JOURNAL OF VIROLOGY, May 1997, p. 3972–3985 Vol. 71, No. 5
0022-538X/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Purification of the Simian Virus 40 (SV40) T Antigen DNA-Binding Domain and Characterization of Its Interactions with the SV40 Origin

WOO S. JOO, XUELIAN LUO, DEBORAH DENIS, HENRY Y. KIM, GODFREY J. RAINEY, CLYDE JONES, K. R. SREEKUMAR, AND PETER A. BULLOCK*

Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts 02111

Received 7 May 1996/Accepted 18 February 1997

To better define protein-DNA interactions at a eukaryotic origin, the domain of simian virus 40 (SV40) large T antigen that specifically interacts with the SV40 origin has been purified and its binding to DNA has been characterized. Evidence is presented that the affinity of the purified T antigen DNA-binding domain for the SV40 origin is comparable to that of the full-length T antigen. Furthermore, stable binding of the T antigen DNA-binding domain to the SV40 origin requires pairs of pentanucleotide recognition sites separated by approximately one turn of a DNA double helix and positioned in a head-to-head orientation. Although two pairs of pentanucleotides are present in the SV40 origin, footprinting and band shift experiments indicate that binding is limited to dimer formation on a single pair of pentanucleotides. Finally, it is demonstrated that the T antigen DNA-binding domain interacts poorly with single-stranded DNA.

Initiation of DNA replication is a complicated process that requires very precise protein-DNA interactions at replication origins (1, 26). The replication of simian virus 40 (SV40) DNA has served as a useful model system for studying initiation events in mammalian cells (for reviews, see references 11, 34, and 80). SV40 has a well-defined origin of replication (see reference 18 and references cited therein) and encodes an 82-kDa protein, termed T antigen (T-ag), that is required for initiation of DNA synthesis (82). SV40 large T-ag binds the viral origin owing to major-groove interactions with GAGGC pentanucleotide recognition sites (21, 35, 84). What is known about SV40 large T-ag and its interactions with the SV40 origin has been reviewed previously (5, 27, 28). It has been observed that upon binding to the SV40 origin, T-ag oligomerizes to form a double hexamer around the origin (14, 16, 46). Assembly of T-ag hexamers around the SV40 origin induces conformational changes (7, 17, 57) that enable subsequent DNA unwinding events to occur (9, 15, 24, 89). DNA unwinding is catalyzed by the inherent helicase activity of T-ag (30, 78).

Analyses of T-ag, including partial proteolysis (67, 71, 72, 91), expression of subfragments of T-ag as recombinant proteins (2, 81), and genetic studies (2, 60, 81), have demonstrated that T-ag is composed of several structural domains (for a review, see reference 28). The best-defined and most extensively studied domain of T-ag is the origin DNA-binding domain (T-ag-bd) (2, 49, 60, 71, 81), also termed the T-ag-obd (44). Currently, there is some uncertainty regarding the exact boundaries of the T-ag-bd; it has been mapped to amino acids 131 to 259 (2), 132 to 246 (49), and 147 to approximately 247 (42). Nevertheless, peptides from all three size classes are known to be sufficient for sequence-specific binding to the SV40 origin region (2, 33, 49, 72).

In addition to sequence-specific binding, mutation studies have demonstrated that the T-ag-bd is involved in many additional activities catalyzed by T-ag. These activities include protein oligomerization (74), binding to single-stranded DNA (49, 75), interactions with cellular factors (31), and DNA unwinding and helicase activities (74, 75, 92). In light of this complexity, it is not surprising that the T-ag-bd is itself organized into subdomains (73). The solution structure of the T-ag-bd region between amino acids 131 and 260 (T-ag-bd$_{131–260}$) has provided preliminary insights into these processes (44).

Previous studies of the T-ag-bd have, in general, been conducted with peptides isolated via immunoprecipitation techniques and not with purified protein preparations. Moreover, certain properties of the T-ag-bd, such as the mechanism by which it interacts with the SV40 origin and single-stranded DNA, have been poorly characterized. Therefore, to provide further insights into the biochemistry of the T-ag-bd, we have overexpressed T-ag-bd$_{131–260}$ in *Escherichia coli*. T-ag-bd$_{131–260}$ has been purified to apparent homogeneity and used to examine its interaction with both double- and single-stranded DNA; the results of these studies are presented herein.

MATERIALS AND METHODS

Commercial supplies of enzymes and oligonucleotides. Restriction endonucleases *Bam*HI and *Eco*RI were purchased from Boehringer Mannheim Biochemicals. Both T4 polynucleotide kinase and *Hae*III-digested ϕx174 RF DNA were from Gibco-BRL. Thrombin was obtained from Haematologic Technologies Inc.

Oligonucleotides were synthesized on an Applied Biosystems 394 DNA synthesizer at the protein chemistry facility at Tufts University, purified by electrophoresis through 10% urea–polyacrylamide gels, and subsequently isolated as described previously (65).

Purification of T-ag. SV40 T-ag was prepared by using a baculovirus expression vector containing the T-ag-encoding SV40 A gene (56) and isolated by immunoaffinity techniques using the PAb 419 monoclonal antibody as previously described (23, 70, 88). Purified T-ag was dialyzed against T-ag storage buffer (20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], 0.1 mM phenylmethylsulfonyl fluoride, 0.2 μg of leupeptin per ml, 0.2 μg of antipain per ml, and 10% glycerol) and frozen at −70°C until use.

Cloning DNA fragments containing T-ag-bd$_{131–260}$ and T-ag-bd$_{112–260}$ into pGEX1λT. (i) pGEX-T-ag-bd$_{131–260}$. The region extending between SV40 nucleotides 4427 and 4041 (encoding T-ag amino acids 131 to 259 [85]) were amplified with pSVLD as the template; this plasmid contains full-length SV40 DNA cloned into the *Bam*HI site of pBR322 (51). Plasmid pSVLD was incubated with oligonucleotide 1 (5′tc**ggatcc**AAGGTAGAAGACCCCAAGGA3′) and oligonucleotide 2 (5′cc**gaattc**TGGATTAAAATCATGCTCCT3′) in a PCR (53). (The boldfaced capital A in oligonucleotide 1 is position 4427, and the boldfaced capital T in oligonucleotide 2 is position 4041). Note that oligonucleotide 1 also contained a *Bam*HI site (in lowercase boldfaced letters) and that oligonucleotide

* Corresponding author. Mailing address: Department of Biochemistry A703, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Phone: (617) 636-6874. Fax: (617) 636-6409. E-mail: PBULLOCK@OPAL.TUFTS.EDU.

2 contained an *Eco*RI site (also in lowercase boldfaced letters). Thus, upon digestion of the PCR product with *Bam*HI and *Eco*RI, DNA coding for T-ag-$bd_{131–259}$ could be readily ligated into *Bam*HI and *Eco*RI-digested pGEX-1λT (76). The first three nucleotides of the *Eco*RI site, GAA, encodes a glutamic acid residue. This is the normal residue at position 260 of T-ag; thus, upon ligation, the expression vector encoded T-ag amino acids 131 to 260. Moreover, during the construction of this molecule, a single nucleotide was spontaneously deleted in the codon following position 260; this resulted in a frameshift mutation that introduced a stop codon in what would have been codon 263.

(ii) pGEX-T-ag-$bd_{112–260}$. The SV40 fragment containing nucleotides 4481 to 4041 (encoding T-ag amino acids 113 to 259) was amplified with pSVLD as the template. This plasmid was combined with oligonucleotide 3 (5′c**ggatcc**GATG ATGAGGCTACTGCTGACTCT3′) and the previously described oligonucleotide 2 (5′cc**gaattc**TGGATTAAAATCATGCTCCT3′) in a PCR (53). (The boldfaced capital G in oligonucleotide 3 is position 4481, and the boldfaced capital T in oligonucleotide 2 is position 4041). Additional cloning procedures were performed as described above. However, in this vector, the normal pGEX-1λT stop codon, located six codons beyond position 260, is utilized. Furthermore, in purified T-ag-$bd_{112–260}$, serine 112 is derived from the vector (see below).

After transformation into *E. coli* BL21 cells, the expression vectors pGEX-T-ag-$bd_{131–260}$ and pGEX-T-ag-$bd_{112–260}$ were isolated by standard methods (65). Dideoxy sequencing reactions (66) were used to confirm the sequences of the constructs. Sequencing reactions (~90,000 cpm of ^{35}S-dATP/lane) were loaded on 8% polyacrylamide gels containing 8 M urea; the gels were electrophoresed and processed as described previously (10).

Expression and purification of T-ag-$bd_{131–260}$ and T-ag-$bd_{112–260}$. T-ag-$bd_{131–260}$ was purified according to the method described by Smith and Johnson (76), adapted by Pharmacia and modified herein. Cultures of *E. coli* BL21 (200 ml) transformed with pGEX-T-ag-$bd_{131–260}$ were grown overnight at 37°C in Luria-Bertani medium containing ampicillin (50 μg/ml). Cultures were subsequently diluted 1:10 into 2 liters of Luria-Bertani plus ampicillin and grown to an optical density at 600 nm of 0.8. Fusion protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM; cells were grown for an additional ~6 h following IPTG addition. Bacteria were harvested by centrifugation and suspended in phosphate-buffered saline (PBS) (15 ml/liter of cells) containing 1 mM DTT and stored overnight at −70°C. The cells were thawed, and lysozyme (Sigma), EDTA, phenylmethylsulfonyl fluoride, leupeptin, antipain, and EGTA were added to final concentrations of 1 mg/ml, 1 mM, 1 mM, 0.3 μg/ml, 0.3 μg/ml, and 75 μM, respectively. The cells were incubated at 4°C for 10 min; Nonidet P-40 and $MgCl_2$ were then added to 1% and 7 mM, respectively. The cells were sonicated (eight times, for 30 s each) in a Branson Sonifier (output 7, continuous pulse) and subsequently centrifuged at 30,000 × *g* for 45 min in a Sorvall JA-20 rotor. To remove nucleic acids, polyethyleneimine (average molecular weight, 750,000); Aldrich Chemical Company, Inc.) was added to the supernatant to a final concentration of 0.2% and the solution was mixed by repeated inversion. After a 5-min incubation at 4°C, the sample was centrifuged at 30,000 × *g* for 30 min in a Sorvall JA-20 rotor. Two milliliters of a 50% slurry of glutathione-Sepharose 4B beads (Pharmacia) was then added to the polyethyleneimine-treated supernatant (~30 ml), and the solution was incubated for 30 min at 4°C on a Clay Adams Nutator. The fusion protein-Sepharose bead complex was purified by centrifugation (1,000 × *g* for 7 min) in a Beckman GS-6R centrifuge. The pellet was washed twice with wash buffer A (PBS [10 ml] supplemented with 1% Nonidet P-40, 1 mM DTT, and 7 mM $MgCl_2$) and twice with 10 ml of wash buffer B (PBS containing 1 mM DTT and 7 mM $MgCl_2$). After the second wash, ~2 ml of wash buffer B was left in the tube containing the glutathione-Sepharose 4B beads complexed to the glutathione *S*-transferase (GST)–T-ag-$bd_{131–260}$ fusion protein. The sample was then incubated with thrombin (~50 μg) overnight at 4°C on a Clay Adams Nutator. The sample was centrifuged (1,000 × *g*) for 10 min in a microcentrifuge at 4°C; the T-ag-$bd_{131–260}$-containing supernatant was subsequently passed over a Sephacryl S-100 sizing column (Pharmacia) equilibrated in column buffer (10 mM KPO_4 [pH 7.2], 100 mM KCl, 2 mM DTT, and 7 mM $MgCl_2$). Fractions containing T-ag-$bd_{131–260}$ were identified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, pooled, and dialyzed against T-ag storage buffer (see above). This procedure resulted in ~10 mg of purified T-ag-$bd_{131–260}$ per liter of culture; protein concentrations were determined by the Bradford reagent (Bio-Rad), using bovine serum albumin as the standard. The T-ag-$bd_{131–260}$ preparation appeared homogeneous; a single band migrating at the position expected for T-ag-$bd_{131–260}$ (15.4 kDa) was the only species present in an SDS-polyacrylamide gel stained with Coomassie blue (data not presented). Finally, upon cleavage with thrombin, two additional amino acids, derived from the vector (Gly and Ser), are present at the amino terminus and two additional amino acids (Ser and Ser) are present at the carboxy terminus. Thus, the purified T-ag-$bd_{131–260}$ contains 134 amino acids.

T-ag-$bd_{112–260}$ (17.8 kDa) was overexpressed and purified by the procedure developed to isolate T-ag-$bd_{131–260}$. As a result of ligating the PCR fragment into the pGEX-1λT vector, five extra amino acids (Phe, Ile, Val, Thr, and Asp) are present at the carboxy terminus of T-ag-$bd_{112–260}$. Upon cleavage of the glutathione–T-ag-$bd_{112–260}$ fusion protein with thrombin, two additional amino acids, derived from the vector (Gly and Ser), are present at the amino terminus. Thus, the purified T-ag-$bd_{112–260}$ contains 155 amino acids.

Band shift assays. Double-stranded oligonucleotides were formed by incubating complementary pairs of oligonucleotides in hybridization buffer as described previously (38). Both single-stranded and double-stranded oligonucleotides were labeled by standard kinase procedures (65). ^{32}P-labeled oligonucleotides were electrophoresed in neutral 10% polyacrylamide gels, and the desired DNA fragments were removed; DNA was eluted in oligonucleotide extraction buffer (65). Following ethanol precipitation and an 80% ethanol wash, the labeled oligonucleotides were resuspended in deionized H_2O (25 fmol/μl).

