Relationship among Location of T-Antigen-Induced DNA Distortion, Auxiliary Sequences, and DNA Replication Efficiency

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T-antigen-induced DNA distortion was studied in a series of simian virus 40 (SV40) plasmid constructs whose relative replication efficiency ranges from 0.2 to 36. Bending was detected in the wild-type SV40 regulatory region consisting of three copies of the GC-rich 21-bp repeat but not in constructs with only one or two copies of the 21-bp repeat. In a construct with enhanced replication efficiency, bending occurred in a 69-bp cellular sequence located upstream of a single copy of the 21-bp repeat. Bending occurred both upstream of *ori* and in the three 21-bp repeats located downstream of *ori* in a construct with reduced replication efficiency. In a construct with no 21-bp repeats, DNA distortion occurred downstream of *ori*. The results indicate that SV40 DNA replication is enhanced when the structure of the regulatory region allows the DNA to form a bent structure upstream of the initial movement of the replication fork.

Simian virus 40 (SV40) DNA replication has been widely used as a model to study DNA replication in eukaryotes (reviewed in references 7 and 19); the events before the initiation of replication are particularly interesting. SV40 has a well-defined origin of replication (2, 10, 22, 43), and the initiation of replication depends on a single viral protein, T antigen (T-ag) (3, 16, 36, 59). Except for T-ag, all proteins required for DNA replication are supplied by the host cell, and with the development of an in vitro SV40 replication system, their identity and function have been well characterized (35, 65; reviewed in references 7, 30, and 53). The SV40 core *ori* is a 64-bp region (20, 27, 43, 54) that consists of three functional domains (13, 36, 45): a 17-bp adenine/thymine-rich (AT) region, a central 27-bp palindrome (PEN), and an early palindrome (EP).

Located proximal to the AT tract are the promoter and enhancer elements of the SV40 regulatory region. The bidirectional promoter element for SV40 transcription consists of three 21-bp tandem repeats, each containing two copies of the conserved 5'-GGGCGG-3' (GC box) sequence. The GC boxes are binding sites for the transcription factor Sp1 (16, 24, 26). The enhancer contains two 72-bp repeats, each with multiple binding sites for transcription factors, including those belonging to the AP family (31).

T-ag binds as a trimer to T-ag binding site I (38) located on the early transcription side of *ori*. The presence of site I facilitates T-ag-dependent unwinding of the DNA (28) and increases the frequency of initiation of DNA replication (25). T-ag binding site II, located at the PEN region in *ori*, is essen-

tial for the initiation of replication (15, 17, 20, 43, 46). T-ag forms double hexameric lobes that surround the PEN region (12, 37), and the helicase activity of the hexamers unwinds the DNA bidirectionally (11, 63). T-ag binding also occurs at site III located within the three 21-bp GC-rich repeats on the late transcription side of *ori* (23, 38, 60).

T-ag binding to *ori* and flanking sequences induces structural distortions of the region. Both dimethyl sulfate protection and potassium permanganate (KMnO₄) oxidation assays show that binding of T-ag to the PEN domain induces melting of approximately 8 bp of the EP and untwisting of the AT tract (4, 5, 45, 46). Unwinding of the AT tract is distinct from the denaturing that occurs in the EP region. Because the amount of dimethyl sulfate methylation of internal hydrogen binding sites is only about 5% of that occurring in the EP region, it is apparent that the AT tract is not denaturing but rather untwisting its double-stranded conformation (4, 5).

To identify the specific elements that regulate replication efficiency in vivo, our laboratory has used plasmid constructs derived either from SV40 evolutionary variants (6, 58) or from rearranged or duplicated portions of the wild-type SV40 regulatory region (33, 34, 61). Figure 1 summarizes the organization of the regulatory regions and the effect on the relative replication efficiency (RRE) of some of these SV40 plasmid constructs (34). While it is clear that various rearrangements of the regulatory region can either help or hinder replication efficiency, the reason for these differences in RRE is not clear.

