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Sequence-Specific Interactions between a Cellular DNA-Binding Protein and the Simian Virus 40 Origin of DNA Replication

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The core origin of simian virus 40 (SV40) DNA replication is composed of a 64-base-pair sequence encompassing T-antigen-binding site II and adjacent sequences on either side. A 7-base-pair sequence to the early side of T-antigen-binding site II which is conserved among the papovavirus genomes SV40, BK, JC, and SA12 was recently shown to be part of a 10-base-pair sequence required for origin activity (S. Deb, A. L. DeLucia, C.-P. Baur, A. Koff, and P. Tegtmeyer, Mol. Cell. Biol. 6:1663–1670, 1986), but its functional role was not defined. In the present report, we have used gel retention assays to identify a monkey cell factor that interacts specifically with double-stranded DNA carrying this sequence and also binds to single-stranded DNA. DNA-protein complexes formed with extracts from primate cells are more abundant and display electrophoretic mobilities distinct from those formed with rodent cell extracts. The binding activity of the factor on mutant templates is correlated with the replication activity of the origin. The results suggest that the monkey cell factor may be involved in SV40 DNA replication.

The 64-base-pair (bp) minimal core origin of DNA replication of simian virus 40 (SV40) has been defined by analysis of deletion and base substitution mutants in vivo and in vitro (2, 11, 31, 44, 45; for a review, see reference 12). The core origin is flanked by elements that enhance the replication activity of the origin, on the early side by T-antigen-binding site I and on the late side by the Sp1 binding sites (11, 21, 22). The core origin itself consists of multiple elements, as defined by mutational analysis: a sequence-specific early domain separated by a spacer region from T-antigen-binding site II, followed by another spacer and the AT-rich domain (8, 20).

The AT-rich domain of the SV40 origin appears to induce an altered conformation of DNA (9) and may be part of a binding site for a protein thought to be required for SV40 DNA replication in vitro (50). Genetic studies show that T-antigen binding to site II is essential in vivo (33). T-antigen helicase activity mediates DNA unwinding at the origin in vitro (7, 43) and may promote the association of DNA polymerase alpha with origin DNA (42). The functional role of the early sequence-specific domain of the origin has not yet been defined.

The origins of DNA replication in the genomes of the closely related primate papovaviruses SV40, BK, JC, and SA12 bear several highly homologous nucleotide sequences (Fig. 1). Some of these sequences, including the 5′-GAGGC-3′ pentanucleotide repeats, which direct the binding of SV40 and polyomavirus T antigens (5, 38, 39, 46) and the AT-rich domain, are also found in the less closely related polyomavirus and lymphotropic papovavirus (LPV) genomes. However, a conserved 12-bp sequence between T-antigen-binding sites I and II in the primate origins is absent in LPV and polyomavirus origin DNA (Fig. 1). This region overlaps a 10-bp sequence specifically required for the replication of SV40 DNA in vivo (8). These results, coupled with the conservation of this sequence among primate papovaviral genomes, raised the question as to whether these sequences could constitute a binding site for a primate-specific protein involved in viral DNA replication.

To address this question, we employed the gel retention technique (17, 19) to assay nuclear extracts for specific binding to wild-type and replication-deficient mutant SV40 origin DNA. This paper reports the specific binding of at least one nuclear protein to wild-type origin DNA. The loss of replication activity induced by mutations in the conserved sequence was accompanied by diminished binding of the nuclear protein. Whereas strong binding activity was observed with primate extracts, rodent cell extracts contained only weak binding activity with a mobility distinct from the activity of the primate extracts.

