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The T-Antigen-Binding Domain of the Simian Virus 40 Core Origin of Replication

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The simian virus 40 origin of replication contains a 27-base-pair palindrome with the sequence 5′-CA-GAGGC-C-GAGGC-G-GCCTC-G-GCCTC-TG-3′. The four 5′-GAGGC-3′/5′-GCCTC-3′ pentanucleotides are known contact sites for simian virus 40 T-antigen binding in vitro. We used oligonucleotide-directed cassette mutagenesis to identify features of this palindrome that are important for the initiation of DNA replication in vivo. Each base pair of a pentanucleotide is crucial for DNA replication. In contrast, sequences adjacent to pentanucleotides have little or no effect on replication. Thus, the pentanucleotide is the basic functional unit, not only for T-antigen binding but also for DNA replication. All four pentanucleotides are indispensable in the initiation process. The spacing of pentanucleotides is crucial because duplication of the single base pair between binding sites has a far greater effect on replication than does substitution of the same base pair. Inversion of any pentanucleotide blocks DNA synthesis. Thus, the pentanucleotide is not a functionally symmetrical unit. We propose that each pentanucleotide positions a monomer of T antigen at the proper distance, rotation, and orientation relative to other T-antigen monomers and to other origin domains and that such positioning leads to subsequent events in replication.

The initiation of DNA replication at a unique origin requires recognition signals for the binding of one or more replication proteins. Site-specific binding, in turn, organizes the proteins for site-specific unwinding of duplex DNA for subsequent synthetic events (2, 9, 22). In some cases, these steps in the initiation of replication are accomplished by a specialized complex of different proteins (9). In the case of tumor virus simian virus 40 (SV40), however, a single initiator protein recognizes and unwinds origin DNA (4a, 8, 28). Hence, the interaction of SV40 T antigen with origin DNA provides a simple system for the characterization of a general mechanism that is of fundamental importance in growth regulation.

The replication origin of SV40 consists of multiple elements (1, 7, 17, 25, 26). Ancillary regions at both ends of the origin increase the efficiency of DNA replication but are not absolutely essential for basal functioning. These regions are part of the SV40 early promoter and operator DNAs (29); possible mechanisms for their action in replication include the regulation of transcription or the maintenance of an open chromatin structure (3, 10, 14, 16). Unlike these ancillary elements, a 64-base-pair (bp) core origin of replication cannot be deleted without loss of basal functioning (5). We have investigated the domain structure within the core origin by using single-base-pair mutagenesis. For operational purposes, we define a functional domain as a contiguous set of sequences in which base substitutions cause a significant decrease in replication. In contrast to functional domains, spacer sequences tolerate base substitutions without a drastic effect on function but do not tolerate insertions or deletions. We have previously identified and mapped two domains that extend from the outer limits of the core origin toward a central T-antigen-binding palindrome from which they are separated by apparent spacer sequences (5, 6).

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In the present study, we undertook an extensive genetic analysis of the T-antigen-binding central domain to define features critical for the initiation of DNA replication. These features determine the precise arrangement of bound T antigen (8, 28) and its subsequent function in the T-antigen-induced unwinding of origin DNA (4a) to allow primer synthesis (13). The T-antigen-binding central palindrome consists of a cluster of four repeated 5′-GAGGC-3′/5′-GCCTC-3′ pentanucleotides separated from each other by a single base pair. The pentanucleotides are arranged as two pairs of direct repetitions that are inverted relative to each other. We have proposed that each repeat serves as a recognition and contact site for a monomer of T antigen in vitro (8, 20). Oligonucleotide-directed mutagenesis of the core origin allowed us to manipulate the pentanucleotides at will. We found that the correct sequence, orientation, spacing, and rotation of all four pentanucleotides are essential for DNA replication. We discuss possible interactions of the T-antigen-binding domain with other functional domains of the core origin.

MATERIALS AND METHODS

Construction of the wild-type plasmid. The wild-type (WT) plasmid pOR1 has been described previously (7). It consists of pML2 (19) sequence 653 to 4361 with a *Hin*dIII linker attached to the 653 position and a polylinker (*Nco*I, *Sal*I, *Bam*HI, *Xma*I, and *Eco*RI) attached to the 4361 position. SV40 sequences from the *Hin*dIII site at 5171 through the *Nco*I site at 39 are inserted between the corresponding sites of the plasmid. This SV40 segment has an internal deletion of T-antigen-binding region I from nucleotides 5178 through 5208 to produce a small core origin capable of autonomous replication.