Our laboratory (64), following the work of Han and Hurley (29) and using similar sequence- and conformation-specific chemical probes that detect DNA in a bent, straight, or unwound conformation, investigated the effect of the GC-rich 21-bp tandem repeats on DNA conformation. The plasmid constructs contained *ori*, T-ag binding site I, three 21-bp repeats, and an AP-1 site on the late gene side of *ori* (pC200); three 21-bp repeats and an AP-1 site on the early gene side of *ori* (p21ds); or no 21-bp repeats (pOri) (Fig. 1). The three 21-bp repeats were bent regardless of their location on the early or late side of *ori*. Because the EP region is an important site of DNA distortion at the initial stages of replication, we

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FIG. 1. RREs of SV40 plasmid constructs with rearranged regulatory regions (34). The RREs were determined by transfecting COS-1 cells with equimolar amounts of the control plasmid (pOri) and the test plasmid (p21ds, pC133, pC163, pC200, or pC139H); replicated DNA was purified and quantified after allowing the plasmids to replicate for 48 h (34). The organization and RRE for each SV40 plasmid construct are indicated. *ori* (shaded arrows) consists of the three functional domains: the EP, PEN, and AT tract; the 21-bp repeats (double filled ovals) each contain two SpI transcription factor-binding sites; the AP-1 transcription factor-binding site (very thick vertical line) is part of the 72-bp transcriptional enhancer; the 69-bp monkey DNA sequence (Host) contains flanking AP-1 sites (thick vertical lines); T-ag binds site I, site II (PEN), and site III (21-bp repeats).

hypothesized that, in constructs like pOri and p21ds with low replicative efficiency, the EP region would not unwind as well as those with high replicative ability. This, however, was not seen in strand-breakage reactions with hedamycin, the chemical probe for unwound DNA. All three constructs, with RREs ranging from barely detectable to 30, showed an increase in unwinding of the EP region when T-ag was present (64).

Since the unwinding of the EP region did not correlate with replication efficiency, we focused our studies on the contribution of auxiliary sequences to conformational changes. Using the chemical probe and the KMnO_4 oxidation assays to detect the *in vitro* DNA distortion that occurs with T-ag, we add further documentation to our earlier work and to the importance of the 21-bp repeats in providing a selective replicative advantage when SV40 constructs are compared in a competitive environment *in vivo*. It is clear that auxiliary sequences affect the location where T-ag induces DNA distortion of the SV40 origin of replication. We propose that the region of structural distortion influences the replication efficiency of SV40 *ori*-containing plasmids by causing the DNA to form a looped structure.

Detection of DNA distortion. To address the hypothesis that the replication efficiency of SV40 *ori*-containing plasmids is influenced by conformational changes upstream and/or downstream of *ori*, we examined T-ag-induced DNA structural distortion of four different SV40 plasmid constructs by the KMnO_4 assay and of two SV40 plasmid constructs by the strand-breakage assay. The plasmids p21ds, pC133, pC163, pC200, pC139H, and pOri (Fig. 1) were previously constructed in our laboratory and range in replication efficiency from 0.2 to

36 (34, 58; M. E. Woodworth, unpublished data). p21ds was examined because of its low RRE. pC133 and pC163 contain one and two copies, respectively, of the three-copy wild-type auxiliary sequence. pC200 contains the SV40 wild-type auxiliary sequences, and pC139H was chosen to determine the sites of T-ag-induced distortion in a highly efficient SV40 DNA replicating molecule where host DNA is substituted for SV40 wild-type auxiliary sequences. pOri was chosen to determine where T-ag distorts DNA in the absence of auxiliary sequences. Distortion was found within the AT tract and the EP for all plasmid constructs (see Table 2), which is consistent with previous studies of the SV40 *ori* (4, 5, 29, 45, 64). Gel mobility shift assays and DNase I footprinting verified that our preparations of T-ag bind to SV40 DNA under our assay conditions (data not shown).

To detect regions of DNA distortion, p21ds, pOri, pC200, and pC139H were each incubated in the absence or presence of T-ag and subjected to chemical modification by KMnO_4 . Potassium permanganate oxidizes the 5,6-double bond of thymine when the DNA is in a single-stranded, bent, or untwisted conformation (5, 52). Oxidation inhibits primer extension by the Klenow fragment of DNA polymerase I, causing the extension products to stop 1 nucleotide upstream of an oxidized thymidine residue. Extension products were separated by gel electrophoresis. Potassium permanganate modification induced by T-ag binding was detected by the appearance of a band or an increase in band intensity. Primers that hybridize upstream or downstream of *ori* were used to detect oxidized thymidines on either DNA strand. The DNA sequences of the oligonucleotide primers and their distances from the AT tract or EP are shown in Table 1. The regulatory region of each construct was sequenced by the dideoxy Sanger method (51), and the sequencing ladders were run next to γ - ^{32}P -labeled primer extension products to identify the sites of DNA distortion. Experiments were done a minimum of three times with each primer, and gels shown are representative (Fig. 2). The data are summarized in Table 2.