MATERIALS AND METHODS

Plasmid constructions and mutagenesis. A 200-bp *Hin*dIII-*Sph*I SV40 core origin DNA fragment was inserted into the large *Hin*dIII-*Sph*I fragment of pAT153 DNA (49). The *Eco*RI-*Sal*I fragment containing the SV40 origin DNA was then cloned into the *Eco*RI and *Sal*I sites of the polylinker in pSDL13 (28) to generate the wild-type origin plasmid pSDL13-HSwt. Single-stranded pSDL13-HSwt DNA was isolated after M13 infection of *Escherichia coli* XS127 (28) carrying this plasmid, as described in detail elsewhere (51; J. Schneider and E. Fanning, manuscript submitted). Oligonucleotide-directed mutagenesis was carried out with this template and the primers M1, M2, and M3 (M1, 5′-ATTC-CAGAGGGAGTGAGGA-3′; M2, 5′-CCAGAAGTCGCGA-GGAGGC-3′; M3, 5′-TGAGCTATTAGAGAAGTAG-3′) to generate the mutant origin plasmids 3/4, 5/6, and 7/8. Mutants were identified by colony hybridization to end-labeled mutant primer DNA and confirmed by supercoil DNA sequencing (4) by using a universal pUC reverse primer (5′-CAGGAAACAGCTATGAC-3′) (a gift of P. Heinrich). The *Eco*RI-*Sal*I fragment was then recloned into pUC18.

SV40 DNA replication. COS1 cells (23) (10^6) in 10-cm-diameter dishes were washed with Tris-saline and transfected with 0.2 μg of plasmid DNA in DEAE-dextran (500 μg/ml in Tris-saline) (34). After 45 min at 37°C, the cells were washed again and fed with fresh medium. To ensure equivalent transfection efficiencies with the different plasmids, the input DNAs were compared by electrophoresis in 1% agarose gels and ethidium bromide staining.

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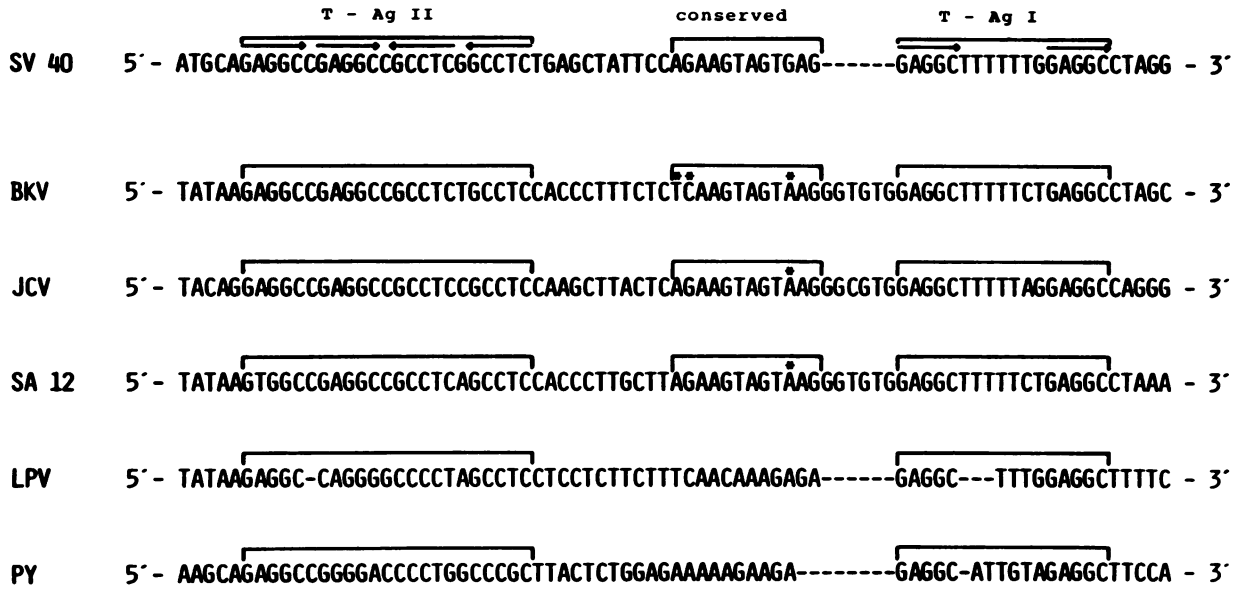


FIG. 1. Conserved nucleotide sequence in primate papovavirus origins of DNA replication. The sequence of SV40 T-antigen-binding sites I and II (bracketed) and the region between them is compared with the origin regions of BK (41), JC (18), SA12 (6), and the more distantly related polyomavirus and LPV DNAs (36, 47). A 12-bp sequence conserved among the closely related viruses and absent in LPV and polyomavirus DNA is bracketed. Variations from the SV40 sequence are designated by asterisks.