Band shift reactions (16, 45, 54), employing both the single-stranded and double-stranded oligonucleotides, were conducted under conditions that are essentially replication conditions (88). However, to minimize secondary-structure formation, the single-stranded oligonucleotides were heated to ~95°C for 4 min prior to use. The reaction mixtures (20 μl) contained 7 mM $MgCl_2$, 0.5 mM DTT, 4 mM ATP, 40 mM creatine phosphate (pH 7.6), 0.48 μg of creatine phosphate kinase, 5 μg of bovine serum albumin, 0.8 μg of *Hae*III-digested φx174 RF DNA (~2.5 pmol; used as a nonspecific competitor), ~50 fmol of either single-stranded or annealed double-stranded labeled oligonucleotide, and various amounts of either T-ag or T-ag-$bd_{131–260}$ (T-ag or T-ag-bd was the last component added to the reaction). In general, after a 20-min incubation at 37°C, glutaraldehyde (0.1% final concentration) was added and the reaction mixtures were further incubated for 5 min. Finally, the reactions were stopped by the addition of 5 μl of 6× loading dye II (15% Ficoll, 0.25% bromophenol blue, and 0.25% xylene cyanol) (65) to the reaction mixtures. Samples were then applied to 3.5 to 12% gradient polyacrylamide gels and electrophoresed in 0.5× Tris-borate-EDTA (TBE) for 2 h (~500 V, 20 mA, 10 W). The gels were dried, subjected to autoradiography, and subsequently placed in a PhosphorImager cassette. Quantitation of the band shifts was performed with a Molecular Dynamics PhosphorImager.

Band shift assays with mixtures of T-ag-$bd_{131–260}$ and T-ag-$bd_{112–260}$ (6 pmol total; mixed at ratios of 4:0, 3:1, 2:2, 1:3, and 0:4) were conducted as described above. However, the resolution between the band-shifted species was improved by employing 12% polyacrylamide gels (19:1 acrylamide-to-bisacrylamide ratio) that were run in 0.5× TBE for 8.5 h (~520 V, ~15 mA, 8 W).

1,10-Phenanthroline–copper footprinting. To prepare asymmetrically labeled substrates for footprinting, single-stranded oligonucleotides were labeled at their 5′ termini by standard methods (65). After the reactions were stopped, the kinase-labeled, single-stranded fragments were hybridized with their complementary strands. The asymmetrically labeled duplex fragments were subsequently isolated from 10% urea–polyacrylamide gels and purified as described previously (65). To provide adequate counts for footprinting, the previously described gel mobility shift assay was scaled up fivefold. Footprinting reactions were carried out as described by Kuwabara and Sigman (39). Briefly, after the samples were loaded on a 6% polyacrylamide gel and electrophoresed in 0.5× TBE for ~1.75 h (~370 V, ~28 mA, 10 W), the gel was rinsed in 50 mM Tris-HCl, pH 8.0. DNA cleavage reactions were performed by soaking the gel in 1,10-phenanthroline (0.17 mM), $CuSO_4$ (38 μM), and 3-mercaptopropionic acid (5 mM) for 20 min at room temperature. The reaction was quenched by adding 2,9-dimethyl-1,10-phenanthroline (final concentration, 2.2 mM) for ~2 min. The gel was rinsed in H_2O, wrapped in plastic wrap, and subjected to autoradiography for 1 h at room temperature. Acrylamide gel slices, containing either free DNA or protein-DNA complexes, were excised and eluted overnight at 37°C in 550 μl of elution buffer (0.3 M sodium acetate [pH 5.2], 0.2% SDS, 10 mM magnesium acetate, 10 μg of proteinase K per ml). After removal of acrylamide gel fragments via centrifugation, DNA-containing solutions were subjected to extractions with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1); DNA fragments were then ethanol precipitated. The DNA pellet was washed with 70% ethanol, dried, and resuspended at ~10,000 cpm/μl in a solution consisting of 80% (vol/vol) formamide, 10 mM NaOH, 1 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. The samples (3 μl) were boiled for 3 min, applied to a 16% polyacrylamide gel, and electrophoresed in 1× TBE at 55 W. Sequencing markers were obtained by conducting Maxam and Gilbert (48) G and G+A reactions on the appropriate asymmetrically labeled duplex DNA fragment.

RESULTS

The SV40 origin region is diagrammed in Fig. 1A; this region contains two strong binding sites for T-ag (sites I and II). Site I stimulates DNA replication both in vivo (see reference 22 for a review) and in vitro (41, 79); however, this site is not required for DNA replication (21, 55). The main physiological function of site I appears to be the autoregulation, via T-ag binding, of early-gene transcription (see reference 28 for a review). The sequences of T-ag binding site I and the SV40 core origin are presented in Fig. 1B; these sequences were included in the oligonucleotide designated 83-bp ori$^+$. The core origin consists of three functional domains: (i) the AT-rich domain, (ii) a palindrome with four GAGGC recognition

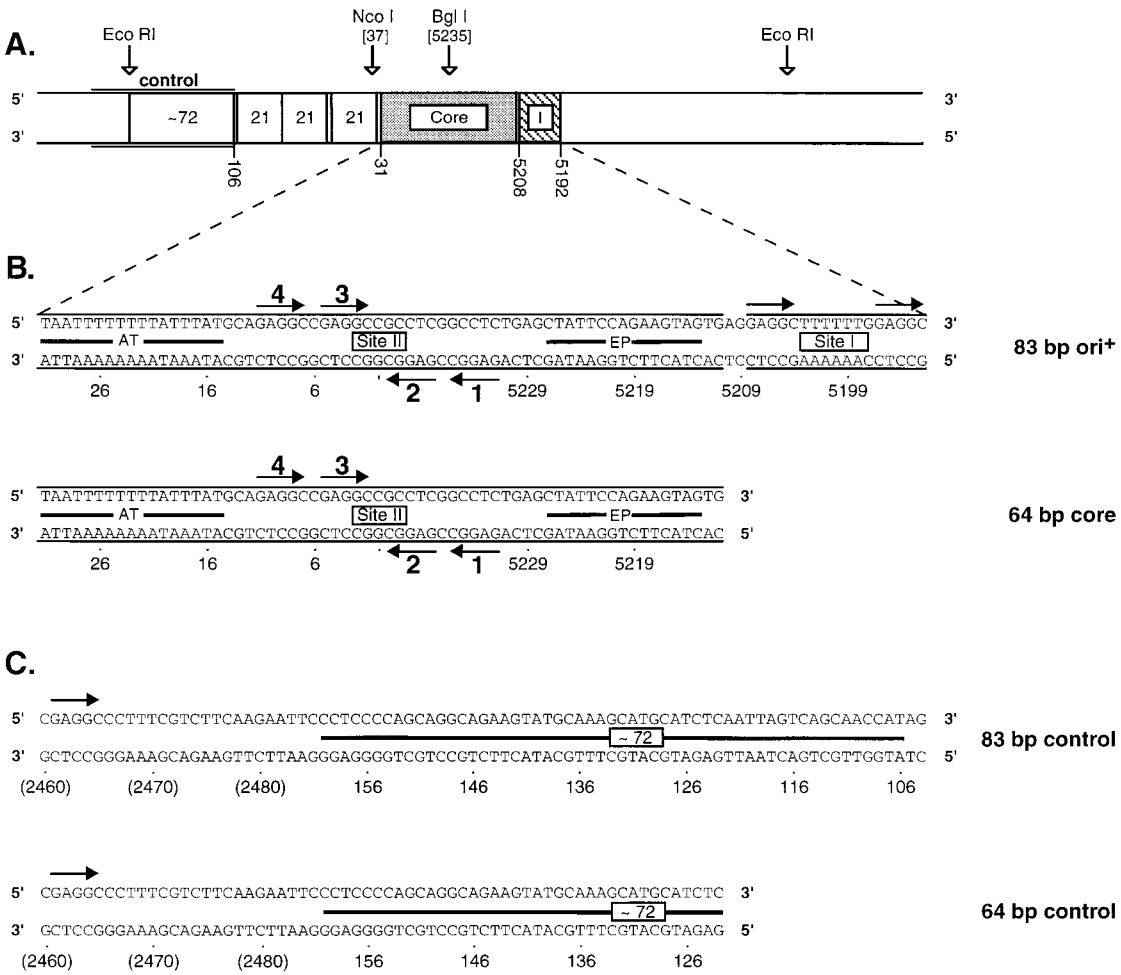

FIG. 1. A map of the SV40 origin region and the oligonucleotide sequences used in this study (the map is based on sequences in pSV01ΔEP, a plasmid used to study SV40 replication 88). (A) The core origin, T-ag binding site I, the 21-bp repeats, and a partial copy of one of the 72-bp enhancer elements are indicated. Also indicated are the template positions from which the control oligonucleotides were derived (control; see below). SV40 nucleotides are numbered as described by Fiers (85). (B) Sequences comprising both the core origin and site I, and exclusively the core origin, are presented (85). These sequences were included in two oligonucleotides termed the 83-bp ori$^+$ and the 64-bp core oligonucleotides. The arrows depict the (GAGGC) pentanucleotide recognition sequences for T-ag; the pentanucleotides were numbered as previously described (40). The locations of the adenine- and thymine-rich region (AT), site II, and the early palindrome regions (EP) are also depicted. (C) This figure presents the sequences of two oligonucleotides, termed the 83-bp control and 64-bp control oligonucleotides, that were used as controls in the band shift assays. The bars depict DNA derived from the SV40 enhancer. The positions of the single pentanucleotide T-ag binding sites are indicated by arrows. Finally, the numbers in parentheses are non-SV40 sequences and are labeled according to the pSV01ΔEP numbering system (88).

pentanucleotides (site II), and (iii) an imperfect inverted repeat (18, 57). Site II is the central region of the core origin; the core origin is both necessary and sufficient for replication (see references 18, 41, and 79 and references cited therein). Figure 1B also presents a second oligonucleotide, designated 64-bp core, that contains sequences from just the core origin. Sequences from the region of DNA that served as a control in the initial studies were included in the oligonucleotides designated 83-bp control and 64-bp control (Fig. 1C). The control oligonucleotides have single GAGGC T-ag recognition sites (Fig. 1C).

Either in the presence or absence of DNA, SV40 T-ag is known to oligomerize in solution (see reference 14 and references cited therein). The domains of T-ag responsible for oligomerization are not yet fully defined. Therefore, we tested whether the purified T-ag-bd$_{131–260}$ (see Materials and Methods) oligomerizes in solution in the absence of DNA. The line widths from one-dimensional nuclear magnetic resonance experiments indicated that T-ag-bd$_{131–260}$ is a monomer even at very high protein concentrations (~50 mg/ml) (43). Additional analytical ultracentrifugation experiments, conducted with purified preparations of T-ag-bd$_{131–260}$, also indicated that this protein is a monomer in solution (25). Thus, while T-ag-bd$_{131–260}$ may be necessary for oligomerization (74), it does not appear to be sufficient.

Interactions of T-ag and T-ag-bd$_{131–260}$ with duplex DNA from the SV40 origin. Gel mobility shift assays were used to test the ability of the purified T-ag-bd$_{131–260}$ to distinguish between origin- and non-origin-containing double-stranded DNA (45, 54). Approximately 50 fmol of kinase-labeled DNA, containing either the 83-bp ori$^+$ or 83-bp control oligonucleotide, were incubated with T-ag or T-ag-bd$_{131–260}$ as described in Materials and Methods. (The sequence of the 83-bp ori$^+$ oligonucleotide is shown in Fig. 1B, and the sequence of the 83-bp control oligonucleotide is presented in Fig. 1C). The samples were cross-linked with glutaraldehyde and loaded on gradient polyacrylamide gels. Inspection of Fig. 2 (lanes 2 to 4) reveals that incubation of 1.5 to 6 pmol of T-ag with the 83-bp

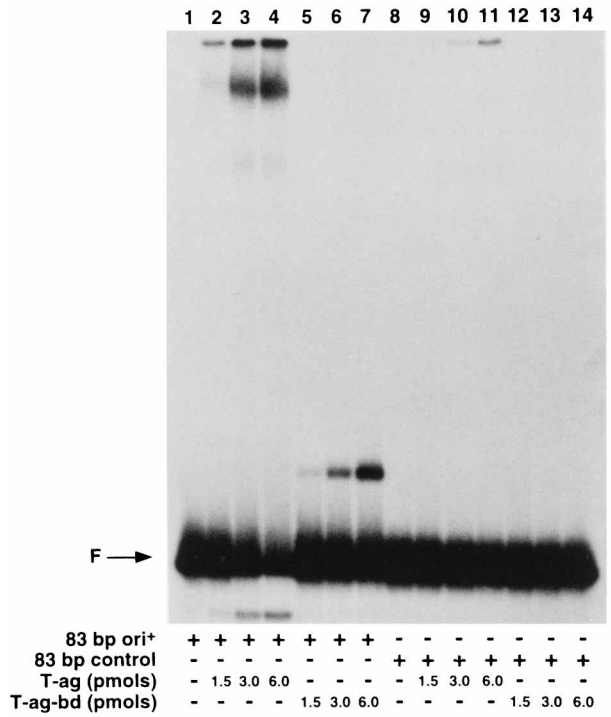

FIG. 2. Gel mobility shift assay used to compare the relative affinities of T-ag and T-ag-bd$_{131–260}$ for the SV40 wild-type origin. Lanes 1 and 8, control band shift assays conducted with the 83-bp ori$^+$ and the 83-bp control oligonucleotides, respectively, in the absence of protein; lanes 2 to 4, band shift assays conducted with the 83-bp ori$^+$ oligonucleotide and increasing concentrations of T-ag (1.5 to 6 pmol); lanes 5 to 7, band shift assays conducted with the 83-bp ori$^+$ oligonucleotide and increasing concentrations of T-ag-bd$_{131–260}$ (1.5 to 6 pmol); lanes 9 to 11, band shift assays conducted with the 83-bp control oligonucleotide and increasing concentrations of T-ag (1.5 to 6 pmol); lanes 12 to 14, band shift assays conducted with the 83-bp control oligonucleotide and increasing concentrations of T-ag-bd$_{131–260}$ (1.5 to 6 pmol). The input or free duplex DNA (F) is indicated by the arrow. Single-stranded DNA, formed as a consequence of T-ag's helicase activity (78), is visible at the bottom of this and subsequent gels. The protein-to-oligonucleotide ratios with 1.5, 3, and 6 pmol of T-ag (or T-ag-bd$_{131–260}$) and 50 fmol of oligonucleotide are 30:1, 60:1, and 120:1, respectively. Finally, formation of the T-ag-bd$_{131–260}$-dependent band shift species required glutaraldehyde but did not require ATP or an ATP regenerating system (data not presented); this is the expected result, given that T-ag-bd$_{131–260}$ lacks the ATP-binding domain present in T-ag (8).