Distortion downstream of *ori* in constructs with reduced replication efficiency. In the presence of T-ag, the KMnO_4 assay detected distortion in the three 21-bp repeats (site III) and an AP-1 site downstream of *ori* in p21ds (Fig. 2A; Table 2). Bending within the 21-bp repeats may cause distortion in the AP-1 site. In pC200, the AP-1 site is located upstream of three monomers of T-ag at a bent site III, and the AP-1 site is not a site of distortion. Sequence- and conformation-specific chemical probe data from our lab (64) also showed that the 21-bp repeats in the regulatory region of p21ds are in a bent conformation; however, distortion within the AP-1 site could not be assessed because the probes were not specific for the AP-1 sequence. Distortion was also detected 45 to 60 bp upstream of the AT tract in p21ds (Fig. 2A). The T-ag double hexamer located at site II may be "locked in" between the bent AP-1 and 21-bp repeats on the early transcription side and the bent upstream DNA sequence at 45 to 60 bp from the AT tract on the late transcription side of *ori*. In contrast, although distortion was detected 66 to 70 bp downstream of *ori* on the early transcription side in pOri (Fig. 2B; Table 2), there are no 21-bp repeats to provide bending on the late transcription side of *ori*. The distortion of the region 66 to 70 bp downstream of *ori* is not detected in pC200 even though the downstream sequences

TABLE 1. Nucleotide sequences and annealing locations of oligonucleotide primers

Primer ^a	DNA sequence	Plasmid	Distance (bp) to:	
			AT tract	EP
CLK1	5'-GGCCCTTTCGTCTTCAAGAATTCTC-3'	pOri	60	
		pC200	154	
		pC139H	157	
		p21ds	72	
CLK2	5'-TTCATACACGGTGCCTGACT-3'	pOri	154	
CLK3	5'-TAGGGGTTCGCGCACATTTCCCG-3'	p21ds	187	
CLK4	5'-GCTGTCGGAATGGACGATATCCCGC-3'			
CCLK1	5'-CACGGTGCCTGACTGCGTTAGCAAT-3'	pC139H		98
CCLK2	5'-TTCATACACGGTGCCTGACT-3'	pC200		103
		pC139H		103
CCLK3	5'-GCTGTCGGAATGGACGATATCCCGC-3'	pOri		219
		pC200		219
		pC139H		219
CCLK4	5'-GGCCCTTTCGTCTTCAAGAATTCTC-3'	p21ds		212

^a CLK designates upstream primer; CCLK designates downstream primer.

are identical. The data suggest that the distortion is dependent on the presence and location of the 21-bp repeats and support a parenthetical statement by DePamphilis (18) that deletion of three 21-bp repeats shifts the beginning of continuous DNA synthesis to the early side of *ori* about 40 bp downstream of the EP. The distortion approximately 45 to 60 bp upstream of the

AT tract on the late transcription side of *ori* in p21ds (Fig. 2A) may be due to the sequential binding of T-ag to sites I and II and further upstream. Our DNase I footprinting assay shows that sequences upstream of *ori* in p21ds are protected from DNase I digestion (data not shown). The sequence- and conformation-specific probes used in the chemical probe assay

FIG. 2. KMnO₄ modification pattern showing conformational changes within p21ds (A), pOri (B), and pC139H (C). Plasmid DNA (0.5 μg) was incubated in the absence (–) and presence (+) of 1 μg of T-ag at 37°C for 60 min. Samples were subjected to KMnO₄ oxidation and primer extension with CCLK4 (A), CLK1 (B), and CCLK2 (C) γ-³²P-labeled primers. A schematic of the regulatory region is adjacent to the autoradiogram of elongation products and dideoxy sequence (51) ladders (A and G). Regulatory elements are as indicated in Fig. 1. Arrows indicate sites of conformational change.

