions of the AT tract and within the early palindrome element were separately quantified and plotted as a function of incubation time (Fig. 5B). The time course of DNA untwisting is presented for comparative purposes (9a). Structural changes in the two AT-tract regions clearly differed, with 50% maximal oxidation occurring at 50 s and 11.5 min for the distal and proximal regions, respectively. The modification of the early palindrome element and the proximal AT tract occurred at a similar rate, with 50% maximal oxidation of the early palindrome calculated to occur at 13.5 min. The kinetic midpoint for DNA untwisting was calculated to be 8 min.

DISCUSSION

Conformational changes induced by T antigen in two critical regions of *ori* DNA, the early palindrome and AT-tract elements, have been demonstrated to be highly and differentially sensitive to reaction conditions. The induction of these structural changes was found to be surprisingly complex. The early palindrome can undergo significant structural transitions at relatively low concentrations of ATP, but these require incubation temperatures above 25°C. The AT tract, on the other hand, can be deformed by T

FIG. 5. Time course of induction of structural changes within *ori*. (A) Representative autoradiograph of the KMnO_4 oxidation pattern in *ori* as a function of the length of incubation of T antigen with *ori*. Form I pSV01ΔEP (0.5 μg) was incubated with 1.0 μg of T antigen and 4 mM ATP for various times (as indicated) at 37°C. The DNA was then modified with KMnO_4 , and the modification sites were probed by primer extension as described in Materials and Methods. SV40 sequence positions are indicated as described in the legend to Fig. 2. (B) Quantitation of *ori* structural changes. KMnO_4 oxidation in the early palindrome and AT-tract regions proximal and distal to the central GAGGC element were determined and normalized as described in Materials and Methods. Symbols: ●, early palindrome oxidation; □, proximal AT-tract oxidation; △, distal AT-tract oxidation; ---, DNA untwisting. The DNA untwisting curve is from Dean and Hurwitz (9a). Maximum DNA untwisting was found at 30 min, at which point 1.0 turn was removed.

antigen at low temperatures (5°C), but these conformational changes required levels of ATP roughly fivefold higher than that needed to induce significant changes in the early-palindrome element. Thus, structural transitions can occur

in one region even in the absence of corresponding changes in the opposite flanking region.

The differential induction of the structural transitions in the two flanking *ori* elements demonstrates that each element has separate environmental requirements for becoming distorted. This likely reflects that each element is composed of different sequence tracts and that each element undergoes distinct conformational changes. The early palindrome element, containing mainly cytosines and thymines on one of the strands, becomes significantly melted by the ATP-dependent complex. Structural changes in the AT tract, on the other hand, do not appear to result from DNA melting but rather represent a distortion of double-stranded DNA (3, 21).

The sharp temperature dependence of KMnO_4 hypersensitivity within the early palindrome element is most likely due to the need for sufficient thermal input for T antigen to melt this DNA. A similar temperature dependence has been observed for melting of the *E. coli* origin of replication (*oriC*) by the DnaA protein (5). We note that the thermal profiles of *oriC* melting and early palindrome modification are nearly identical both in shape and temperature range. This pronounced thermal dependence is also reminiscent of the profiles seen for melting of promoter elements by *E. coli* RNA polymerase (16). The similarities in response to temperature in these three diverse systems suggest that such sharp thermal responses may be typical of many protein-mediated DNA melting events such as at other origins of replication or, possibly, within eukaryotic transcription complexes.

Formation of the ATP-dependent complex rendered the AT tract susceptible to KMnO_4 oxidation over a broad temperature range, with a thermal profile quite distinct from that of the early palindrome (Fig. 3C). The structural transitions occurring in the AT tract thus do not appear to require a high thermal input. The actual conformational change taking place within the AT tract is at present poorly understood, though the lack of pronounced thermal dependence again indicates that the primary conformational change is not DNA melting. We have previously suggested that the DNA becomes untwisted by T antigen (3), and this proposal would be consistent with the observation that T antigen can untwist the SV40 *ori*.

The T-antigen-mediated induction of structural changes in the AT tract was found to require fivefold-higher levels of ATP than changes induced in the early palindrome. Because the T antigen bound to each flanking element contacts DNA of dissimilar sequence, these interactions may induce T antigen to have different affinities for ATP. An alternative explanation is that distortion of the AT tract requires a higher energetic input by T antigen than melting of the early palindrome element and that this extra "work" is driven by the presence of higher concentrations of ATP. In support of this model, melting of the early palindrome element by T antigen has been shown to take place efficiently in the presence of nonhydrolyzable analogs of ATP, while complete distortion of the AT tract requires ATP hydrolysis (3).