Oligonucleotide-directed mutagenesis. WT and mutant origins were synthesized as previously described (6, 12). The

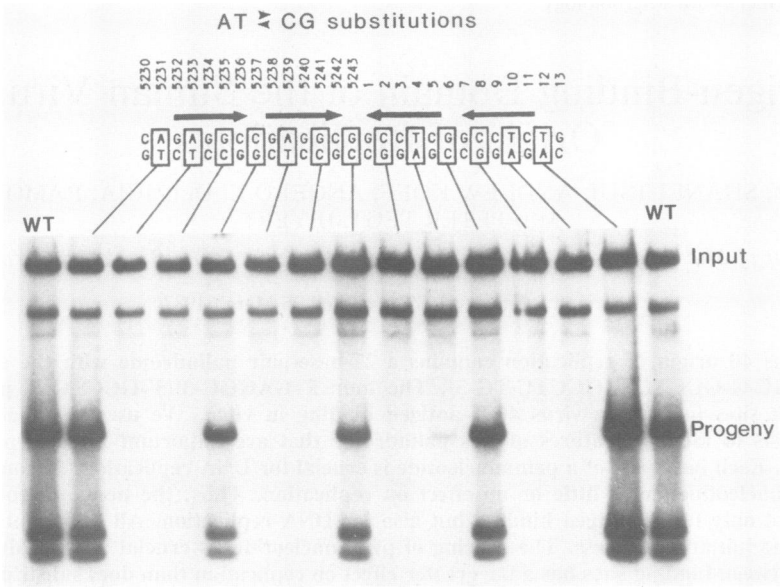


FIG. 1. Mutational mapping of the T-antigen-binding domain. The sequence of the 27-bp central palindrome of the SV40 core origin of replication is shown at the top of the figure. The numbers indicate nucleotide positions in the SV40 genome (29). Single exchanges of A · T and C · G base pairs were synthesized at every other position in the palindrome (shown by boxes). The autoradiogram shows the replication efficiency of WT and mutant plasmids in COS-1 cells in a representative experiment. Input and progeny DNAs were distinguished by their *Mbo*I sensitivities. The data are quantitated and summarized in Fig. 6.

region of the core origin between the *Hin*dIII and *Nco*I sites was reconstructed with 12 overlapping oligonucleotides that had been phosphorylated in 70 mM Tris hydrochloride (pH 7.5)–10 mM $MgCl_2$–5 mM dithiothreitol–1 mM ATP with T4 polynucleotidyl kinase. The oligonucleotides (0.5 pmol) were annealed in 9 μl of the same buffer for 1 h at 37°C and then for 1 h at 23°C to produce a double-stranded segment with *Hin*dIII and *Nco*I protruding ends. The annealed oligonucleotides were ligated to each other and to 0.1 pmol of the large, dephosphorylated, *Hin*dIII-*Nco*I fragment of plasmid pOR1 by the addition of 400 U of T4 DNA ligase (New England BioLabs, Inc.) in the same buffer and incubation overnight at 12°C. The transformation of HB101 cells and the preparation of cloned plasmid DNA have been described previously (7). The construction of directed mutants required the synthesis of only two additional oligonucleotides with complementary base changes for ligation with 10 of the 12 WT oligonucleotides. All mutations were verified by dideoxy sequencing.

DNA replication. The assay for DNA replication in COS-1 cells has been described previously (7). To ensure equivalence in transfection efficiencies with each plasmid preparation, we quantitated the input DNAs by densitometry of form I DNA separated in agarose gels and stained with ethidium bromide.

RESULTS

Identification and mapping of functional sequences within the T-antigen-binding domain. Gluzman et al. (11) and Shortle and Nathans (23) showed that deletions or base substitutions within the 27-bp palindrome inhibit DNA replication to variable extents. However, the number of mutants examined was insufficient to establish the substructure of this T-antigen-binding domain. We first screened the entire region by exchanging A · T and C · G base pairs at every other position in the palindrome (Fig. 1). This pattern of mutations allowed an inspection of the equivalent second and fourth positions within each of the four pentanucleotide repeats and of every position adjacent to a pentanucleotide. The A · T-to-C · G exchanges were chosen because these substitutions would be expected to have the greatest effects on potential interactions with proteins (21). We tested the replication efficiency of the mutant origins in COS-1 cells, which constitutively express T antigen and support the replication of WT core origins. After 72 h of incubation at 36°C, DNA was extracted and digested with *Mbo*I to distinguish methylated input DNA from unmethylated progeny DNA. DNAs were analyzed by gel electrophoresis, blotting, and hybridization with radiolabeled pBR322 DNA. The residual input DNA, shown in the autoradiograms, served as an internal standard for the amount of plasmid DNA added to the cells. We used *Mbo*I progeny fragments to quantitate the efficiency of replication.