TABLE 2. Summary of potassium permanganate and chemical probe data

Plasmid	No. of 21-bp repeats	Location and/or type of T-ag-induced DNA distortion				
		Site I	EP	AT	21-bp	AP-1
p21ds	3		Yes ^a	Yes ^a	Yes ^a	Yes ^a
pOri	0		Yes ^a	Yes ^a		
pC133	1	Not bent ^b	Unwound ^c	Bent/straight ^{b,d}	Not bent ^b Less unwound ^c	Yes ^a
pC163	2	Not bent ^b	Unwound ^c	Bent/straight ^{b,d}	Not bent ^b	
pC200	3		Yes ^a	Yes ^a	Yes ^a	
pC139H	1		Yes ^a	Yes ^a		

^a Detected by KMnO₄ assay.^b Detected by adozelesin assay.^c Detected by hedamycin assay.^d Detected by bizelesin assay.

were not specific for the upstream sequence in p21ds or the downstream sequence in pOri, so distortion in these regions was not previously detected (64).

Distortion upstream of *ori* in constructs with increased replication efficiency. A bent, untwisted, or single-stranded conformation induced by T-ag in the SV40 wild-type auxiliary sequence (three 21-bp repeats and an AP-1 site) upstream of *ori* in pC200 was detected by the KMnO₄ assay (Table 2). The overall structural features of the multimeric complex between transcription factor Sp1 and T-ag binding to the 21-bp repeat region have been examined using different experimental techniques (29, 56). The looping structure may facilitate T-ag-induced DNA distortion of the EP and AT tract within the SV40 *ori*, and the fact that this DNA-protein complex forms upstream, rather than downstream, of *ori* may be an important factor in explaining why plasmid pC200 replicates 30-fold more efficiently than does pOri.

Auxiliary sequences different from those in wild-type SV40 result in new sites of T-ag-induced DNA distortion upstream of *ori*. KMnO₄ oxidation analysis of pC139H detected T-ag-induced DNA distortion at two sites within a cellular DNA sequence upstream of *ori* (Fig. 2C; Table 2). pC139H contains the SV40 *ori*, T-ag site I, one 21-bp repeat containing two transcription factor-binding sites for Sp1, and a 69-bp cellular sequence that has a transcription factor-binding site for AP-1 at each end. Gel mobility shift experiments with an SV40 DNA fragment containing one AP-1-binding site and no sequence motifs specific to T-ag showed nonspecific T-ag–DNA complex formation (data not shown). To explain the DNA distortion in pC139H, we propose that the binding of T-ag molecules to host cell DNA leads to a trimer complex that bends DNA into an upstream looping structure. Formation of a DNA structural loop could stabilize the SV40 origin of replication as a replication bubble and facilitate more efficient access by the replication machinery to the DNA.

Lack of distortion upstream of *ori* in constructs with intermediate levels of replication efficiency. To detect DNA in a bent, straight, or single-stranded conformation, the 210-bp regulatory region of p133 and the 240-bp regulatory region of p163 were each incubated in replication buffer containing 4 mM ATP in the presence and absence of T-ag and subjected to modification with adozelesin, bizelesin, or hedamycin. Adozelesin alkylates DNA in a bent conformation (48) containing the sequences 5'-(A/T)(A/T)A*-3' and 5'-(A/T)(G/C)(A/T)

A*-3' (32, 62), where the asterisk indicates the covalently modified adenine. Bizelesin forms interstrand links between adenines 6 or 7 bp apart on regions of straight DNA (41) with the target sequences 5'-TAAAAA*-3'/3'-A*TTTTT-5' and 5'-TAATTA*-3'/3'-A*TTAAT-5' (21, 32, 55), where the asterisk indicates the covalently modified adenine. Hedamycin intercalates DNA and alkylates the N-7 position of guanines in all unwound sequences of DNA (57); treatment with heat and piperidine causes strand breakage 1 nucleotide before the alkylated base. A γ -³²P-labeled 591-bp fragment was added before phenol extraction and ethanol precipitation as a control for DNA recovery. Fragments were separated by electrophoresis and visualized by autoradiography. Band intensities were quantified by image analysis (NIH Image 1.61; National Institutes of Health; <http://rsb.info.nih.gov/nih-image>). All experiments were done a minimum of three times for each chemical probe and each construct. Representative gels are shown in Fig. 3; data are summarized in Table 2.