After 48 to 60 h, low-molecular-weight DNA was isolated (27), extracted with phenol, ethanol precipitated, and cleaved with *Eco*RI and *Dpn*I. The digestion products were analyzed by electrophoresis in 1% agarose gels and Southern blot hybridization to nick-translated pUC18 vector DNA.

Nuclear extracts and cell lines. Nuclear extracts were made from tissue culture cell lines essentially as described by Dignam et al. (13), except that nuclear extraction buffer C contained 0.35 M rather than 0.43 M NaCl. All buffers contained 1 mM phenylmethylsulfonyl fluoride and 10 mM $Na_2S_2O_4$ as protease inhibitors. Extracts were stored in aliquots at −70°C. The following cell lines were used: TC7 (37), a monkey kidney line; HeLa, a human cervical carcinoma line; 293 (24), an adenovirus 5-transformed human embryo kidney line; BHK, a Syrian hamster fibroblast line; 3T3, a Swiss mouse fibroblast line; Rat 2 (48), a rat fibroblast line; BL64, a Burkitt lymphoma line (25); IARC 549, a human lymphoblastoid line immortalized with Epstein-Barr virus (25); and 143tk$^-$, a human fibroblast line (1). Nuclear extracts generally contained 2 to 4 mg of protein per ml as measured by the Bradford assay (3) with bovine serum albumin as a standard (Bio-Rad Laboratories, Munich, Federal Republic of Germany).

Preparation of labeled oligonucleotides and restriction fragments. Oligonucleotides were synthesized on an Applied Biosystems model 380A DNA synthesizer and purified by denaturing polyacrylamide gel electrophoresis as described previously (15). Synthetic oligonucleotides (10 pmol) were 5′ end labeled with 20 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham, Braunschweig, Federal Republic of Germany) and T4 polynucleotide kinase (Pharmacia-PL, Freiburg, Federal Republic of Germany) in 50 mM Tris hydrochloride (pH 9.5)–1 mM $MgCl_2$–5 mM dithiothreitol–3.5% glycerol in a 10-μl reaction mixture for 45 min at 37°C. Labeled oligonucleotides were separated from unincorporated nucleotide by gel filtration (Nick columns; Pharmacia). Pairs of oligonucleotides were heated at 68°C and reannealed at 25°C for 15 min. The 109-bp *Hin*dIII-*Nco*I SV40 cs1097 origin DNA fragment (14) was purified by agarose gel electrophoresis (16) and 5′ end labeled in a similar fashion.

Alternatively, synthetic oligonucleotide pairs (1 pmol) were heated to 68°C for 10 min, annealed at 25°C for 15 min, and labeled with Klenow polymerase (Pharmacia) and either [α-^{32}P]dCTP or [α-^{32}P]dTTP or both (3,000 Ci/mmol; Amersham), together with unlabeled dGTP and, if appropriate, dCTP or dTTP to ensure that the recessed ends were filled in (32). Labeled oligonucleotides were purified by gel filtration. Double-stranded unlabeled oligonucleotides were purified by electrophoresis in 15% nondenaturing polyacrylamide gels (32).

Gel retention assay. Binding-reaction mixtures included 500 ng of poly(dA-dT)–poly(dA-dT) (Pharmacia), 10 fmol of labeled oligonucleotides in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–KOH (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 10% glycerol, and 6 μg of nuclear protein adjusted to 0.15 M NaCl. The amounts of protein and labeled oligonucleotide were varied in some experiments, as stated in the figure legends. The total volume of each reaction mixture was 30 μl. The reaction was allowed to proceed for 15 min, generally at 25°C, and then analyzed by electrophoresis in 11% nondenaturing polyacrylamide gels exactly as described earlier (40).