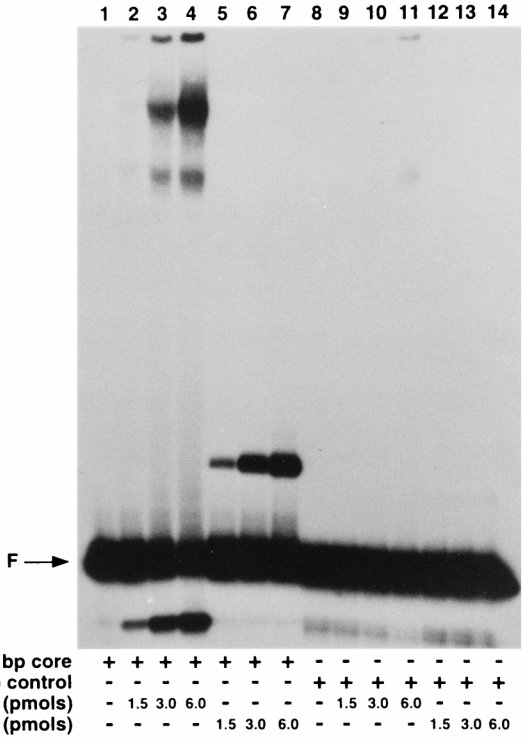

FIG. 3. Gel mobility shift assay used to compare the relative affinities of T-ag and T-ag-bd$_{131–260}$ for the SV40 core origin. Lanes 1 and 8, control band shift assay conducted with the 64-bp core and 64-bp control oligonucleotides, respectively, in the absence of protein; lanes 2 to 4, band shift assays conducted with the 64-bp core oligonucleotide and increasing concentrations of T-ag (1.5 to 6 pmol); lanes 5 to 7, band shift assays conducted with the 64-bp core oligonucleotide and increasing concentrations of T-ag-bd$_{131–260}$ (1.5 to 6 pmol); lanes 9 to 11, band shift assays conducted with the 64-bp control oligonucleotide and increasing concentrations of T-ag (1.5 to 6 pmol); lanes 12 to 14, band shift assays conducted with the 64-bp control oligonucleotide and increasing concentrations of T-ag-bd$_{131–260}$ (1.5 to 6 pmol). The input or free DNA (F) is indicated by the arrow.

ori$^+$ oligonucleotide resulted in two distinct species (the lower species is more easily observed in subsequent figures [see Fig. 3 and 7]). These two species were previously characterized (14, 59, 87); the faster-migrating species consists of a single hexamer bound to the SV40 origin, while the more slowly migrating species contains a double hexamer. In contrast to results with the 83-bp ori$^+$ oligonucleotide, T-ag failed to significantly shift the 83-bp control oligonucleotide (lanes 9 to 11). Analogous results were obtained in band shift reactions conducted with the purified T-ag-bd$_{131–260}$. When incubated with the 83-bp ori$^+$ oligonucleotide, T-ag-bd$_{131–260}$ (1.5 to 6 pmol) induced the formation of one very distinct band shift species (lanes 5 to 7). However, this species was not observed when T-ag-bd$_{131–260}$ was incubated with the 83-bp control oligonucleotide (lanes 12 to 14). These studies demonstrate that as with T-ag, T-ag-bd$_{131–260}$ binds preferentially to the SV40 origin of replication.

Both in vivo and in vitro, replication of SV40 DNA depends upon the specific binding of T-ag to site II within the SV40 core origin (55, 69, 79). Binding of T-ag to site II is known to be regulated by ATP (6, 16, 20). However, assays based on immunoaffinity techniques have indicated that certain T-ag-bd-containing polypeptides purified from *E. coli* have difficulty recognizing either site I or site II (33, 49, 81). Therefore, it was of interest to determine whether the purified T-ag-bd$_{131–260}$ interacted with isolated site II and site I and whether the interaction with these sites was altered relative to the wild-type SV40 origin.

The interaction of the purified T-ag-bd$_{131–260}$ with an oligonucleotide containing the SV40 core origin is presented in Fig. 3. (The sequences of the oligonucleotides designated 64-bp core and 64-bp control are presented in Fig. 1B and C, respectively.) As in the experiment shown in Fig. 2, T-ag (1.5 to 6 pmol) served as a positive control for binding to both the 64-bp core (lanes 2 to 4) and 64-bp control (lanes 9 to 11) oligonucleotides. It is apparent that although T-ag bound to the 64-bp core oligonucleotide, it failed to significantly shift the 64-bp control oligonucleotide. Analogous results were obtained in band shift reactions conducted with T-ag-bd$_{131–260}$. When incubated with the 64-bp core oligonucleotide, T-ag-bd$_{131–260}$ (1.5 to 6 pmol) induced the formation of one distinct species (lanes 5 to 7). However, this species was not observed when T-ag-bd$_{131–260}$ was incubated with the 64-bp control oligonucleotide (lanes 12 to 14). Thus, it is clear that T-ag-bd$_{131–260}$ has a much higher affinity for the core origin than the control DNA fragment. Furthermore, the interaction of T-ag-bd$_{131–260}$

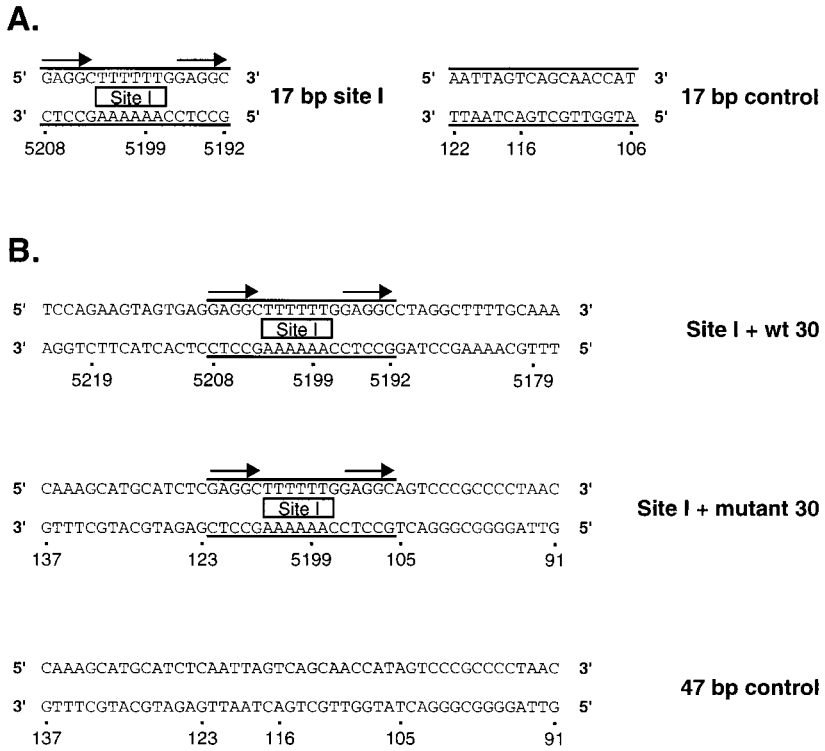

FIG. 4. Oligonucleotides used to compare the relative affinities of T-ag and T-ag-bd$_{131–260}$ for site I. (A) The 17-bp site I and 17-bp control oligonucleotides depicted in this figure were used in initial experiments designed to measure the relative affinities of T-ag and T-ag-bd$_{131–260}$ for site I. (B) Since it was not possible to detect a band shift with the initial set of site I oligonucleotides, a second set of site I-containing oligonucleotides was designed. One 47-bp oligonucleotide (designated site I + wt 30) contained site I (delineated by two parallel lines) flanked on either side by 15 bp of sequence normally found in SV40. The second 47-bp site I-containing oligonucleotide (designated site I + mutant 30) contained site I flanked on either side by 15 bp of sequence derived from the control oligonucleotide. Also depicted is the 47-bp control oligonucleotide that served as a negative control in these assays. The arrows depict two T-ag (GAGGC) pentanucleotide recognition sequences in site I.

with the 64-bp core oligonucleotide (Fig. 3) was at least as efficient as its interaction with the 83-bp ori$^+$ oligonucleotide (Fig. 2; quantitated in Table 1 [see below]). These experiments indicate that T-ag-bd$_{131–260}$ molecules bound to site I do not stimulate the binding of T-ag-bd$_{131–260}$ molecules to site II. This observation is consistent with previous studies of T-ag which indicated that there was little or no cooperativity between T-ag molecules bound to these two sites (7, 83). Finally, the experiments presented in Fig. 2 and 3 were conducted after a 20-min incubation, a time interval long enough to include both early and late binding events (17). Therefore, a time course experiment was conducted employing the 64-bp core oligonucleotide. These studies indicated that the single T-ag-bd$_{131–260}$-dependent band shift species (Fig. 2 and 3) is the product of protein-DNA interactions that take place soon ($\leq$1 min) after mixing (36) (data not presented).

Gel mobility assays were also conducted to assess the ability of both T-ag and T-ag-bd$_{131–260}$ to interact with an oligonucleotide containing site I. Site I contains two high-affinity GAGGC recognition sites arranged as direct repeats (64). Initially, a 17-bp oligonucleotide containing site I was used in gel shift assays (Fig. 4A). However, when using the oligonucleotide designated 17-bp site I, we failed to detect a band shift signal with either T-ag or T-ag-bd$_{131–260}$ (data not shown). Therefore, the experiments were repeated with additional oligonucleotides containing 15 bp of flanking sequence on either side of site I (Fig. 4B). In one oligonucleotide, designated site I + wt 30, the additional 30 bp were those normally surrounding site I in the SV40 origin. In the second oligonucleotide, designated site I + mutant 30, the additional 30 bp were arbitrarily selected from the control oligonucleotide (SV40 residues 91 to 105 and 123 to 137 [Fig. 1C]). Figure 5 presents data on the interaction of T-ag and T-ag-bd$_{131–260}$ with the oligonucleotides designated site I + wt 30 and 47-bp control. It is apparent from Fig. 5 that T-ag (3 and 6 pmol) interacted with the oligonucleotide designated site I + wt 30 (lanes 2 and 3) but not with the 47-bp control oligonucleotide (lanes 7 and 8). In a similar manner, T-ag-bd$_{131–260}$ (3 and 6 pmol) interacted with the oligonucleotide designated site I + wt 30 (lanes 4 and 5) but not with the 47-bp control oligonucleotide (lanes 9 and 10). It is concluded that T-ag-bd$_{131–260}$, like T-ag, preferentially interacts with site I-containing oligonucleotides. Furthermore, since nearly identical results were obtained with the oligonucleotide designated site I + mutant 30 (data not shown), it is concluded that stable binding of either T-ag or T-ag-bd$_{131–260}$ to site I requires additional flanking sequences; however, the exact sequence composition of the flanking sequences does not appear to be important. This observation is consistent with previous studies of the interaction of T-ag with site I (64). Finally, we are uncertain of the identities of the three bands observed in the positive controls conducted with T-ag (lanes 2 and 3); they may correspond to bound monomers, dimers, and trimers (47) or to higher oligomeric forms of T-ag.