Conformational changes in the regulatory regions of pC133 and pC163. In the absence of T-ag, the AT tract of pC133 was alkylated by adozelesin, a chemical probe that modifies bent DNA (Fig. 3A, lanes 3 to 6). Alkylation of the AT tract by adozelesin was decreased when T-ag was added (Fig. 3A, lanes 7 to 10), indicating that some of the molecules found in a bent conformation without T-ag straightened when T-ag was added. In the absence of T-ag, site I was alkylated by adozelesin (Fig. 3A, lanes 3 to 6); however, T-ag binding site I was not detected in a bent conformation after T-ag addition (Fig. 3A, lanes 7 to 10). Presumably, binding of T-ag trimers stabilizes the naturally bent site I sequence into a straight conformation. No bending occurred in the 21-bp repeat region of pC133 either with or without T-ag (Fig. 3A, lanes 3 to 10). Similar results were obtained when the regulatory region of pC163 was probed with adozelesin in the presence and absence of T-ag (data are summarized in Table 2). This lack of bending is dramatically different from the conformational changes seen by some of us (64) and by others (29) when the regulatory region contained three copies of the 21-bp repeat (pC200): the 21-bp-repeat region is bent in the absence of T-ag, and bending increases after the addition of T-ag.

The only sequence-specific site for bizelesin in the regulatory regions of pC133 and pC163 is the AT-rich region. Chemical modification of this AT tract by bizelesin, which targets straight DNA, was unchanged when T-ag was added (Fig. 3B,

FIG. 3. Conformational changes in the regulatory regions of pC133 (A to C) and pC163 (D). Approximately 0.1 ng of ^{32}P -labeled fragment was probed with adozelesin, the chemical probe for bent DNA (A); bizelesin, the chemical probe for straight DNA (B); or hedamycin, the chemical probe for unwound DNA (C and D). Reactions were done in the absence (lanes 3 to 6) or presence (lanes 7 to 10) of 1 μg of T-ag. Reaction times for adozelesin and bizelesin were 0, 5, 10, and 30 min; reaction times for hedamycin were 0, 10, 30, and 60 min. Lanes 1 and 2 contain Maxam-Gilbert (40) A>C and G reactions to provide landmarks within the regulatory region of pC133 or pC163. To the left of each autoradiogram is a diagram showing the regulatory region with the binding sites for adozelesin or bizelesin indicated. Regulatory elements are as indicated in Fig. 1. Symbols: $\diamond$, adozelesin; $\blacklozenge$, bizelesin.

lanes 3 to 10), showing that molecules detected in a straight conformation without T-ag remained in that conformation. Similar results were obtained with pC163 (summarized in Table 2). Because adozelesin detects bent DNA and bizelesin detects straight DNA, it was clear that the AT tract could be either bent or straight without T-ag. However, when T-ag was present the alkylation patterns of the AT tract demonstrated that fewer molecules were detected in a bent conformation than when no T-ag was present.

The inability of the region to bend could hinder oligomerization events. One T-ag monomer at the 21-bp repeat in pC133 or two T-ag monomers at the pair of 21-bp sequences in pC163 were not sufficient to bend the DNA. Without bending of the 21-bp region and corresponding protein-protein interaction, the regulatory regions of pC133 and pC163 are apparently less able to stimulate initiation events.

When hedamycin was used as a probe, there was some alkylation of guanines throughout the regulatory region in the absence of T-ag (Fig. 3C, lanes 3 to 6). It should be noted that, due to a lack of guanines, the AT-rich region could not be monitored. After the addition of T-ag, alkylation of guanines in the PEN region was decreased (Fig. 3C, lanes 7 to 10). It is reasonable that T-ag reduces unwinding of the PEN region because it is the site of T-ag double hexamer formation and is consistent with previous models for regulatory regions that contain three copies of the 21-bp repeat (29, 64). Hedamycin alkylation could be inhibited by this large protein-DNA complex. The EP region in both pC133 (Fig. 3C) and pC163 (Fig.

3D) showed increased reactivity with hedamycin after the addition of T-ag.