Our results revealed a distinct four-subdomain arrangement. All mutations within pentanucleotides drastically reduced replication, whereas all mutations outside pentanucleotides had lesser effects. Mutations at either end of the pentanucleotide cluster allowed replication at WT levels. Mutations between pentanucleotides reduced replication to approximately one-half of WT levels. Thus, the same pentanucleotide sequence that is the simplest sequence common to known T-antigen-binding sites (8, 30) also is a functional subdomain in the central origin palindrome. Sequences between the subdomains may contribute to T-antigen binding and DNA replication also, but to a much lesser extent. The lesser role of these intervening sequences is probably related to T-antigen binding rather than to cruciform formation because the central base pair at position 5243 would have no effect on the inverted symmetry of the palindrome. If cruciform structures are involved in replica-

tion, the single base substitutions of nucleotides 5231, 5237, 6, and 12 must have little effect on their structures in vivo.

To determine whether every base pair in a pentanucleotide is important in replication, we made single transversions of each base pair in the first pentanucleotide (Fig. 2A). All mutations reduced replication more than 10-fold. Mutations in the first two positions of the 5′-GAGGC-3′ sequence allowed low but detectable levels of replication. This functional hierarchy of bases within the pentanucleotide for replication is similar to the hierarchy that we previously identified for the binding of T antigen to pentanucleotides that occur in nonorigin DNA (Fig. 2B). We found that T antigen is bound to pentanucleotides with alternative bases at positions 1, 2, and 5 but not at positions 3 and 4 (8, 30).

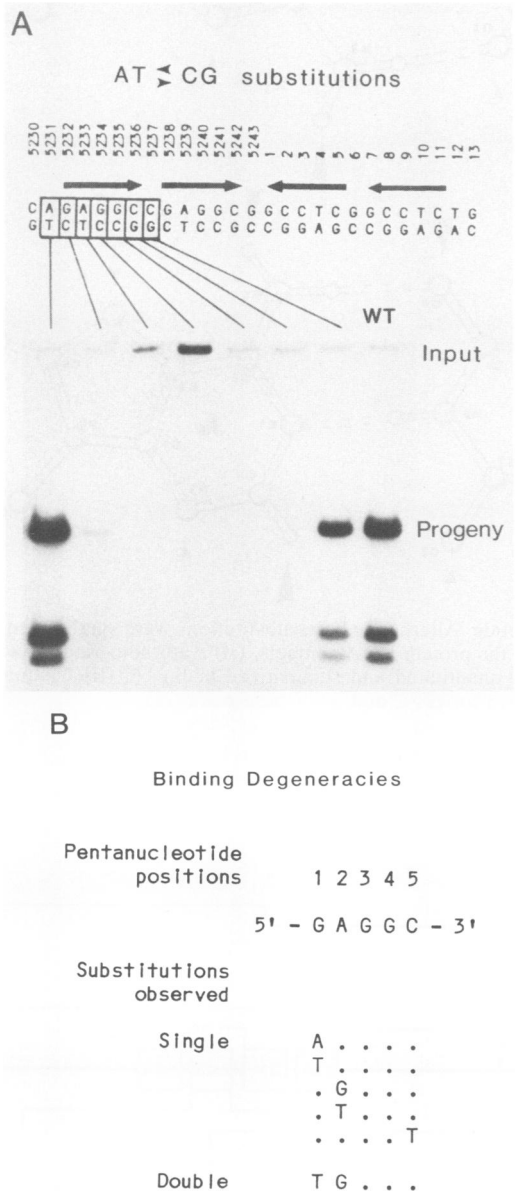


FIG. 2. Fine mapping of a single pentanucleotide site. To determine the precise structure and limits of subdomains within the T-antigen-binding palindrome, we made A · T and C · G exchanges at the seven consecutive positions indicated by the boxes in the sequence. (A) Autoradiogram showing the replication efficiency of mutants in a representative experiment. The data are quantitated and summarized in Fig. 6. (B) Alternative recognition sequences for T-antigen binding taken from previous studies (8, 30).