The results in Fig. 2, 3, and 5 were quantitated with a PhosphorImager, and the results are summarized in Table 1. PhosphorImager analyses of the data presented in Fig. 2, a measure of the relative affinities of T-ag and T-ag-bd$_{131–260}$ for the

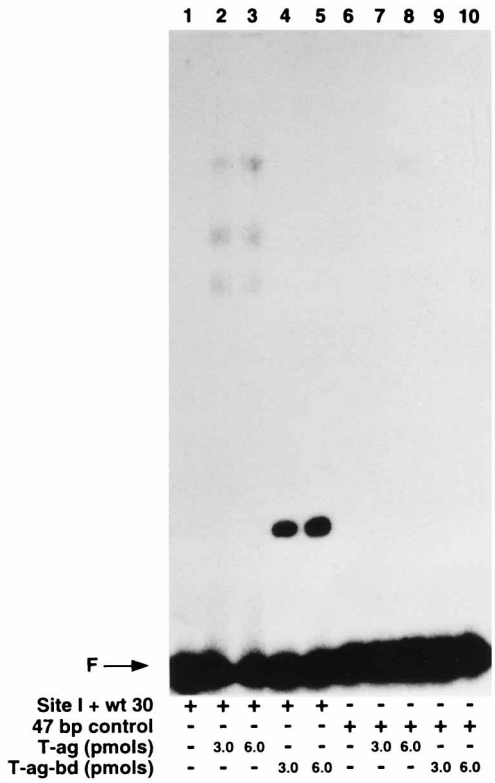

FIG. 5. Gel mobility shift assay used to compare the relative affinities of T-ag and T-ag-bd$_{131–260}$ for site I-containing oligonucleotides. Lanes 1 and 6, control band shift assays conducted in the absence of protein with the oligonucleotides designated site I + wt 30 and 47-bp control, respectively; lanes 2 and 3, band shift assays conducted with the oligonucleotide designated site I + wt 30 and either 3 or 6 pmol of T-ag; lanes 4 and 5, band shift assays conducted with the oligonucleotide designated site I + wt 30 and either 3 or 6 pmol of T-ag-bd$_{131–260}$; lanes 7 and 8, band shift assays conducted with the 47-bp control oligonucleotide and either 3 or 6 pmol of T-ag; lanes 9 and 10, band shift assays conducted with the 47-bp control oligonucleotide and either 3 or 6 pmol of T-ag-bd$_{131–260}$. The position of the 47-bp free DNA (F) is indicated by the arrow.

83-bp ori$^+$ oligonucleotide, are presented in the left-hand column. With 1.5, 3, and 6 pmol of T-ag, there was, respectively, 20-, 28-, and 9.7-fold greater interaction with the 83-bp ori$^+$ oligonucleotide than with the 83-bp control oligonucleotide. Similar analyses of T-ag-bd$_{131–260}$ were difficult to perform since it was not possible to detect a band shift with the 83-bp control oligonucleotide (discussed below). PhosphorImager analyses of the data presented in Fig. 3, a measure of the relative affinities of T-ag and T-ag-bd$_{131–260}$ for the core origin, are also presented in Table 1 (middle column). With 1.5, 3, and 6 pmol of T-ag, there was 20-, 31-, and 27-fold greater interaction, respectively, with the 64-bp core oligonucleotide than with the 64-bp control oligonucleotide. Furthermore, with 1.5, 3, and 6 pmol of T-ag-bd$_{131–260}$, there was 23-, 59-, and 47-fold greater interaction, respectively, with the 64-bp core oligonucleotide than with the 64-bp control oligonucleotide. PhosphorImager analyses of the data presented in Fig. 5, a measure of the relative affinity of T-ag and T-ag-bd$_{131–260}$ for a fragment of DNA containing site I, are presented in the right-hand column of Table 1. With 3 and 6 pmol of T-ag, there was, respectively, 17- and 6-fold greater interaction with the oligonucleotide designated site I + wt 30 than with the 47-bp control oligonucleotide. As with the 83-bp control oligonucleotide, quantitation of the preferential binding of T-ag-bd$_{131–260}$ to the site I-containing oligonucleotide was difficult, since it was not possible to detect a band shift with the 47-bp control oligonucleotide. Collectively, these analyses demonstrate that T-ag-bd$_{131–260}$ is at least as discriminating as T-ag in its origin-specific DNA binding. Moreover, inspection of the data presented in Table 1 indicates that on a molar basis, T-ag has a slightly (two- to threefold) better ability to interact with DNA from the SV40 origin than does T-ag-bd$_{131–260}$. This may reflect a requirement for additional domains of T-ag for high-affinity interactions with the SV40 origin.

It is interesting that T-ag-bd$_{131–260}$ interacts very poorly with the non-origin-containing DNA control fragments. This observation is consistent with studies by Lin et al. (42), who concluded that a second region of T-ag, extending between residues 269 and 522, defines a region that is important for nonspecific DNA binding. Since T-ag-bd$_{131–260}$ lacks this region, this molecule would be expected to interact poorly with non-origin-containing DNA. However, since mutations that interfere with nonspecific binding have been described in T-ag-bd (75) and T-ag-bd$_{131–260}$ is capable of low, but detectable, nonspecific DNA binding, T-ag-bd does have a role in nonspecific binding. Finally, at high T-ag–to–DNA ratios, nonspecific binding may explain the drop in origin-specific binding observed with the 83-bp ori$^+$ and site I + wt 30 oligonucleotides.

TABLE 1. Quantitation of relative affinities of T-ag and the T-ag-bd$_{131–260}$ for binding to DNA fragments from the SV40 origin[a]

Protein (concn, in pmol)	% of total DNA and relative affinity with oligonucleotide:								
	ori$^+$			Core			I + 30 wt		
	ori$^+$ fragment	Control fragment	Relative affinity	Core fragment	Control fragment	Relative affinity	I + 30 wt fragment	Control fragment	Relative affinity
T-ag (1.5)	8.6	0.44	20	1.2	0.06	20	NT[b]	NT	NT
T-ag (3.0)	28	1.0	28	15	0.49	31	20	1.2	17
T-ag (6.0)	38	3.9	9.7	32	1.2	27	18	3.0	6.0
T-ag-bd (1.5)	1.3	~0		2.3	0.10	23	NT	NT	NT
T-ag-bd (3.0)	5.3	~0		9.4	0.16	59	14	~0	
T-ag-bd (6.0)	12	~0		20	0.43	47	18	~0	

[a] SV40 origin DNA fragments tested include ori$^+$ (Fig. 1B), core (Fig. 1B), and site I flanked by 15 bp of wild-type DNA (I + 30 wt [Fig. 4B]). The results shown in Fig. 2, 3, and 5 were quantitated with a Molecular Dynamics PhosphorImager, and the percentage of the total DNA in a given lane that is in a band-shifted species is given. For T-ag-bd$_{131–260}$, this was a single distinct species; for T-ag, quantitation was conducted on all species above the presumptive hexamer species and included DNA present in the wells. The relative affinity values represent the affinity of either T-ag or T-ag-bd$_{131–260}$ for the origin-containing fragment relative to the affinity for the control fragment. As discussed in the text, there was very little indication of nonspecific binding of T-ag-bd$_{131–260}$ to control DNA fragments. Finally, the protein-to-oligonucleotide ratios with 1.5, 3, and 6 pmol of T-ag (or T-ag-bd$_{131–260}$) and 50 fmol of oligonucleotide were 30:1, 60:1, and 120:1, respectively.

[b] NT, not tested.

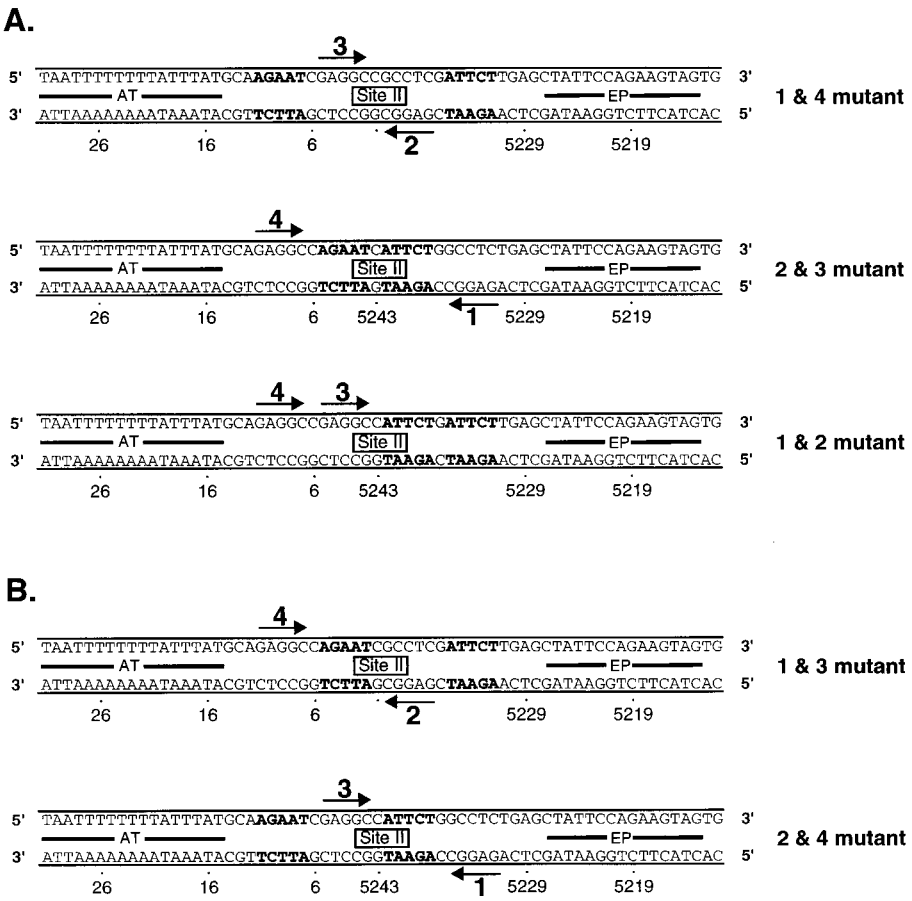

FIG. 6. Sequences of the core origin oligonucleotides having transition mutations in particular pairs of pentanucleotide binding sites. (A) The inactive set of pentanucleotide double mutants is presented. The oligonucleotides are named after the mutated pair of pentanucleotide binding sites. The transition mutations are indicated with boldfaced letters. The arrows delineate the intact pair of pentanucleotides. (B) The active set of pentanucleotide double mutants is presented. The symbols used to designate the mutated and active pairs of pentanucleotides are the same as described for panel A. AT, adenine- and thymine-rich regions; EP, early palindrome regions.

Interactions of T-ag and T-ag-bd$_{131–260}$ with DNA containing mutant pentanucleotide binding sites. The experiments presented in Fig. 2 and 3 demonstrated that only a single, rapidly formed species is detected in gel mobility shift assays conducted with T-ag-bd$_{131–260}$ and oligonucleotides containing the SV40 origin. This single species could reflect complete occupancy of the four pentanucleotide binding sites or binding to a subset of these sites. To distinguish between these possibilities, band shift assays were conducted with core origin oligonucleotides containing various combinations of mutant pentanucleotides.

Previous experiments indicated that the two pentanucleotides proximal to the early palindrome (EP) (pentanucleotides 1 and 2; Fig. 1B) nucleate T-ag hexamer assembly (59). Related experiments indicated that pentanucleotide 1 is the strongest binding site in the core origin palindrome (12, 40, 83). In view of these observations, the band shift assays were initially conducted with T-ag-bd$_{131–260}$ and oligonucleotides containing transition mutations in either pentanucleotide 1 or 2. It was obvious from these experiments that relative to the 64-bp core oligonucleotide there were few quantitative or qualitative differences in the band shift experiments conducted with either single pentanucleotide mutant (data not shown). Thus, formation of the distinct band shift species (Fig. 2 and 3) is not dependent upon complete occupancy of all four pentanucleotide binding sites by T-ag-bd$_{131–260}$. Therefore, to further characterize the pentanucleotide requirements for binding to the SV40 origin, oligonucleotides containing transition mutations in particular pairs of pentanucleotide recognition sites were designed (Fig. 6). A representative band shift assay conducted with the pentanucleotide double mutants designated 1&4 and 1&3 is presented in Fig. 7. Lanes 1, 6, and 11 show control reactions conducted in the absence of protein. Positive controls are presented in lanes 2 to 5; they contain the products of band shift reactions conducted with the 64-bp core oligonucleotide (Fig. 1B) and either T-ag (lanes 2 and 3) or T-ag-bd$_{131–260}$ (lanes 4 and 5). Lanes 6 to 10 show reactions conducted with the 1&4 pentanucleotide double mutant. It is clear from lanes 9 and 10 that the 1&4 pentanucleotide double mutant is inactive in band shift assays conducted with T-ag-bd$_{131–260}$. Lanes 11 to 15 contain the products of reactions conducted with the 1&3 pentanucleotide double mutant. A comparison of lanes 4 and 5 with lanes 14 and 15 demonstrates that the 1&3 pentanucleotide double mutant is as good a substrate for binding by the T-ag-bd$_{131–260}$ as an oligonucleotide containing a wild-type copy of site II. Additional assays demonstrated that the 1&4 pentanucleotide double mutant is a member of a set of mutants (the inactive set of pentanucleotide double mutants [Fig. 6A]) that were completely inactive in band shift assays with T-ag-bd$_{131–260}$. In contrast, the 1&3 and