T-ag had no effect on the unwinding of the two 21-bp repeats in pC163 or the three 21-bp repeats in pC200 (64) but appeared to inhibit unwinding in the single 21-bp repeat of pC133. Reactions with hedamycin showed that alkylation of guanines in the single 21-bp repeat in pC133 was inhibited by the addition of T-ag; there is an overall decrease in band intensity across the 21-bp region from lanes 3 to 6 to lanes 7 to 10 (Fig. 3C). Conversely, hedamycin alkylation of the double 21-bp repeat region of pC163 showed that T-ag had no significant effect on the number of molecules with already unwound 21-bp repeats (Fig. 3D). T-ag also had no significant effect on the amount of alkylation within the triple 21-bp repeat in pC200 (64). The conformation of the 21-bp repeat region of pC133 is distinct from that of pC163 and from the changes that some of us (64) and others (29) observed in pC200, pointing to the importance of bending and unwinding of this region.

Gutierrez et al. (28) found that the 21-bp repeat region facilitates unwinding of the regulatory region. While their work did not investigate deletions of individual copies of the 21-bp repeat, their findings still support the importance of the 21-bp repeat region in unwinding the regulatory region. One possible explanation for the decreased RRE of pC133, compared to that of pC163, is the reduced ability of the 21-bp repeat to unwind.

The unwinding of the EP region, presumably a vital step in the initiation process, was observed in both pC133 and pC163,

FIG. 4. Model for conformation of regulatory regions upstream and downstream of *ori*. In panel A DNA looping on both sides of *ori* inhibits replication to a greater extent than in panel B, where DNA looping occurs only on the downstream side of *ori*. In panel C lack of DNA looping is associated with an intermediate level of replication efficiency that is greater than that seen in panel A or B but less than that seen in panel D, where DNA looping occurs upstream of *ori* and facilitates uninhibited, initial fork movement in the downstream direction.

constructs with RREs that are intermediate between those of pOri and pC200. The data confirm that the ease of unwinding of the EP region does not correlate with differences in replication efficiency or with differences in the number of 21-bp repeats.

KMnO₄ and chemical probe assays as tools for determining conformational changes within DNA. A notable difference between the KMnO₄ assay and the chemical probe assay is that the former detects distortion within a whole plasmid whereas the latter detects distortion within a fragment of DNA. Due to the large number of sequence-specific sites in a genome that would be recognized in the chemical probe assay, distortion was assessed in a fragment of DNA containing the regulatory region. The KMnO₄ assay is advantageous in that the plasmid more closely resembles an *in vivo* system of replication. It is apparent that both methods detect T-ag-induced DNA distortion within similar regions of the SV40 regulatory region of various constructs, including the EP, the AT tract, and the 21-bp repeats. Additional areas of distortion were detected within the AP-1 site and within additional sequences both upstream and downstream of *ori* with the KMnO₄ assay. Because the chemical probes were not specific for these regions, distortion could not be assessed. Both systems have their advantages, provide complementary data, and taken together provide important insights into the relationship between DNA conformation and replication efficiency.

DNA looping. The importance of DNA bending and unwinding is not limited to replication events. Conformational changes play a crucial role in regulating the initiation of both replication and transcription. Eukaryotic transcription is mediated by DNA bending and interactions between proteins bound to DNA at distant sites (8, 39, 49), resulting in formation of a DNA loop encircling over 20 interacting transcriptional proteins (9, 42, 47). Transcriptional events proceed from this large, activated complex (9, 42). Both *cis*-acting regulatory sequences near *ori* and proteins acting *in trans* appear to have an important function in controlling replication (4, 5, 14, 29, 45, 50, 61). Bent DNA may facilitate interaction between *cis*-DNA elements and *trans*-acting factors by organizing local chromatin infrastructure (44). Our data are consistent with the reported role that chromatin structure and nucleosome destabilization play in DNA replication (1, 19). Just as DNA bending must occur for an activated transcriptional complex, it appears that DNA bending at the triplet of 21-bp repeats results in a more efficient replication complex. When only one or two of these repeat sequences are present, bending does not occur and replication efficiency is weakened.

We believe that DNA looping plays a major role in the initiation of DNA replication (Fig. 4). The bending of the 21-bp repeats in pC200 may allow T-ag bound at site III to interact with T-ag located at PEN within *ori*. Additional interactions between T-ag and other cellular replication machinery would be facilitated by the DNA loop, and the initiation of DNA replication would be enhanced. In contrast, the proposed DNA looping that occurs in p21ds would not enhance the initiation of DNA replication because the DNA is bent on both sides of *ori*, providing an unfavorable conformation for bidirectional movement of the T-ag hexamers located at PEN.

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