RESULTS

Binding of cellular proteins to the SV40 origin of DNA replication. To determine whether the SV40 core origin of DNA replication carries binding sites for cellular proteins, we used an end-labeled restriction fragment bearing T-antigen-binding site II and the early- and late-region domains required for replication (8, 9), but lacking T-antigen-binding site I and the enhancer region, as a template in a gel retention assay (17, 19, 40). Specific complexes formed between the labeled DNA and factors present in nuclear extracts of primate and rodent cells were detected by their slower

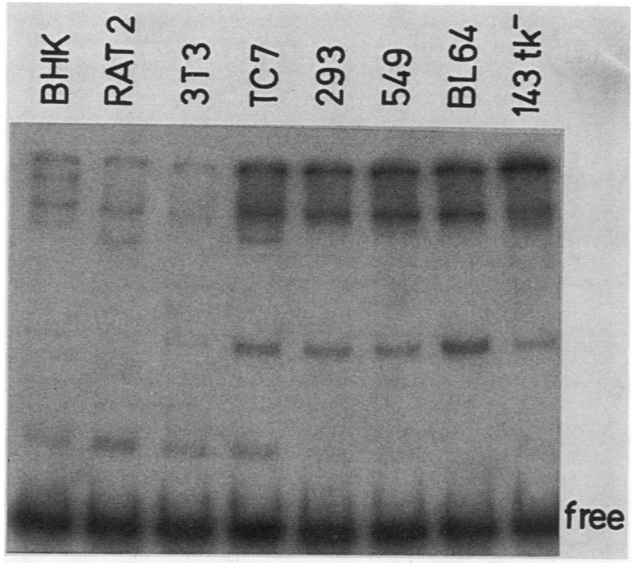


FIG. 2. Occurrence of origin binding activity in nuclear extracts of primate and rodent cell lines. Binding of 6 μg of nuclear proteins from the indicated cell lines to the 5'-end-labeled *Hin*dIII-*Nco*I origin fragment of cs1097 SV40 DNA was tested in a gel retention assay. The position of the free unbound DNA fragment is indicated.

electrophoretic migration relative to unbound DNA in nondenaturing gels.

Two prominent protein-DNA complexes, as well as several minor ones, were formed in each of the primate extracts (Fig. 2). The complexes formed with lymphoid, fibroblastic, and epithelioid cell extracts were similar to one another. Complexes were also observed with several rodent extracts, though at a much lower abundance. A smaller restriction fragment from the origin region (*Hin*dIII to *Bgl*I), which spans the region between T-antigen-binding sites I and II, was used in an initial attempt to identify the DNA sequences recognized by these cellular proteins. The pattern of DNA-protein complexes detected with this template resembled that obtained with the intact core origin fragment (data not shown), suggesting that these proteins interact with the early origin domain.

A panel of oligonucleotides spanning the region between T-antigen-binding sites I and II was synthesized to test for specific binding of cellular proteins to this domain of the origin (Fig. 3). One oligonucleotide pair carried the wild-type origin sequence. Two pairs of oligonucleotides (W3W4 and W5W6) carried mutations in the central 7-bp sequence conserved in SV40, BK, JC, and SA12 origin DNA (Fig. 1). One pair of oligonucleotides (W7W8) carried mutations outside of the 12-bp conserved sequence.

Figure 4A shows that three DNA-protein complexes (bands a to c) similar to those observed using labeled SV40 origin DNA were detected with each of the SV40 oligonucleotide templates. In some experiments a fourth DNA-protein complex (a') migrating more slowly than band a was also detected. Band c was significantly weaker with templates W3W4 and W5W6 than with the wild-type template. A heterologous template derived from the human c-*myc* gene promoter (40), used as a control, formed a major DNA-protein complex different from those formed on SV40 DNA. Free oligonucleotides migrated in band d.