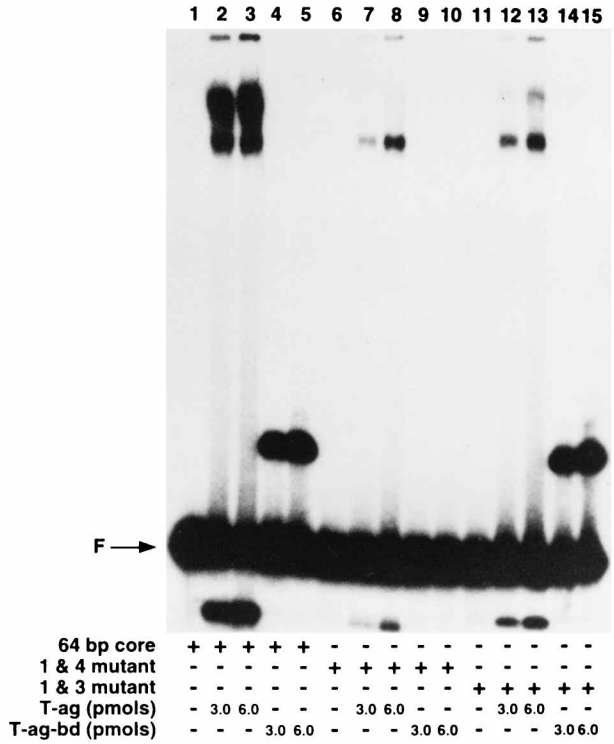

FIG. 7. A representative gel mobility assay used to assess the pentanucleotide requirements for binding of T-ag-bd$_{131–260}$ to the SV40 core origin. Control band shift assays were conducted in the absence of protein with the 64-bp core oligonucleotide (lane 1), the 1&4 mutant oligonucleotide (lane 6), and the 1&3 mutant oligonucleotide (lane 11). Additional control reactions included band shift assays conducted with the 64-bp core oligonucleotide and either T-ag (3 [lane 2] and 6 [lane 3] pmol) or T-ag-bd$_{131–260}$ (3 [lane 4] and 6 [lane 5] pmols). Band shift assays conducted with the 1&4 mutant and T-ag (3 and 6 pmol, respectively) are presented in lanes 7 and 8. Band shift reactions conducted with the same oligonucleotide and T-ag-bd$_{131–260}$ (3 and 6 pmol, respectively) are presented in lanes 9 and 10. Band shift assays conducted with the 1&3 mutant and T-ag (3 and 6 pmol, respectively) are presented in lanes 12 and 13. Band shift reactions conducted with the same oligonucleotide and T-ag-bd$_{131–260}$ (3 and 6 pmol, respectively) are presented in lanes 14 and 15. As in previous figures, the position of free DNA (F) is indicated by the arrow.

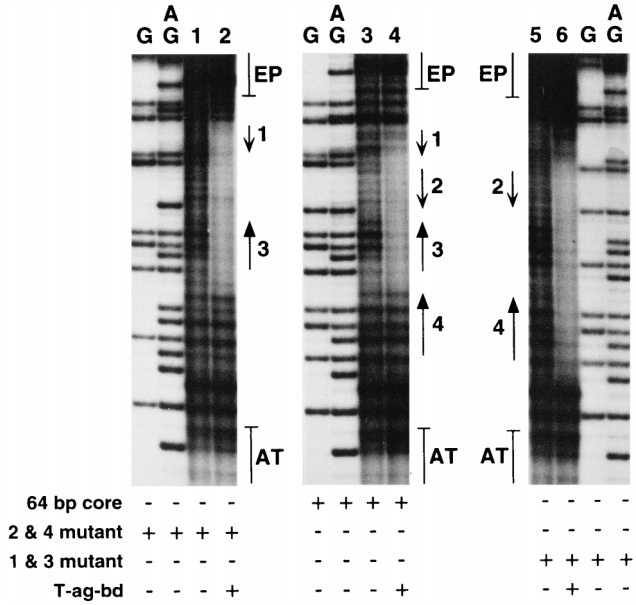

FIG. 8. In situ footprinting of T-ag-bd$_{131–260}$–core origin complexes by using the nuclease activity of 1,10-phenanthroline–copper ion. Lanes 1, 3, and 5, control experiments conducted with free DNA isolated from samples containing the 2&4 mutant, 64-bp core, and 1&3 mutant oligonucleotides, respectively; lanes 2, 4, and 6, products of footprinting reactions conducted with the same oligonucleotides and T-ag-bd$_{131–260}$. Size markers were generated by subjecting the indicated oligonucleotides to the G and G+A sequencing reactions described by Maxam and Gilbert (48). Flanking each panel is a map of the relative positions of the early palindrome region (EP), pentanucleotides 1 to 4 (arrows), and the adenine- and thymine-rich region (AT). The arrows associated with the smaller arrowheads represent the complementary sequences of given pentanucleotides. The experiments presented in this figure were conducted at a 240:1 protein-to-oligonucleotide ratio; however, similar results were obtained at a 120:1 protein-to-oligonucleotide ratio.

2&4 pentanucleotide double mutants constitute a second set (the active set of pentanucleotide double mutants [Fig. 6B]) that were quantitatively and qualitatively indistinguishable from the wild-type site II-containing oligonucleotide in the band shift assays conducted with this polypeptide.

We next addressed whether both pentanucleotide recognition sites in the active pentanucleotide double mutants (Fig. 6B) were required for stable binding. To conduct these assays, oligonucleotides containing a third pentanucleotide mutation were synthesized. For instance, with regard to the 2&4 double mutant (Fig. 6B; pentanucleotides 1 and 3 intact), additional pentanucleotide mutations in pentanucleotide 1 (the 1-2-4 triple-mutant oligonucleotide) or pentanucleotide 3 (the 2-3-4 triple-mutant oligonucleotide) were synthesized and used in band shift assays. These triple-pentanucleotide mutant oligonucleotides were completely inactive in band shift assays (data not shown). Thus, it is concluded that substrates containing pairs of pentanucleotide recognition sites, positioned in a head-to-head orientation and separated by approximately one helical turn, are required for stable binding by T-ag-bd$_{131–260}$.

Evidence that binding of T-ag-bd$_{131–260}$ to the core origin is limited to dimer formation. The similar mobility of the band-shifted species formed in reactions containing the 64-bp core and active-set pentanucleotide double mutants (Fig. 7) suggested that all four pentanucleotides in the 64-bp core oligonucleotide were not occupied by T-ag-bd$_{131–260}$. To test this hypothesis, we employed the gel retardation–1,10-phenanthroline–copper ion footprinting procedure described by Kuwabara and Sigman (39). This technique permits protein-DNA complexes to be footprinted within the gel matrix, and the resulting DNA fragments can then be resolved on a sequencing gel.

In one set of experiments, the 64-bp core, 1&3 mutant, and 2&4 mutant oligonucleotides were analyzed; these molecules were asymmetrically labeled (see Materials and Methods) at the 5′ termini of the top strands (Fig. 1 and 6). Inspection of Fig. 8 (lane 4) reveals that when an oligonucleotide containing the core origin was used in the reaction, an ~18-nucleotide (nt) region was protected by T-ag-bd$_{131–260}$. The protected region initiates over pentanucleotide 3 and extends into a region containing sequences complementary to pentanucleotide 1. The footprint does not include any of the sequences that comprise pentanucleotide 4. The footprint obtained when the 2&4 mutant (pentanucleotides 1 and 3 present [Fig. 6]) oligonucleotide was used in an identical assay is presented in lane 2. It is clear that relative to the 64-bp core oligonucleotide, nearly identical regions of site II are protected in the 2&4 mutant oligonucleotide. Moreover, the DNA region containing the transition mutations substituting for pentanucleotide 4 was not protected in the 2&4 mutant oligonucleotide. The footprint obtained when the 1&3 mutant (pentanucleotides 2 and 4 present) oligonucleotide was analyzed by the gel retardation–1,10-phenanthroline–copper ion procedure is presented in lane

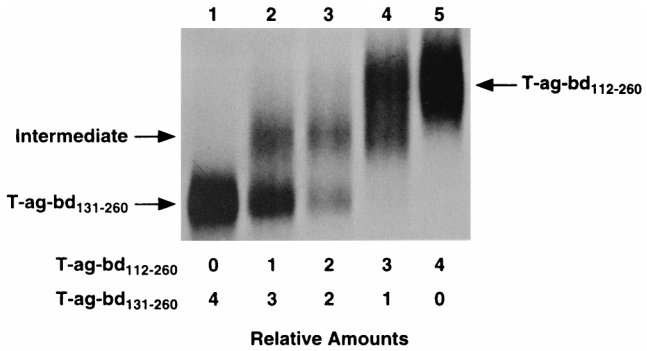

FIG. 9. Evidence from a mixing experiment that T-ag-bd-containing polypeptides form a dimer on the SV40 core origin. The T-ag-$bd_{131–260}$ and T-ag-$bd_{112–260}$ polypeptides were mixed at various ratios with the 64-bp core oligonucleotide and analyzed via gel mobility assays. Lane 1, a band shift assay conducted with T-ag-$bd_{131–260}$ (6 pmol); lane 5, a band shift assay conducted with the T-ag-$bd_{112–260}$ (6 pmol); lanes 2 to 4, band shift experiments conducted with 3:1, 2:2, and 1:3 mixtures of the T-ag-$bd_{131–260}$ and T-ag-$bd_{112–260}$ polypeptides, respectively (6 pmol of total protein [120:1 molar ratio of protein to oligonucleotide]). The location of a single novel intermediate species, the predicted outcome for dimer formation, is indicated. If trimers or tetramers were assembled on the core origin, two or three intermediate species should form, at ratios of 1:3:3:1 and 1:4:6:4:1, respectively. If it is argued that the intermediate species are not resolved in this gel system, one would predict relative intensities of 1:6:1 for the trimer intermediate and 1:14:1 for the tetramer intermediate. PhosphorImager analyses of this and several related gels suggested that the intermediate is formed at the ratio expected for dimer formation (~1:2:1).

6. As with the previous two examples, the footprint extends ~18 nt. However, in this instance, the footprint extends between pentanucleotides 2 and 4. Thus, T-ag-$bd_{131–260}$ is capable of binding pentanucleotide 4, but it binds only when pentanucleotides 1 and 3 are not present. Finally, control DNAs (i.e., free DNA obtained from reactions lacking T-ag-$bd_{131–260}$) are presented in lanes 1, 3, and 5. The incomplete cleavage of certain regions within the control DNAs may be related to the sequence preferences exhibited by 1,10-phenanthroline–copper (86).

These experiments were repeated with the same oligonucleotides which were asymmetrically labeled on the bottom strand (data not presented). These experiments also revealed that the footprint obtained with the 64-bp core oligonucleotide (~18 nt) was nearly identical to that obtained with the 2&4 mutant oligonucleotide. Moreover, the footprint obtained with the 1&3 mutant (~18 nt) extended between pentanucleotides 2 and 4. In summary, these studies provided direct evidence that T-ag-$bd_{131–260}$ does not simultaneously bind all four pentanucleotides. Moreover, they demonstrated that T-ag-$bd_{131–260}$ prefers to bind to pentanucleotides 1 and 3 but can bind pentanucleotides 2 and 4 when pentanucleotides 1 and 3 are not available.

The nearly identical footprints obtained with the 2&4 mutant oligonucleotide and the oligonucleotide containing the core origin are most easily explained by dimer formation on both DNA substrates. To test this hypothesis, a gel shift assay was conducted to analyze the products formed in a mixing experiment employing T-ag-$bd_{131–260}$ and a T-ag-bd derivative containing an additional 21 amino acids (T-ag-$bd_{112–260}$) (see Materials and Methods). Theoretically, mixing experiments should generate a single intermediate species if a dimer is formed between T-ag-$bd_{131–260}$ and T-ag-$bd_{112–260}$ (at a ratio of 1:2:1; T-ag-$bd_{131–260}$, T-ag-$bd_{131–260}$-T-ag-$bd_{112–260}$ intermediates, T-ag-$bd_{112–260}$, respectively). Alternatively, if trimers or tetramers are assembled on the core origin, two or three intermediate species should form at a ratio of 1:3:3:1 (T-ag-$bd_{131–260}$, T-ag-$bd_{131–260}$-T-ag-$bd_{112–260}$ intermediates, T-ag-$bd_{112–260}$, respectively) or 1:4:6:4:1 (T-ag-$bd_{131–260}$, T-ag-$bd_{131–260}$-T-ag-$bd_{112–260}$ intermediates, T-ag-$bd_{112–260}$, respectively).

Results from mixing experiments employing T-ag-$bd_{131–260}$ and T-ag-$bd_{112–260}$ are presented in Fig. 9. The distinct T-ag-$bd_{131–260}$-dependent band shift species is presented in lane 1, while the slightly larger T-ag-$bd_{112–260}$-dependent species is shown in lane 5. The similar amounts of product formed in these band shift reactions indicate that T-ag-$bd_{112–260}$ and T-ag-$bd_{131–260}$ have similar affinities for the core origin. The products formed when the band shift reactions were conducted with 3:1, 2:2, or 1:3 mixtures of T-ag-$bd_{131–260}$ and T-ag-$bd_{112–260}$, respectively, are shown in lanes 2 to 4. A single novel species was formed in these reactions whose mobility was roughly intermediate between those formed with the individual polypeptides. It is concluded that T-ag-$bd_{131–260}$ is binding as a dimer to two of the four available pentanucleotides in the core origin.