The levels of *ori* DNA untwisting and KMnO_4 oxidation of the early palindrome and AT-tract elements were each linearly dependent on the amount of added T antigen. No plateau was found for any of these three quantities up to the highest levels of T antigen tested. The higher levels of T antigen (i.e., 2.0 μg) nominally represent a fivefold molar excess over that required to completely fill T-antigen sites I and II (little binding to site III occurs under these conditions) (2). The linear dependence of *ori* structural changes on T antigen indicates that the core *ori* is not saturated with T antigen, even in the upper range of the protein titration. This conclusion is reinforced by the observations of Mastrangelo et al. (17), who showed that while a double hexamer of T antigen is the maximal ATP-dependent complex bound to the core *ori*, the majority of T-antigen-*ori* complexes found at moderate levels of T antigen (i.e., 0.4 μg) are smaller. An increase in the amount of T antigen might therefore shift more of the protein into the double-hexamer form, leading to greater distortion of *ori* structure. Whether T antigen contained in structures smaller than a double hexamer can distort the *ori* structure is as yet unclear, but quite possible.

Changes in reaction conditions had similar effects on T-antigen-mediated DNA untwisting and on the induction of structural transitions in the early palindrome and AT-tract regions. In each of the four reaction parameters studied, DNA untwisting was found generally to parallel or fall intermediate between KMnO_4 oxidation of the two flanking *ori* regions (three regions in the time course of T-antigen binding). These results suggest that DNA untwisting and the localized structural distortions are closely related manifestations of the changes that occur when T antigen binds to *ori*. The melting of 8 bp in the early palindrome element and the distortion of the AT tract over a region of 16 bp appears to be consistent with the untwisting of one to two helical turns.

The formation of the T-antigen ATP-dependent complex with the SV40 *ori* occurred in at least two discrete steps. In the first step, T antigen bound to *ori* induced conformational changes in the distal portion of the AT tract. T antigen then structurally deformed the remaining portion of the AT tract and the early palindrome element. The first step likely requires the previous or simultaneous binding of T antigen to the central GAGGC repeats. Others have shown that distortion of the AT tract is not observed in the absence of the central GAGGC element (21). The T-antigen complex therefore minimally spans the entire AT tract in this initial step. As this step precedes complete distortion of the complete AT tract, distortion of the distal portion of the AT tract may be a required substep serving to initiate changes in the remainder of the tract.

The early palindrome element did not show any evidence of multiple structural transitions during the initial few minutes of binding. The kinetic profile of KMnO_4 modification in the early palindrome instead resembled the curve obtained for the proximal AT tract (Fig. 5). Structural distortion of the distal AT tract does not appear to be a prerequisite for distortion of the early palindrome element, since Parsons et

FIG. 6. Model of events occurring during the initial steps of SV40 DNA replication.

al. (21) have shown that T antigen can melt the early palindrome in the absence of the AT tract and, at high concentrations of T antigen, in DNA constructs lacking both the AT tract and central GAGGC elements. These investigators also postulated that early palindrome melting precedes distortion of the AT tract. However, in light of the kinetic experiments presented above, this suggestion may require revision.

These data combined with work by a number of investigators (see references 1, 6, and 23 and references therein) lead to an updated model of the events occurring during the initial steps of SV40 DNA replication (Fig. 6). T antigen in the presence of ATP binds to the central GAGGC element of the SV40 *ori*. Simultaneously with or soon after this initial binding, T antigen contacts and distorts the distal portion of the AT tract and causes limited untwisting of the helical structure of *ori*. Conformational changes then occur in the T-antigen-*ori* complex that result in distortion of the remainder of the double-stranded AT tract, melting of the early palindrome, and more-significant untwisting of the DNA. This complex contains two structural lobes which, in the presence of human SSB, separate and bidirectionally unwind the DNA outward from *ori*. The SSB constrains the single strands, allowing the DNA polymerase machinery entry for catalysis of nascent-strand synthesis.

Conversion of DNA into a template suitable for DNA synthesis is seen to be an intricate process. Large-scale denaturation of the DNA helix entails an elaborate series of protein-protein and protein-DNA interactions. The SV40 model system is remarkable in that these complex reactions are catalyzed predominantly by a single protein, T antigen.

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