The amounts of labeled SV40 oligonucleotides and nuclear extract were varied with little effect on the pattern of protein-DNA complexes detected (data not shown). Reduction in the amount of unlabeled poly(dA-dT)–poly(dA-dT) led to increased amounts of labeled DNA unable to migrate into the gel, with a concomitant decrease in complexes (bands a to c) and free oligonucleotide (data not shown). A pattern similar to that observed with the wild-type oligonucleotide W1W2 was also obtained using W1W2 template labeled with Klenow polymerase (cf. Fig. 6 and 8B) as the template. Nuclear extracts denatured in sodium dodecyl sulfate or heated for 10 min at 70°C did not form DNA-protein complexes that could be detected with this assay. The extent of complex formation at 0°C was similar to that formed at 25°C, but reduced at 37°C. Mild proteolytic treatment (in the presence of phenylmethylsulfonyl fluoride

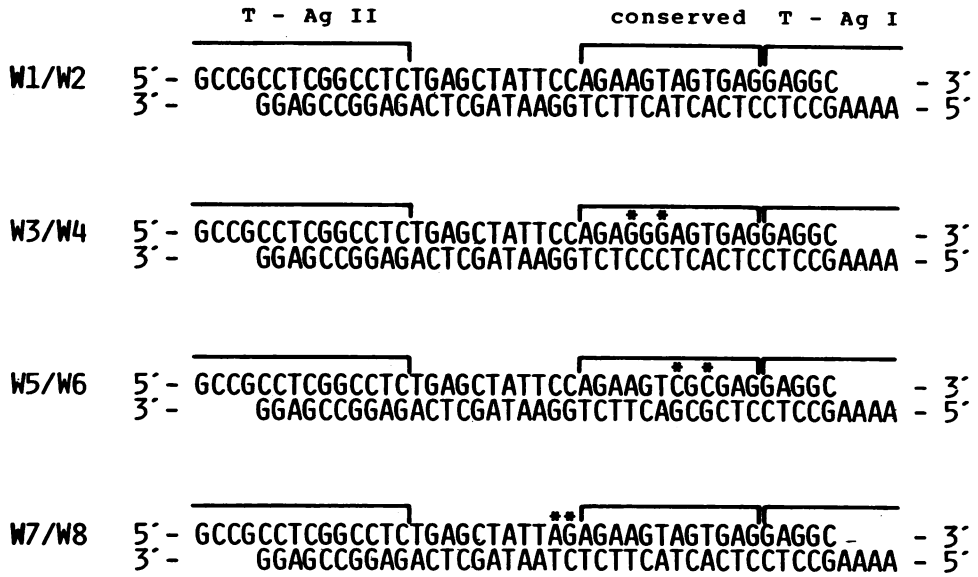
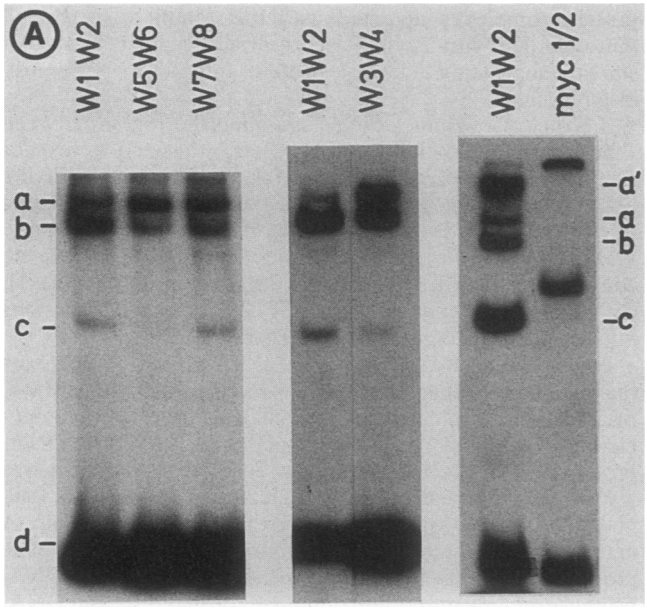


FIG. 3. Synthetic oligonucleotides for DNA binding. The conserved 12-bp sequence and portions of T-antigen-binding sites I and II are bracketed. Mutations relative to the wild-type sequence W1W2 are indicated by asterisks.