Interactions of T-ag and T-ag-$bd_{131–260}$ with single-stranded DNA. T-ag is known to bind single-stranded DNA in a non-sequence-specific manner. Indeed, studies have indicated that it has a higher affinity for single-stranded DNA than for double-stranded DNA (3, 77). Moreover, previous experiments indicated that T-ag-bd-containing polypeptides are capable of binding single-stranded DNA (49). Therefore, we tested whether T-ag-$bd_{131–260}$ bound to single-stranded DNA (Fig. 10). In these assays, the top strands of the 83-bp ori^+ and 83-bp control oligonucleotides were used as single-stranded DNA templates (Fig. 1). In Fig. 10, the reactions in lanes 1 to 9 were conducted in the presence of competitor DNA (0.8 μg/reaction); those in lanes 10 to 15 were conducted in the absence of competitor DNA. As positive controls, band shift reactions were performed with the 83-bp ori^+ double-stranded oligonucleotide and either T-ag (lane 2) or T-ag-$bd_{131–260}$ (lane 3). It is apparent that both the T-ag and the T-ag-$bd_{131–260}$ preparations used in these assays were active. As a negative control, single-stranded DNA was incubated in the absence of protein under replication conditions and the products were analyzed (lanes 4, 7, 10, and 13). Inspection of lanes 4 and 10 revealed that a protein-independent band is detected, owing to the secondary structure of the 83-nt ori^+ single-stranded oligonucleotide. Products from band shift reactions containing T-ag (6 pmol) and the 83-nt ori^+ single-stranded oligonucleotide are shown in lanes 5 and 11; it is apparent that T-ag interacted with this DNA. The interactions of T-ag with the single-stranded 83-nt control oligonucleotide (Fig. 1C) was also examined (lanes 8 and 14). It is obvious that T-ag bound to the control oligonucleotide and that the interaction of T-ag with single-stranded DNA was not sequence specific. The interaction of T-ag-$bd_{131–260}$ (6 pmol) with the same single-stranded DNA templates was also examined. In contrast to T-ag, T-ag-$bd_{131–260}$ failed to interact with single-stranded DNA from the upper strand of the SV40 origin (lanes 6 and 12). Furthermore, there was no indication of a band shift when T-ag-$bd_{131–260}$ was incubated with the single-stranded 83-nt control oligonucleotide (lanes 9 and 15). Nearly identical results (not presented) were obtained when these experiments were repeated with origin-containing and non-origin-containing DNA from the lower strand (Fig. 1). Collectively, these studies indicated that under replication conditions, T-ag-$bd_{131–260}$ is not sufficient for a stable interaction with single-stranded DNA.

The reactions in lanes 1 to 9 were conducted in the presence of competitor DNA (see Materials and Methods) that was included in an effort to eliminate nonspecific protein-DNA interactions. We were concerned that the competitor DNA may have competed with the single-stranded DNA for binding by T-ag-$bd_{131–260}$. Therefore, the experiments were repeated in

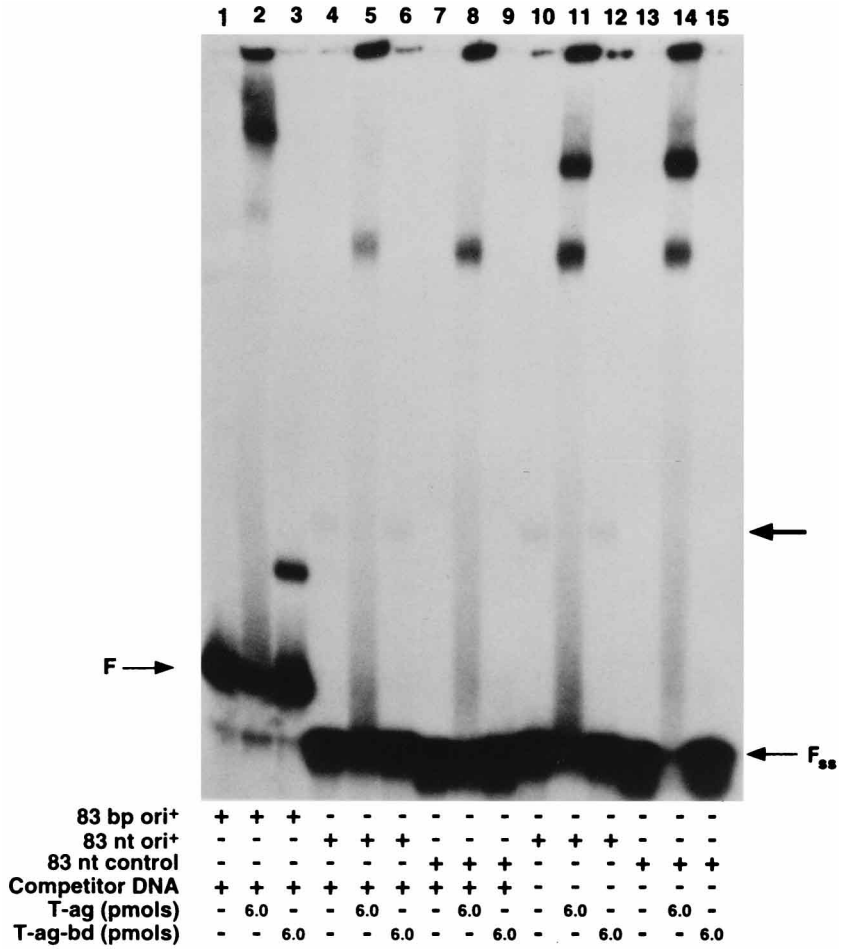

FIG. 10. Gel mobility shift assay used to compare the relative affinities of T-ag and T-ag-$bd_{131–260}$ for single-stranded DNA. Lanes 1 to 3, control reactions conducted with the 83-bp ori^+ oligonucleotide and either no protein (lane 1), T-ag (6 pmol) (lane 2), or T-ag-$bd_{131–260}$ (6 pmol) (lane 3). The reaction mixtures in lanes 4 to 6 and 10 to 12 contained the single-stranded 83-nt ori^+ oligonucleotide from the upper strand of the SV40 origin (Fig. 1B); those in lanes 7 to 9 and 13 to 15 contained the single-stranded 83-nt control oligonucleotide from the upper strand of the control oligonucleotide (Fig. 1C). The reactions in lanes 4 to 9 were conducted in the presence of double-stranded competitor DNA; those in lanes 10 to 15 were performed in the absence of competitor DNA. The band shift assays in lanes 4, 7, 10, and 13 were conducted in the absence of protein. The band shift assays presented in lanes 5, 8, 11, and 14 were conducted in the presence of T-ag (6 pmol); those in lanes 6, 9, 12, and 15 were conducted in the presence of T-ag-$bd_{131–260}$ (6 pmol). The positions of free duplex DNA (F) and free single-stranded DNA (F_{ss}) are indicated. The unlabeled arrow indicates the position of a protein-independent, alternative DNA conformation adopted by the single-stranded 83-nt ori^+ oligonucleotide during electrophoresis. Finally, the protein-to-oligonucleotide ratio with 6 pmol of T-ag (or T-ag-$bd_{131–260}$) and 50 fmol of single-stranded oligonucleotide is 120:1.

the absence of competitor DNA (lanes 10 to 15). It is apparent that the inability of T-ag-$bd_{131–260}$ to bind single-stranded DNA is not a function of the presence of competitor DNA. However, it is interesting that in the absence of competitor DNA, T-ag forms a slower-mobility complex with both single-stranded DNA substrates. This complex presumably reflects the higher effective concentration of T-ag in the absence of competitor DNA. These experiments indicate that T-ag can oligomerize on single-stranded templates in a sequence-independent manner.

DISCUSSION

Studies by Lin et al. (42), and those presented herein, indicate that nonspecific DNA-binding events require a T-ag domain that is distinct from the T-ag-bd. Indeed, others have reported that while the T-ag-bd contains the essential origin-specific DNA-binding domain, additional regions of the protein are involved in stabilizing (2, 33, 49, 52, 72), or even destabilizing (49, 81), the interaction of the T-ag-bd with the SV40 origin. These observations raise the possibility that previous conclusions regarding the mechanism by which T-ag interacts with the SV40 origin were based on composite signals reflecting both nonspecific and specific DNA-binding events. Therefore, to more clearly establish assembly events at a eukaryotic origin of replication, we initiated studies of the interaction of T-ag-$bd_{131–260}$ with the SV40 origin.

With various DNA substrates, the sequence specificity of purified T-ag-$bd_{131–260}$ was analyzed. T-ag-$bd_{131–260}$ is at least as good as T-ag at distinguishing between an oligonucleotide containing the entire SV40 origin (core and site I) and a control oligonucleotide of the same size. In related experiments, it was demonstrated that purified T-ag-$bd_{131–260}$ is at least as good as T-ag at distinguishing between an oligonucleotide containing the core origin and a control oligonucleotide. Therefore, it is concluded that purified T-ag-$bd_{131–260}$ is a good model polypeptide for studies of site-specific binding to the SV40 origin.

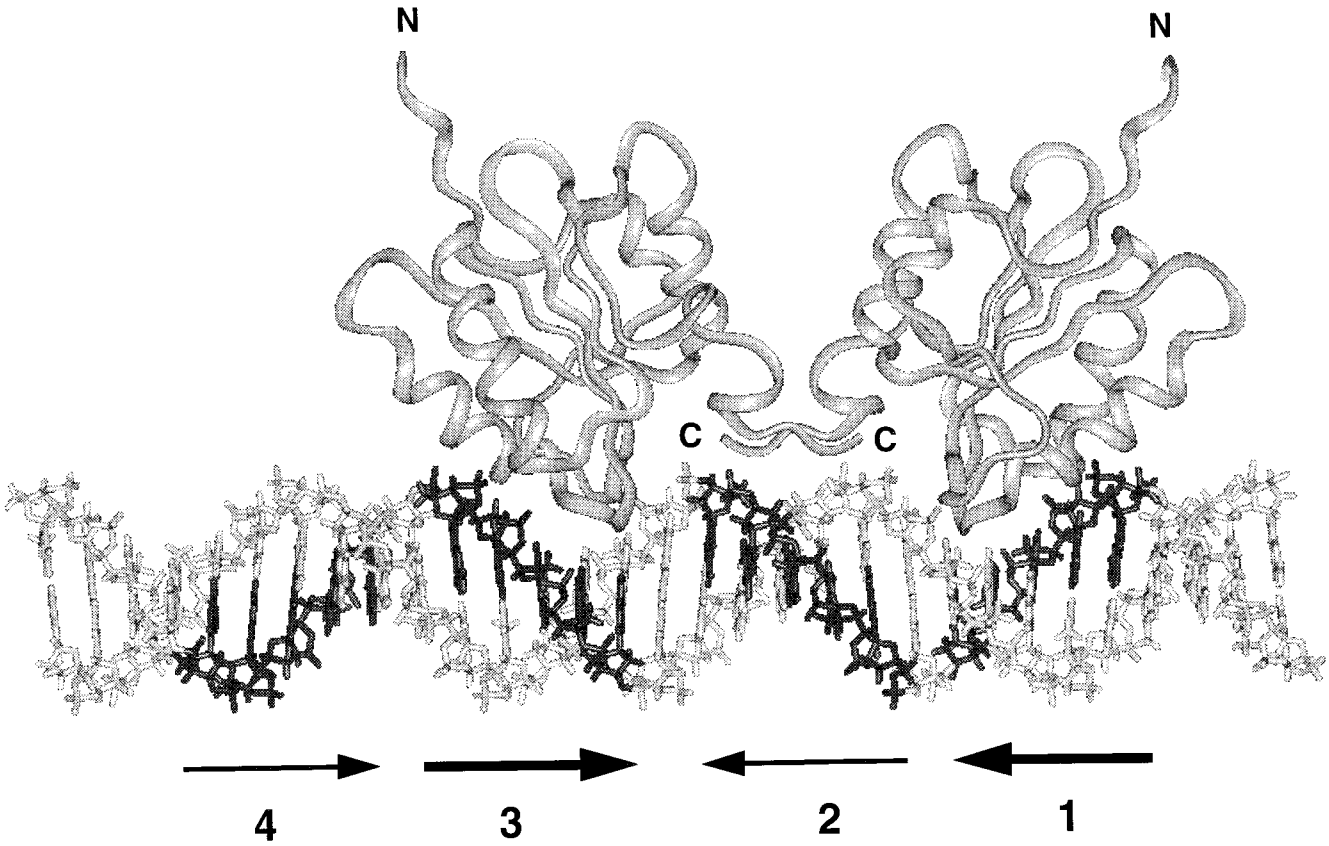

FIG. 11. A model of T-ag-bd$_{131–260}$ dimer formation on a 31-bp segment of B-DNA containing site II. The model was prepared by using the program INSIGHT (Biosym, San Diego, Calif.) and depicts the interaction of two T-ag-bd$_{131–260}$ molecules with pentanucleotides 1 and 3. The N and C termini of the T-ag-bd$_{131–260}$ molecules are indicated. Individual pentanucleotides in the B-DNA model are printed in boldface. The arrows below the figure are used to indicate the individual pentanucleotides; the active pairs of pentanucleotide recognition sites (1-3 and 2-4) are indicated by matching pairs of arrows. The costructure of T-ag-bd$_{131–260}$ bound to a pentanucleotide has not been established. Therefore, to model this interaction, Arg-204 of T-ag-bd$_{131–260}$, a critical residue for T-ag binding (75), was hydrogen bonded to the third G in a given GAGGC sequence. Since individual molecules are free to rotate around this bond, the relative orientation of the individual T-ag-bd$_{131–260}$ molecules is arbitrary. Many alternative models were generated; this version was selected since there were minimal steric clashes and good protein-DNA contacts.