To investigate further the functional importance of alternative bases at the first and fourth positions in a 5′-GAGGC-3′ pentanucleotide, we made additional substitutions (Fig. 3A). The G · C-to-T · A mutation at the first position reduced replication 10- to 20-fold. In contrast, the G · C-to-A · T substitution at the same location failed to reduce replication. This comparison suggests that, in the first position of the 5′-GAGGC-3′ sequence, T antigen makes contacts with molecular sites common to G · C and A · T base pairs and does not depend on contacts with sites unique to the G · C base pair. For example, Fig. 3B shows potential binding sites for amino acids on G · C and A · T base pairs. T antigen could bind the equivalent N7 atoms of either the guanine or adenine bases and is unlikely to depend on bonds to sites unique to the G · C base pair (shown by the large arrowheads). The results were quite different at the fourth position, where all three possible base substitutions reduced replication more than 100-fold. Thus, at least one essential contact site with T antigen must be unique to the G · C base pair. This kind of genetic analysis will be of importance in determining the fidelity of cocrystals of T antigen and origin DNA when they become available.

Importance of spacing within the T-antigen-binding domain. The above studies of single-base-pair mutations have identified four pentanucleotide subdomains in the 27-bp central palindrome of the core origin. We wished to investigate possible functional interactions between these protein-binding sites by changing their spatial relationships. Duplication of the single base pair between these subdomains allowed the smallest possible change in the distance and rotation among the pentanucleotides (Fig. 4A). The duplication maintained the proper sequence adjacent to each pentanucleotide, whereas other insertions or deletions would not. Each of the three insertions drastically reduced replication. Thus, the position of each protein recognition site relative to the other three sites is crucial to the replication function. The same result has been shown for a duplication of the central base pair in the palindrome in the complete viral origin rather than in the core origin (4). Figure 4B shows a proposed arrangement of four monomers of T antigen bound to each of the four pentanucleotides of the core origin (27). The close proximity of the recognition sites would lead to precise positional relationships among bound T-antigen monomers; single-base-pair insertions would interfere with these interactions in three dimensions.

Importance of the orientation of pentanucleotides in the T-antigen-binding domain. The pentanucleotide is a partially symmetrical sequence. Inversion of the sequence would create three base substitutions at the center of each pentanucleotide. Our results with single-base-pair substitutions suggested that inversion of a pentanucleotide in the core origin would severely inhibit replication. However, on the basis of a comparison of T-antigen binding to different arrangements of pentanucleotides in origin regions I and II, Jones and Tjian have suggested that a single species of T antigen may interact with the guanine clusters of a recognition sequence in either orientation (15). Thus, it was important to test rigorously the importance of pentanucleotide polarity by using a sensitive in vivo functional assay. Figure 5A shows that inversion of any one of the four pentanucleotides abolished replication completely. We concluded that this effect was not caused by loss of the inverted symmetry