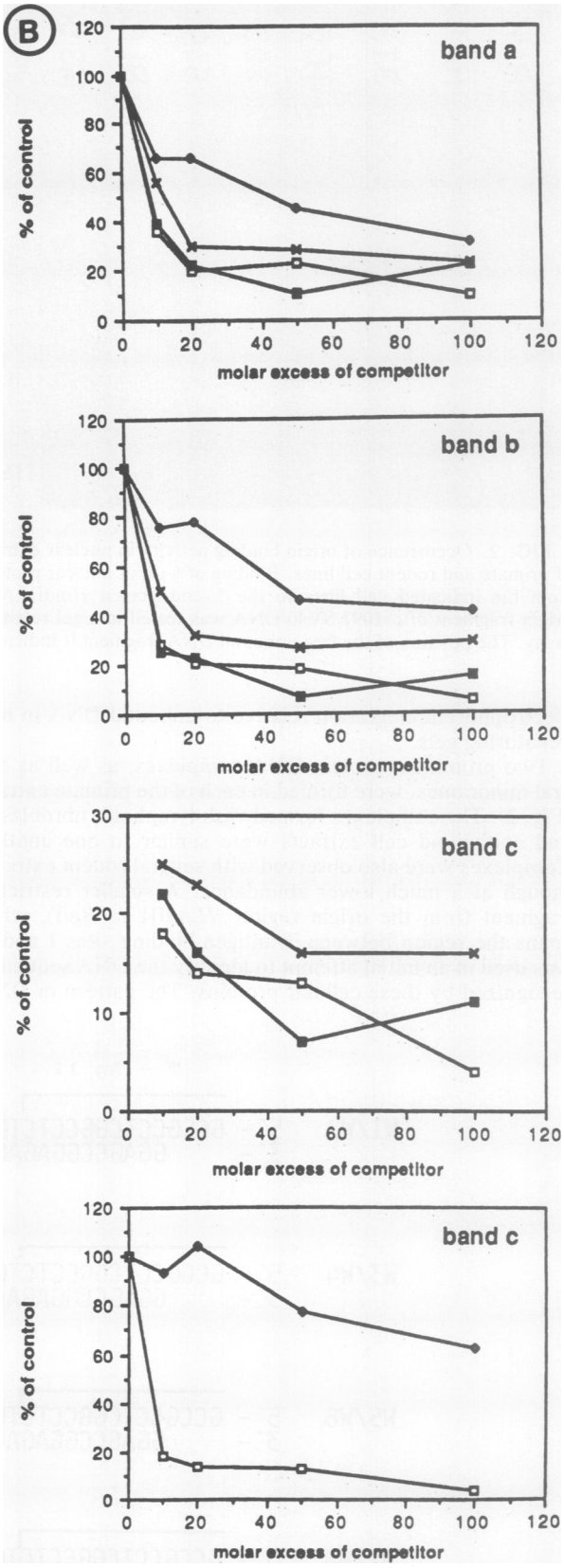


FIG. 4. Specific binding of nuclear factors to double-stranded wild-type and mutant SV40 DNAs and to heterologous DNA. (A) Nuclear protein (6 μg) from TC7 cells was mixed with 10 fmol of 5′-end-labeled reannealed oligonucleotides as indicated, and binding was analyzed by gel retention. Myc 1 and 2 bear sequences from the human c-*myc* promoter (40). Myc 1, 5′-TCGACCTCCCCAC-CTTCCCCACCCTCCCCACCCTCCCCAT; myc 2, 5′-CTAGATG-GGGAGGGTGGGGAGGGTGGGGAAGGTGGGGAGG. (B) Binding of 3 μg of TC7 nuclear protein to 20 fmol of Klenow-labeled reannealed W1W2 was assayed in the presence of 10-, 20-, 50-, and 100-fold amounts of purified double-stranded competitor oligonucleotides. DNA-protein complex bands detected by autoradiography were evaluated quantitatively by microdensitometry. Symbols: □, W1W2; ×, W3W4; ●, W5W6; ■, W7W8.

and sodium bisulfite) prevented formation of complexes a and b, whereas traces of complex c and a new faster-migrating band (visible in Fig. 8B) remained. The DNA-binding activity was stable to RNase A digestion (data not shown). The addition of 5 mM EDTA or EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] to the binding assay had no effect on protein-DNA complex formation.