The interaction of T-ag-bd$_{131–260}$ with site I was also examined. It is known that T-ag preferentially interacts with site I in the absence of ATP (see reference 28 for a review). Nevertheless, when a 17-bp site I-containing oligonucleotide was used in the band shift assay, we failed to detect a signal. When a larger oligonucleotide was used, containing 15 bp on either side of site I, a strong band shift was observed. The source of the additional 15 bp on either side of site I was not a critical determinant for detecting a band shift. Thus, under these reaction conditions, a stable interaction of T-ag-bd$_{131–260}$ with site I requires sequences flanking site I. It is interesting that the yeast origin recognition complex failed to interact with an A element oligonucleotide unless sequences adjacent to the A element were included (61).

Having established that purified T-ag-bd$_{131–260}$ is a good model polypeptide for SV40 origin-specific binding, we next addressed the pentanucleotide requirements for a stable interaction with T-ag-bd$_{131–260}$. Previous studies demonstrated that a single pentanucleotide is sufficient to bind and orient a T-ag monomer (40, 83). Nuclear magnetic resonance experiments were used to confirm that T-ag-bd$_{131–260}$ binds to a single GAGGC pentanucleotide, albeit at millimolar DNA concentrations (44). Nevertheless, oligonucleotides containing single pentanucleotide binding sites (such as the 64-bp 1-2-4 and 2-3-4 triple pentanucleotide mutants) were inactive in band shift assays conducted with T-ag-bd$_{131–260}$. It should be noted that the oligonucleotides used in these assays contained the inverted-repeat region of the core origin. It has been reported that this region constitutes a second, albeit low-affinity, binding site for T-ag (57). In our assays, stable binding to the SV40 origin occurred only with those substrates containing at least one pair of pentanucleotide recognition sites separated by approximately one turn of a double-stranded helix and positioned in a head-to-head orientation (Fig. 7). These observations suggest that binding of T-ag-bd$_{131–260}$ monomers to suitably arranged pentanucleotides promotes dimer formation and that dimerization stabilizes binding to the SV40 origin.

The intensity and relative position of the band shift species generated with oligonucleotides containing either the 64-bp core or active-set pentanucleotide mutants were nearly identical (Fig. 7). Given that the 64-bp core oligonucleotide has four pentanucleotide binding sites and oligonucleotides for the active set contain two pentanucleotides, this was a surprising result. One possibility is that even with the 64-bp core oligonucleotide, T-ag-bd$_{131–260}$ binding may be limited to formation of a single dimer. Support for this hypothesis was obtained with the 1,10-phenanthroline–copper ion footprinting procedure (39). These experiments revealed that T-ag-bd$_{131–260}$ does not protect all four pentanucleotides in the 64-bp core oligonucleotide. They also demonstrated that the footprint obtained with

the 64-bp core oligonucleotide was nearly identical to the footprint obtained with the 2&4 mutant oligonucleotide. Additional evidence that T-ag-bd$_{131–260}$ binding to the core origin is limited to dimer formation was provided by the mixing experiments (Fig. 9). Taken together, the studies presented in Fig. 7 to 9 provide direct evidence that T-ag-bd$_{131–260}$ binding to the core origin is limited to dimer formation and that pentanucleotides 1 and 3 are the preferred binding sites.

Previous studies with T-ag demonstrated that a major stabilizing factor for binding is a second nearby GAGGC pentanucleotide (21, 35, 40, 68, 83, 84, 90). Other studies with DNA containing deletions and insertions in the SV40 origin have also indicated that the orientation and spacing between nearby pentanucleotides help determine protein-protein interactions that stabilize bound subunits of T-ag (12, 19, 40, 58). In related studies with T-ag, it was concluded that the stability of DNA binding depends on interactions between bound protein molecules (19, 40, 47). Indeed, a model for the binding of T-ag to the SV40 origin has been proposed (19, 50, 83) that is consistent with the data presented herein for the binding of T-ag-bd$_{131–260}$ to the core origin. Furthermore, if one incorporates the observation that the T-rich spacer between the pentanucleotide recognition elements in site I is known to bend (63), it is possible to invoke the "head-to-head" model to explain binding to site I. Moreover, because of similarities in the arrangement of GAGGC pentanucleotides, the head-to-head model can be used to predict how T-ag interacts with the JC (29) and BK (62) virus core origins. Finally, at least for the origin region, the head-to-head model can be used to suggest how T-ag interacts with the polyomavirus origin of replication (13).

Genetic and biochemical studies of T-ag have implicated T-ag-bd in single-stranded DNA binding events (49, 75). However, it is apparent from the experiments presented in Fig. 10 that T-ag-bd$_{131–260}$ has a very low affinity for single-stranded DNA compared with its affinity for duplex DNA. These results raise the possibility that along with T-ag-bd$_{131–260}$, additional domains of T-ag are required for binding to single-stranded DNA. McVey et al. (49) also noted that wild-type levels of binding to single-stranded DNA required additional sequences missing from their T-ag-bd constructs. Alternatively, in the purified polypeptide, a region(s) of T-ag-bd$_{131–260}$ required for binding to single-stranded DNA may be incorrectly folded or blocked due to novel structural features of the polypeptide. Finally, the band shift experiments conducted with T-ag and single-stranded DNA (Fig. 10, lanes 5, 8, 11, and 14) confirmed earlier studies (3) showing that T-ag binds single-stranded DNA without any sequence specificity. However, it is interesting that T-ag readily assembles into presumptive hexamers and double hexamers on single-stranded DNA.

To gain further insights into T-ag-bd binding to the core origin, this interaction was modeled by using the T-ag-bd$_{131–260}$ structure (44) and the program INSIGHT (Biosym, San Diego, Calif.). In view of the data presented in Fig. 7 to 9, two T-ag-bd$_{131–260}$ molecules were docked to pentanucleotides 1 and 3. Inspection of Fig. 11 demonstrates that active pairs of pentanucleotides, and molecules bound to these sites, are on the same B-DNA face. The model also suggests that there are no steric clashes between the monomers. Furthermore, in keeping with the data presented in Fig. 8, the model predicts that upon binding to an active pair of pentanucleotides, an ~17- to 18-bp region of DNA should be covered by two T-ag-bd$_{131–260}$ molecules. However, the model does not account for several additional observations. For example, the model does not provide insights into the failure of T-ag-bd$_{131–260}$ to bind to the second pair of pentanucleotides in the wild-type core origin. One hypothesis is that as with DNA binding by the bovine papillomavirus type 1 E2 (32) and Epstein-Barr virus (4) DNA-binding domains, T-ag-bd$_{131–260}$ binding and dimer formation may result in significant deformation of the DNA target. An additional possibility, not conveyed by the model in Fig. 11, is that T-ag-bd$_{131–260}$ regions may protrude through the DNA into the lower plane and thereby block binding of the second pair of molecules. Moreover, the model suggests that the DNA between the active pentanucleotides should be susceptible to cleavage by agents such as 1,10-phenanthroline–copper; however, the experiments presented in Fig. 8 demonstrate that this is not the case. Furthermore, the model fails to account for the preferential binding to pentanucleotides 1 to 3. Additional studies are required to address these and related issues.

Finally, an interesting question concerns the extent to which T-ag-bd$_{131–260}$ serves as a useful model for interactions of T-ag with the core origin. This topic will be addressed at length in a subsequent publication (37). Nevertheless, it is noted that a correlation exists between those pentanucleotide pairs that support stable T-ag-bd$_{131–260}$ binding and those that support T-ag double-hexamer formation (e.g., compare lanes 7 to 10 of Fig. 7 with lanes 12 to 15). These studies suggest that T-ag-bd$_{131–260}$ dimer formation may mimic nucleation of T-ag double hexamers.

ACKNOWLEDGMENTS

We thank W. Bachovchin, J. M. Kaguni, B. Schaffhausen, and D. Sanford for helpful discussions. We also acknowledge S. Helmling for assistance with protein purification, H. Shih for assistance with initial band shift assays, and J. Sudmeier for help with computer modeling.

This study was funded by grants from the National Institutes of Health (RO1 AI30032) and an American Cancer Society Junior Faculty Award (JFRA 396).

REFERENCES

1. **Alberts, B.** 1984. The DNA enzymology of protein machines. Cold Spring Harbor Symp. Quant. Biol. **49:**1–12.
2. **Arthur, A. K., A. Höss, and E. Fanning.** 1988. Expression of simian virus 40 T antigen in *Escherichia coli*: localization of T-antigen origin DNA-binding domain to within 129 amino acids. J. Virol. **62:**1999–2006.
3. **Auborn, K. J., R. B. Markowitz, E. Wang, Y. T. Yu, and C. Prives.** 1988. Simian virus 40 (SV40) T antigen binds specifically to double-stranded DNA but not to single-stranded DNA or DNA/RNA hybrids containing the SV40 regulatory sequences. J. Virol. **62:**2204–2208.
4. **Bochkarev, A., J. A. Barwell, R. A. Pfuetzner, E. Bochkareva, L. Frappier, and A. M. Edwards.** 1996. Crystal structure of the DNA-binding domain of the Epstein-Barr virus origin-binding protein, EBNA1, bound to DNA. Cell **84:**791–800.
5. **Borowiec, J. A., F. B. Dean, P. A. Bullock, and J. Hurwitz.** 1990. Binding and unwinding—how T antigen engages the SV40 origin of DNA replication. Cell **60:**181–184.
6. **Borowiec, J. A., and J. Hurwitz.** 1988. ATP stimulates the binding of the simian virus 40 (SV40) large tumor antigen to the SV40 origin of replication. Proc. Natl. Acad. Sci. USA **85:**64–68.
7. **Borowiec, J. A., and J. Hurwitz.** 1988. Localized melting and structural changes in the SV40 origin of replication induced by T-antigen. EMBO J. **7:**3149–3158.
8. **Bradley, M. K., T. F. Smith, R. H. Lathrop, D. M. Livingston, and T. A. Webster.** 1987. Consensus topography in the ATP binding site of the simian virus 40 and polyomavirus large tumor antigens. Proc. Natl. Acad. Sci. USA **84:**4026–4030.
9. **Bullock, P. A., Y. S. Seo, and J. Hurwitz.** 1989. Initiation of simian virus 40 DNA replication in vitro: pulse-chase experiments identify the first labeled species as topologically unwound. Proc. Natl. Acad. Sci. USA **86:**3944–3948.
10. **Bullock, P. A., S. Tevosian, C. Jones, and D. Denis.** 1994. Mapping initiation sites for simian virus 40 DNA synthesis events in vitro. Mol. Cell. Biol. **14:**5043–5055.
11. **Challberg, M. D., and T. J. Kelly.** 1989. Animal virus DNA replication. Annu. Rev. Biochem. **58:**671–717.
12. **Cohen, G. L., P. J. Wright, A. L. DeLucia, B. A. Lewton, M. E. Anderson, and P. Tegtmeyer.** 1984. Critical spatial requirement within the origin of simian virus 40 DNA replication. J. Virol. **51:**91–96.
13. **Cowie, A., and R. Kamen.** 1986. Guanine nucleotide contacts within viral DNA sequences bound by polyomavirus large T antigen. J. Virol. **57:**505–514.