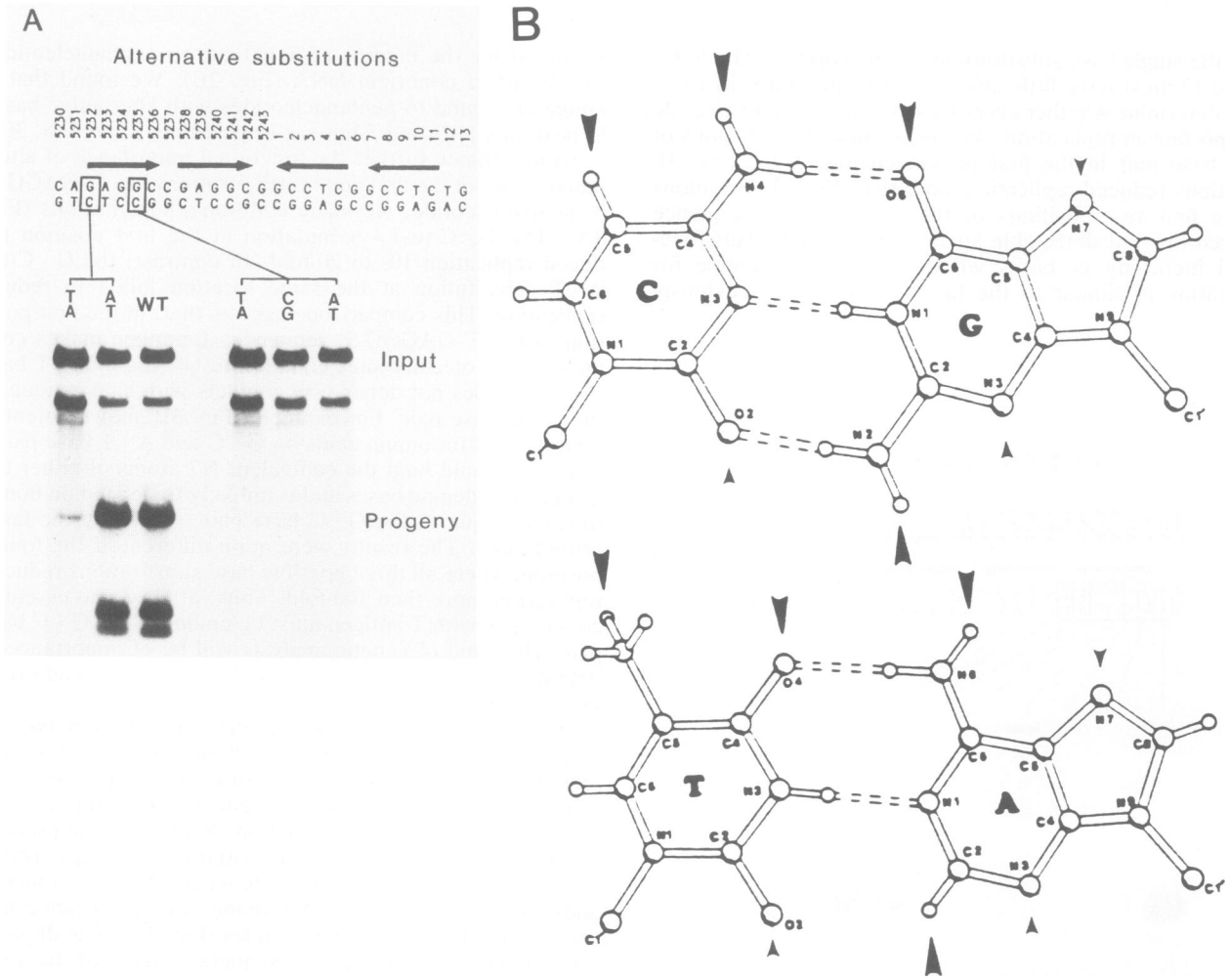
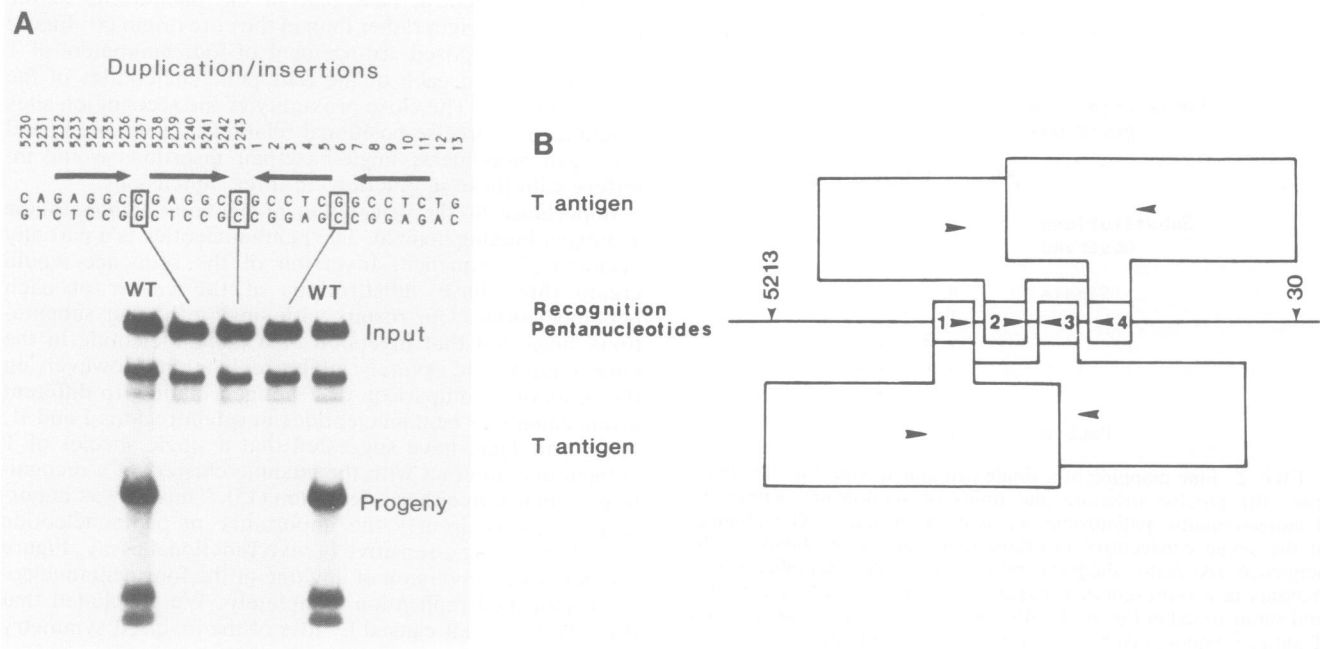