The specificity of the protein-origin DNA interactions was tested in competition assays. Binding of a nuclear protein to the labeled template is diminished if the unlabeled competitor DNA carries a specific binding site for the protein. A quantitative evaluation of data from several competition experiments (Fig. 4B) confirms the specific binding of the band c protein to W1W2. Oligonucleotide W7W8 competed for binding to W1W2 as efficiently as the homologous sequence. W3W4 competed with a somewhat lower efficiency, and W5W6 failed to compete efficiently, even at a 100-fold molar excess over the labeled template. The heterologous oligonucleotides myc 1 and 2 also failed to compete at this concentration (data not shown). The patterns of competition obtained for the band a and band b proteins were similar, except that oligonucleotide W5W6 competed more efficiently and W3W4 competed less efficiently than with band c.

The results indicate that at least one, perhaps more, nuclear proteins bind specifically to DNA sequences present in templates W1W2 and W7W8, more weakly to template

W3W4, and very weakly to template W5W6. Since the latter two templates, taken together, differ from the wild-type template in only four nucleotides that span six of the seven highly conserved nucleotides in this origin domain (Fig. 1 and 3), we suggest that the 7-bp motif constitutes part or all of a specific recognition sequence for a cellular protein.

Protein interactions with single-stranded SV40 origin DNA. Since many of our experiments were carried out with 5'-end-labeled oligonucleotide templates that may contain residual single-stranded oligonucleotides, we also tested for the binding of nuclear proteins to radiolabeled single-stranded oligonucleotides. Figure 5A shows that monkey proteins bind to the heterologous oligonucleotides myc 1 and myc 2, used as controls. Three or four prominent complexes, different from the myc complexes, were formed (bands a' to c) with SV40 oligonucleotides, depending on the template used. Curiously, intense bands of complex c were observed with even-numbered oligonucleotides and to a lesser extent with W1, but much weaker bands were seen with the other complementary strands. The intensity of band a' appeared to parallel that of band c, but the relative intensities of bands a' to c varied slightly among different preparations of extract. Binding activity of the nuclear extracts was resistant to RNase, EDTA, and EGTA, but sensitive to protease digestion and to heating at 70°C for 10 min (data not shown).

The apparent preference of the single-stranded DNA-binding activity for particular oligonucleotide sequences was further tested in competition assays. In Fig. 5B, binding of nuclear proteins to the single-stranded W2 wild-type sequence was carried out in the presence of the other homologous mutant oligonucleotides. The replication-competent sequence in oligonucleotide W8 competed as well as the wild-type W2, but competition by the W4 and W6 sequences was markedly reduced (Fig. 5B). When oligonucleotide W1 was used as the labeled template and the odd-numbered oligonucleotides were used as competitor, W1 competed but W3, W5, and W7 did not (results not shown). Myc 1 and 2 also failed to compete (data not shown).

To investigate the relationship of the single-stranded DNA-binding activity (Fig. 5) to the double-strand activity (Fig. 4), we performed competition experiments. Reannealed wild-type template W1W2 was labeled with Klenow polymerase at the 3' end of W2 (Fig. 6) or at the 3' end of W1 (data not shown) in the presence of unlabeled nucleotide triphosphates to ensure that a completely double-stranded template was generated. Binding of nuclear extracts to the radiolabeled double-stranded template was carried out in the presence of unlabeled single-stranded competitor oligonucleotide. Figure 6 demonstrates that, in general, the even-numbered oligonucleotides competed efficiently for nuclear proteins binding to the double-stranded template, whereas the odd-numbered oligonucleotides competed less well. W6 competed considerably less effectively than the other even-numbered oligonucleotides. Among the odd-numbered oligonucleotides, W1 competed best. Myc 1 and 2 did not compete for binding to W1W2 (data not shown). Thus, the pattern of competition of single-stranded DNA with the double-stranded template (Fig. 6) closely resembles the