14. **Dean, F. B., J. A. Borowiec, T. Eki, and J. Hurwitz.** 1992. The simian virus 40 T antigen double hexamer assembles around the DNA at the replication origin. J. Biol. Chem. **267:**14129–14137.
15. **Dean, F. B., P. Bullock, Y. Murakami, C. R. Wobbe, L. Weissbach, and J. Hurwitz.** 1987. Simian virus 40 (SV40) DNA replication: SV40 large T antigen unwinds DNA containing the SV40 origin of replication. Proc. Natl. Acad. Sci. USA **84:**16–20.
16. **Dean, F. B., M. Dodson, H. Echols, and J. Hurwitz.** 1987. ATP-dependent formation of a specialized nucleoprotein structure by simian virus 40 (SV40) large tumor antigen at the SV40 replication origin. Proc. Natl. Acad. Sci. USA **84:**8981–8985.
17. **Dean, F. B., and J. Hurwitz.** 1991. Simian virus 40 large T antigen untwists DNA at the origin of DNA replication. J. Biol. Chem. **266:**5062–5071.
18. **Deb, S., A. L. DeLucia, C.-P. Baur, A. Koff, and P. Tegtmeyer.** 1986. Domain structure of the simian virus 40 core origin of replication. Mol. Cell. Biol. **6:**1663–1670.
19. **Deb, S., S. Tsui, A. Koff, A. L. DeLucia, R. Parsons, and P. Tegtmeyer.** 1987. The T-antigen-binding domain of the simian virus 40 core origin of replication. J. Virol. **61:**2143–2149.
20. **Deb, S. P., and P. Tegtmeyer.** 1987. ATP enhances the binding of simian virus 40 large T antigen to the origin of replication. J. Virol. **61:**3649–3654.
21. **DeLucia, A. L., B. A. Lewton, R. Tjian, and P. Tegtmeyer.** 1983. Topography of simian virus 40 A protein-DNA complexes: arrangement of pentanucleotide interaction sites at the origin of replication. J. Virol. **46:**143–150.
22. **DePamphilis, M. L.** 1993. Eukaryotic DNA replication: anatomy of an origin. Annu. Rev. Biochem. **62:**29–63.
23. **Dixon, R. A. F., and D. Nathans.** 1985. Purification of simian virus 40 large T antigen by immunoaffinity chromatography. J. Virol. **53:**1001–1004.
24. **Dodson, M., F. B. Dean, P. Bullock, H. Echols, and J. Hurwitz.** 1987. Unwinding of duplex DNA from the SV40 origin of replication by T antigen. Science **238:**964–967.
25. **Eaton, S., P. A. Bullock, and T. M. Lave.** Unpublished data.
26. **Echols, H.** 1986. Multiple DNA-protein interactions governing high-precision DNA transactions. Science **233:**1050–1055.
27. **Fanning, E.** 1992. Simian virus 40 large T antigen: the puzzle, the pieces, and the emerging picture. J. Virol. **66:**1289–1293.
28. **Fanning, E., and R. Knippers.** 1992. Structure and function of simian virus 40 large tumor antigen. Annu. Rev. Biochem. **61:**55–85.
29. **Frisque, R. J.** 1983. Nucleotide sequence of the region encompassing the JC virus origin of DNA replication. J. Virol. **46:**170–176.
30. **Goetz, G. S., F. B. Dean, J. Hurwitz, and S. W. Matson.** 1988. The unwinding of duplex regions in DNA by the simian virus 40 large tumor antigen-associated DNA helicase activity. J. Biol. Chem. **263:**383–392.
31. **Gruda, M. C., J. M. Zabolotny, J. H. Xiao, I. Davidson, and J. C. Alwine.** 1993. Transcriptional activation by simian virus 40 large T antigen: interactions with multiple components of the transcription complex. Mol. Cell. Biol. **13:**961–969.
32. **Hegde, R. S., S. R. Grossman, L. A. Laimins, and P. B. Sigler.** 1992. Crystal structure at 1.7 Å of the bovine papillomavirus-1 E2 DNA-binding domain bound to its DNA target. Nature **359:**505–512.
33. **Höss, A., I. F. Moarefi, E. Fanning, and A. K. Arthur.** 1990. The finger domain of simian virus 40 large T antigen controls DNA-binding specificity. J. Virol. **64:**6291–6296.
34. **Hurwitz, J., F. B. Dean, A. D. Kwong, and S.-H. Lee.** 1990. The in vitro replication of DNA containing the SV40 origin. J. Biol. Chem. **265:**18043–18046.
35. **Jones, K. A., and R. Tjian.** 1984. Essential contact residues within SV40 large T antigen binding sites I and II identified by alkylation-interference. Cell **36:**155–162.
36. **Joo, W. S., and P. A. Bullock.** Unpublished data.
37. **Joo, W. S., H. Y. Kim, K. R. Sreekumar, and P. A. Bullock.** 1997. Unpublished data.
38. **Kadonaga, J. T., and R. Tjian.** 1986. Affinity purification of sequence-specific DNA binding proteins. Proc. Natl. Acad. Sci. USA **83:**5889–5893.
39. **Kuwabara, M. D., and D. S. Sigman.** 1987. Footprinting DNA-protein complexes in situ following gel retardation assays using 1,10-phenanthroline-copper ion: *Escherichia coli* RNA polymerase-lac promoter complexes. Biochemistry **26:**7234–7238.
40. **Lewton, B. A., A. L. DeLucia, and P. Tegtmeyer.** 1984. Binding of simian virus 40 A protein to DNA with deletions at the origin of replication. J. Virol. **49:**9–13.
41. **Li, J. J., K. W. C. Peden, R. A. F. Dixon, and T. Kelly.** 1986. Functional organization of the simian virus 40 origin of DNA replication. Mol. Cell. Biol. **6:**1117–1128.
42. **Lin, H.-J. L., R. H. Upson, and D. T. Simmons.** 1992. Nonspecific DNA binding activity of simian virus 40 large T antigen: evidence for the cooperation of two regions for full activity. J. Virol. **66:**5443–5452.
43. **Luo, X., D. G. Sanford, P. A. Bullock, and W. W. Bachovchin.** Unpublished data.
44. **Luo, X., D. G. Sanford, P. A. Bullock, and W. W. Bachovchin.** 1996. Structure of the origin specific DNA binding domain from simian virus 40 T-antigen. Nature Struct. Biol. **3:**1034–1039.
45. **Lusky, M., J. Hurwitz, and Y.-S. Seo.** 1993. Cooperative assembly of the bovine papillomavirus E1 and E2 proteins on the replication origin requires an intact E2 binding site. J. Biol. Chem. **268:**15795–15803.
46. **Mastrangelo, I. A., P. V. C. Hough, J. S. Wall, M. Dodson, F. B. Dean, and J. Hurwitz.** 1989. ATP-dependent assembly of double hexamers of SV40 T antigen at the viral origin of DNA replication. Nature **338:**658–662.
47. **Mastrangelo, I. A., P. V. C. Hough, V. G. Wilson, J. S. Wall, J. F. Hainfield, and P. Tegtmeyer.** 1985. Monomers through trimers of large tumor antigen bind in region I and monomers through tetramers bind in region II of simian virus 40 origin of replication DNA as stable structures in solution. Proc. Natl. Acad. Sci. USA **82:**3626–3630.
48. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
49. **McVey, D., M. Strauss, and Y. Gluzman.** 1989. Properties of the DNA-binding domain of the simian virus 40 large T antigen. Mol. Cell. Biol. **9:**5525–5536.
50. **McVey, D., B. Woelker, and P. Tegtmeyer.** 1996. Mechanisms of simian virus 40 T-antigen activation by phosphorylation of threonine 124. J. Virol. **70:**3887–3893.
51. **Miller, J., P. Bullock, and M. Botchan.** 1984. Simian virus 40 T antigen is required for viral excision from chromosomes. Proc. Natl. Acad. Sci. USA **81:**7534–7538.
52. **Mohr, I. J., M. P. Fairman, B. Stillman, and Y. Gluzman.** 1989. Large T-antigen mutants define multiple steps in the initiation of simian virus 40 DNA replication. J. Virol. **63:**4181–4188.
53. **Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich.** 1986. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harbor Symp. Quant. Biol. **51:**263–273.
54. **Murakami, Y., and J. Hurwitz.** 1993. DNA polymerase α stimulates the ATP-dependent binding of simian virus tumor T antigen to the SV40 origin of replication. J. Biol. Chem. **268:**11018–11027.
55. **Myers, R. M., and R. Tijan.** 1980. Construction and analysis of simian virus 40 origins defective in tumor antigen binding and DNA replication. Proc. Natl. Acad. Sci. USA **77:**6491–6495.
56. **O'Reilly, D. R., and L. K. Miller.** 1988. Expression and complex formation of simian virus 40 large T antigen and mouse p53 in insect cells. J. Virol. **62:**3109–3119.
57. **Parsons, R., M. E. Anderson, and P. Tegtmeyer.** 1990. Three domains in the simian virus 40 core origin orchestrate the binding, melting, and DNA helicase activities of T antigen. J. Virol. **64:**509–518.
58. **Parsons, R., and P. Tegtmeyer.** 1992. Spacing is crucial for coordination of domain functions within the simian virus 40 core origin of replication. J. Virol. **66:**1933–1942.
59. **Parsons, R. E., J. E. Stenger, S. Ray, R. Welker, M. E. Anderson, and P. Tegtmeyer.** 1991. Cooperative assembly of simian virus 40 T-antigen hexamers on functional halves of the replication origin. J. Virol. **65:**2798–2806.
60. **Paucha, E., D. Kalderon, R. W. Harvey, and A. E. Smith.** 1986. Simian virus 40 origin DNA-binding domain on large T antigen. J. Virol. **57:**50–64.
61. **Rao, H., and B. Stillman.** 1995. The origin recognition complex interacts with a bipartite DNA binding site within yeast replicators. Proc. Natl. Acad. Sci. USA **92:**2224–2228.
62. **Ryder, K., A. L. DeLucia, and P. Tegtmeyer.** 1983. Binding of SV40 A protein to the BK virus origin of DNA replication. Virology **129:**239–245.
63. **Ryder, K., S. Silver, A. L. DeLucia, E. Fanning, and P. Tegtmeyer.** 1986. An altered DNA conformation in origin region 1 is a determinant for the binding of SV40 large T antigen. Cell **44:**719–725.
64. **Ryder, K., E. Vakalopoulou, R. Mertz, I. Mastrangelo, P. Hough, P. Tegtmeyer, and E. Fanning.** 1985. Seventeen base pairs of region I encode a novel tripartite binding signal for SV40 T antigen. Cell **42:**539–548.
65. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
66. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
67. **Schwyzer, M., R. Weil, G. Frank, and H. Zuber.** 1980. Amino acid sequence analysis of fragments generated by partial proteolysis from large simian virus 40 tumor antigen. J. Biol. Chem. **255:**5627–5634.
68. **Shalloway, D., T. Kleinberger, and D. M. Livingston.** 1980. Mapping of SV40 DNA replication origin region binding sites for the SV40 T antigen by protection against exonuclease III digestion. Cell **20:**411–422.
69. **Shortle, D., and D. Nathans.** 1979. Regulatory mutants of simian virus 40: constructed mutants with base substitutions at the origin of DNA replication. J. Mol. Biol. **131:**801–817.
70. **Simanis, V., and D. P. Lane.** 1985. An immunoaffinity purification procedure for SV40 large T antigen. Virology **144:**88–100.
71. **Simmons, D. T.** 1986. DNA-binding region of the simian virus 40 tumor antigen. J. Virol. **57:**776–785.
72. **Simmons, D. T.** 1988. Geometry of the simian virus 40 large tumor antigen-DNA complex as probed by protease digestion. Proc. Natl. Acad. Sci. USA **85:**2086–2090.
73. **Simmons, D. T., G. Loeber, and P. Tegtmeyer.** 1990. Four major sequence elements of simian virus 40 large T antigen coordinate its specific and

nonspecific DNA binding. J. Virol. **64:**1973–1983.
74. **Simmons, D. T., R. Upson, K. Wun-Kim, and W. Young.** 1993. Biochemical analysis of mutants with changes in the origin-binding domain of simian virus 40 tumor antigen. J. Virol. **67:**4227–4236.
75. **Simmons, D. T., K. Wun-Kim, and W. Young.** 1990. Identification of simian virus 40 T-antigen residues important for specific and nonspecific binding to DNA and for helicase activity. J. Virol. **64:**4858–4865.
76. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. Gene **67:**31–40.
77. **Spillman, T., D. Giacherio, and L. P. Hager.** 1979. Single strand DNA binding of simian virus 40 tumor antigen. J. Biol. Chem. **254:**3100–3104.
78. **Stahl, H., P. Droge, and R. Knippers.** 1986. DNA helicase activity of SV40 large tumor antigen. EMBO J. **5:**1939–1944.
79. **Stillman, B., R. D. Gerard, R. A. Guggenheimer, and Y. Gluzman.** 1985. T antigen and template requirement for SV40 DNA replication in vitro. EMBO J. **4:**2933–2939.
80. **Stillman, B. W.** 1989. Initiation of eukaryotic DNA replication *in vitro*. Annu. Rev. Cell Biol. **5:**197–245.
81. **Strauss, M., P. Argani, I. J. Mohr, and Y. Gluzman.** 1987. Studies on the origin-specific DNA-binding of simian virus 40 large T antigen. J. Virol. **61:**3326–3330.
82. **Tegtmeyer, P.** 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. **10:**591–598.
83. **Tegtmeyer, P., B. A. Lewton, A. L. DeLucia, V. G. Wilson, and K. Ryder.** 1983. Topography of simian virus 40 A protein-DNA complexes: arrangement of protein bound to the origin of replication. J. Virol. **46:**151–161.
84. **Tjian, R.** 1978. The binding site on SV40 DNA for a T-antigen related protein. Cell **13:**165–179.
85. **Tooze, J.** 1981. DNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
86. **Veal, J. M., and R. L. Rill.** 1988. Sequence specificity of DNA cleavage by bis(1,10-phenanthroline) copper(I). Biochemistry **27:**1822–1827.
87. **Virshup, D. M., A. A. R. Russo, and T. J. Kelly.** 1992. Mechanism of activation of simian virus 40 DNA replication by protein phosphatase 2A. Mol. Cell. Biol. **12:**4883–4895.
88. **Wobbe, C. R., F. Dean, L. Weissbach, and J. Hurwitz.** 1985. *In vitro* replication of duplex circular DNA containing the simian virus 40 DNA origin site. Proc. Natl. Acad. Sci. USA **82:**5710–5714.
89. **Wold, M. S., J. J. Li, and T. J. Kelly.** 1987. Initiation of simian virus 40 DNA replication *in vitro*: large-tumor-antigen- and origin-dependent unwinding of the template. Proc. Natl. Acad. Sci. USA **84:**3643–3647.
90. **Wright, P. J., A. L. DeLucia, and P. Tegtmeyer.** 1984. Sequence-specific binding of simian virus 40 A protein to nonorigin and cellular DNA. Mol. Cell. Biol. **4:**2631–2638.
91. **Wun-Kim, K., and D. T. Simmons.** 1990. Mapping of helicase and helicase substrate-binding domains on simian virus 40 large T antigen. J. Virol. **64:**2014–2020.
92. **Wun-Kim, K., R. Upson, W. Young, T. Melendy, B. Stillman, and D. T. Simmons.** 1993. The DNA-binding domain of simian virus 40 tumor antigen has multiple functions. J. Virol. **67:**7608–7611.