FIG. 3. Sequence specificity for the first and fourth positions in a pentanucleotide. Alternative base substitutions were synthesized at two positions (shown by boxes) in the first pentanucleotide to examine the nature of the protein-DNA contacts. (A) Autoradiogram showing the replication efficiency of mutants in a representative experiment. The data are quantitated and summarized in Fig. 6. (B) Unique (large arrowheads) and common (small arrowheads) binding sites for amino acids shown for G · C and A · T base pairs (21).



of the entire 27-bp palindrome, because the simultaneous inversion of the first and last pentanucleotides also destroyed origin function completely (data not shown). Thus, a pentanucleotide is not functionally symmetrical in DNA replication. Figure 5B shows a cylindrical projection of the first and third pentanucleotides in the core origin. Only the positions of the guanines, known to contact T antigen by dimethyl sulfate protection studies (8), are shown. Inversion of the first pentanucleotide would change the positions of two of the four guanines (shown by open circles). This inverted arrangement could have a number of different effects, including the following: failure to bind T antigen, inversion of one monomer of bound T antigen, or distortion of bound amino acids projecting into the major groove. Any of these alterations could block a subsequent step in DNA replication.

DISCUSSION

We have shown that the central 27-bp palindrome of the SV40 core origin of replication can be divided into four distinct 5′-GAGGC-3′ subdomains that are essential for the initiation of DNA replication. Furthermore, each of the 5 bp in the first pentanucleotide repeat is important for the replication function. This organizational pattern is entirely consistent with the results of previous studies of T-antigen binding to origin and nonorigin DNAs. Footprinting analysis with dimethyl sulfate indicated that all binding sites contain one or more pentanucleotides (8, 30). Furthermore, both replication and binding studies revealed the same hierarchy of sequence importance at various positions within the 5′-GAGGC-3′ repeat. This striking correlation between in vivo and in vitro assays strongly argues that the pentanucleotide is a primary component common to both replication and T-antigen binding. Our finding that all five positions in the pentanucleotide are important for DNA replication indicates that a number of amino acids interact with each pentanucleotide.

Our present results do not support the need for a cruciform structure formed by the central palindrome in the process of replication. Our mutations would introduce pyrimidine-pyrimidine or purine-purine base pairs into the stem of the potential cruciform DNA. These mismatches cause maximal disruption within known cruciform structures (18). Two of the mutations at nucleotide positions 5231 and 12 have no effect on DNA replication, even though these substitutions shorten the stem of a putative cruciform by 2 bp or more. Two other mutations, those at positions 5237 and 6, interrupt the stem of a potential cruciform near its center. These mutations decrease DNA replication by approximately 50%. The mutation at position 5243 also decreases DNA replication to one-half of WT efficiency. This mutation does not affect the stem structure. Thus, these latter three mutations probably have a weak effect on T-antigen binding or subsequent events rather than on the formation of a DNA cruciform.

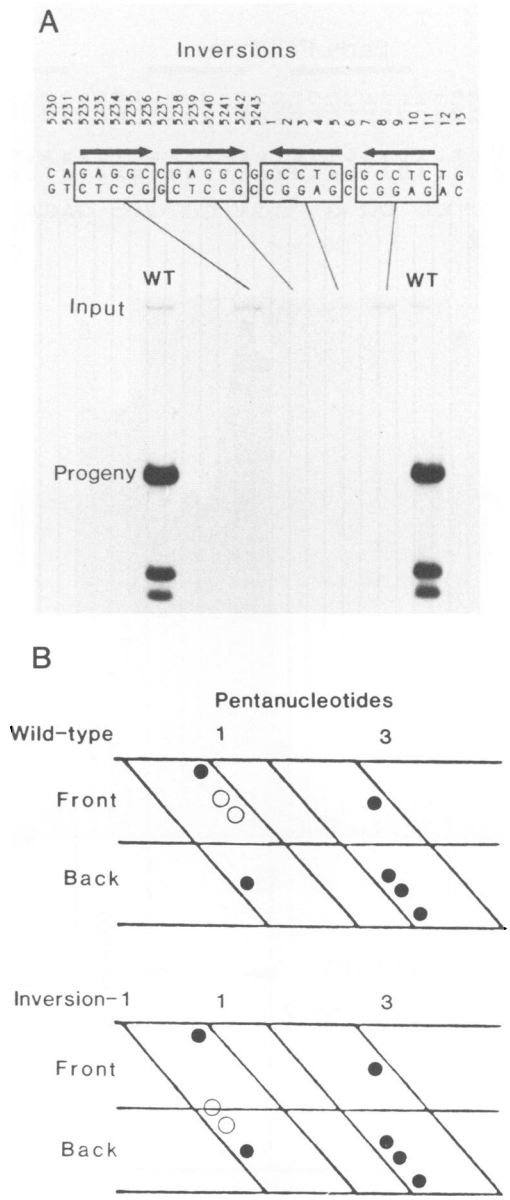


FIG. 5. Importance of pentanucleotide orientation. We synthesized core origins with inversions of single pentanucleotides (shown by boxes). (A) Autoradiogram showing the replication efficiency of WT and mutant plasmids in a representative experiment. Prolonged exposure of the autoradiogram failed to reveal any replication signal. (B) Cylindrical projection showing the guanine contact sites (open and closed circles) for T-antigen binding to the first and third pentanucleotides. The first projection has a WT sequence; the second has an inversion of the first pentanucleotide.

FIG. 4. Positional constraints within the T-antigen-binding palindrome. Each of the single base pairs between pentanucleotides was duplicated to create a single-base-pair insertion while maintaining the sequence integrity immediately adjacent to each pentanucleotide. (A) Autoradiogram showing the replication efficiency of mutants in a representative experiment. (B) Model for the binding of T antigen to the core origin taken from Tegtmeyer et al. (27). The four pentanucleotides, numbered in an early-to-late direction, are shown as small boxes on the DNA, and their orientations are shown by arrowheads. The large rectangles represent monomers of T antigen bound to each pentanucleotide. Their lengths approximate the extent to which they interfere with DNase I in a protection assay (27, 30) and their physical lengths determined by scanning transmission electron microscopy (20). The orientation of each pentanucleotide determines the orientation of the bound subunit of T antigen.

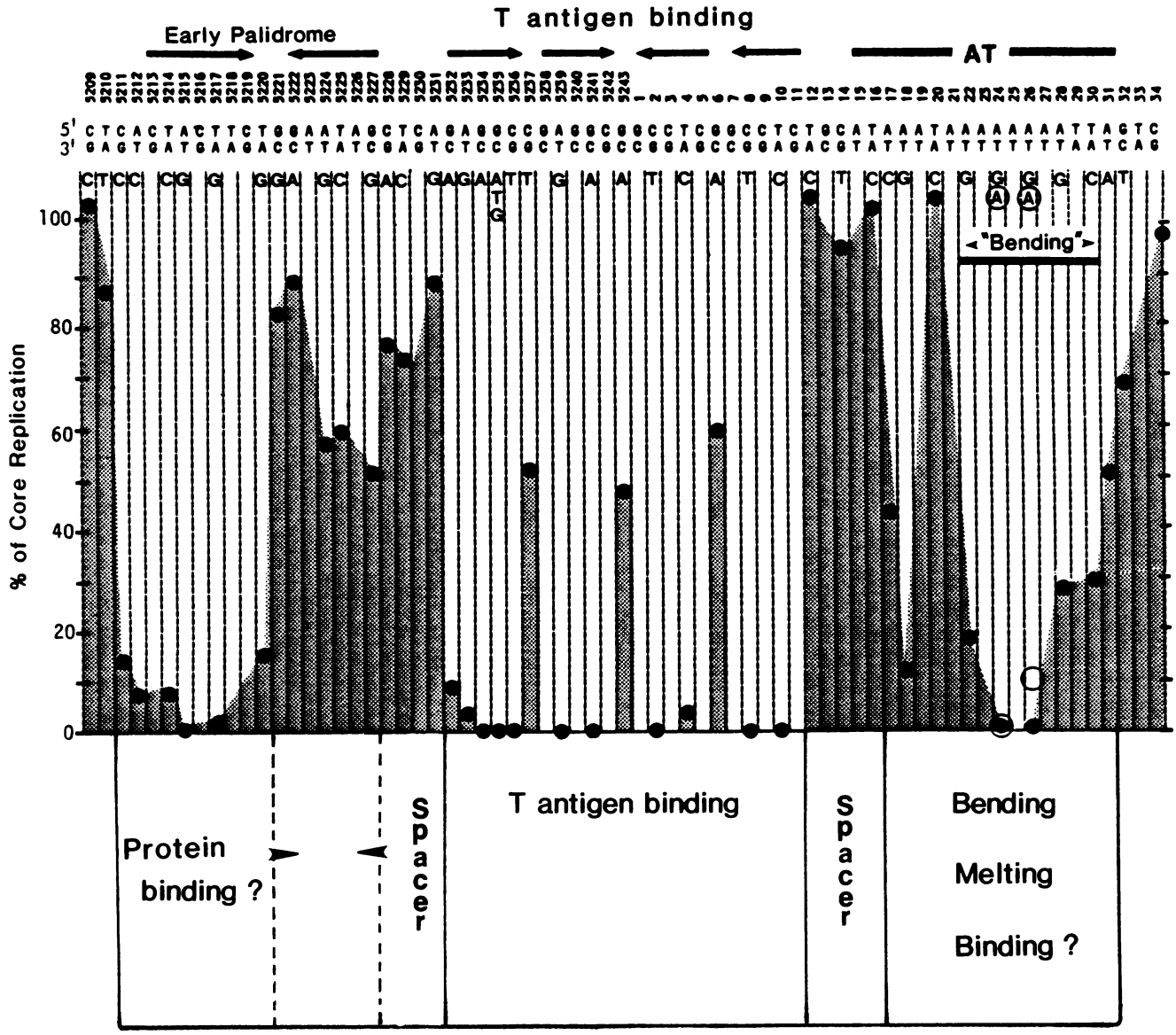


FIG. 6. Domain structure of the SV40 core origin of replication. The core origin consists of nucleotides 5211 through 31. Structural features of the DNA are shown at the top of the figure. Single base substitutions corresponding to the lower strand of SV40 DNA are indicated below the WT sequence. The histogram shows the mean replication efficiency of these mutants determined in three independent experiments. The open circles at nucleotides 24 and 26 indicate the replication levels of adenine-to-thymine substitutions. Data corresponding to the early and late ends of the core origin were taken from our previous studies (5, 6).

We propose that the primary role of the sequences within the inverted repetitions that contain the pentanucleotides is to bind T antigen and to position it in the proper location and orientation for a subsequent functional activity. The drastic effect of minimal changes in spacing between pentanucleotides or of inversion of pentanucleotides strongly supports this idea. Mastrangelo et al. (20) showed that the core origin binds four monomer equivalents of T antigen. Previous studies showed that T antigen binds to the core DNA in a stepwise fashion (27). Thus, the four pentanucleotides within the palindrome of the core origin may assemble monomers or dimers in the tightly packed, symmetrical arrangement shown in Fig. 4B. The spacing between the centers of the binding sites would place alternating monomers of T antigen on opposite faces of the DNA. Such an arrangement may allow T antigen to separate double-stranded DNA in and around its contact sites. Dean et al. (4a) showed recently that purified T antigen unwinds plasmid DNA in the presence of single-stranded binding protein and topoisomerase only if the plasmid contains a functional origin. Presumably, melting begins within origin sequences, but the initial melting site remains to be determined. This origin-specific unwinding activity may be related to a less specific helicase function of T antigen that is important in the subsequent propagation of DNA replication (24).

The correct binding arrangement and orientation of T antigen would promote interactions with other proteins bound to adjacent functional domains of the core origin. Figure 6 summarizes the domain structure of the origin that has been determined in our present and previous studies (5,

6). Three segments of contiguous or nearly contiguous DNA encode sequence-specific core functions. These are separated by spacer DNAs with relaxed sequence requirements but with precise positional constraints (5). The early functional domain extends 10 bp from positions 5211 through 5220. It corresponds to one arm of an imperfect inverted repetition. We do not know the function of this segment, but it may be a recognition site or part of a recognition site for a cellular protein. This sequence-specific domain is followed by an 11-bp segment from positions 5221 through 5231. This segment serves a spacer function (5) but also has a minor sequence-specific domain that corresponds to the second arm of the early imperfect palindrome. Perhaps it is the second and weaker binding site for a protein dimer that interacts with the early palindrome. The central domain consists of four T-antigen-binding pentanucleotides which play a key role in T-antigen binding and DNA replication. The central domain is followed by a 5-bp apparent spacer segment without a demonstrable sequence requirement. A late sequence-specific. A · T-rich, bipartite domain extends from nucleotides 17 through 31. The subdomain from nucleotides 20 through 31 corresponds exactly to a locus that determines DNA bending and may be a structural signal for protein binding or DNA melting (6). Thus, the origin consists of three or more sequence-specific functional domains that are interaction sites for proteins, structural signals, or both. The orientation of the domains and the spacers between them would position these functional regions at the proper distances, rotations, and directions to allow coordinated interactions in the initiation of DNA replication.

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