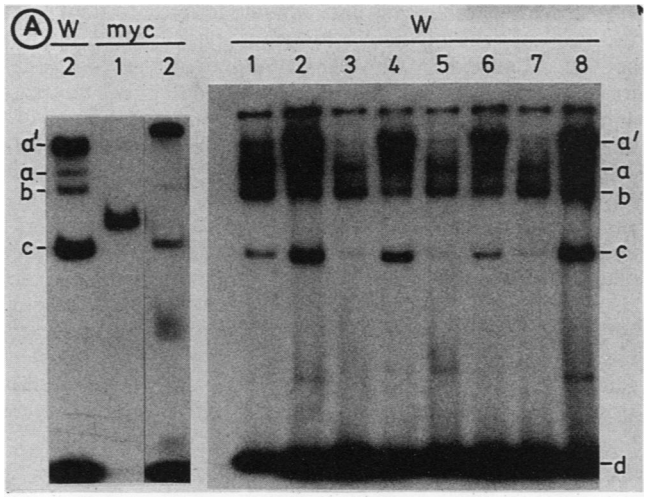
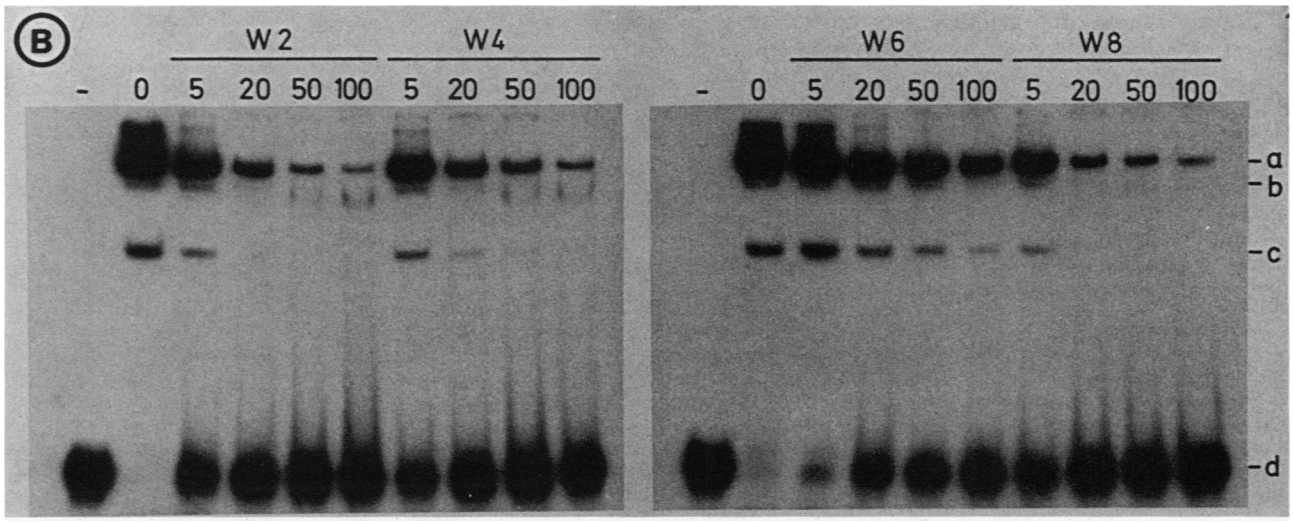


FIG. 5. Binding of nuclear protein to single-stranded wild-type and mutant SV40 DNAs and heterologous DNA. (A) Binding of 10 fmol of 5'-end-labeled oligonucleotides W1 through W8, and the heterologous oligonucleotides myc 1 and myc 2, with proteins in 6 μg of TC7 nuclear extract was analyzed by gel retention. (B) Binding of nuclear proteins (6 μg) from TC7 cells to 5'-end-labeled oligonucleotide W2 (10 fmol) was assayed as in panel A in the absence (lane 0) and presence of a 5-, 20-, 50-, or 100-fold molar amount of unlabeled single-stranded oligonucleotide, as indicated. The lane designated (−) contained no nuclear protein.



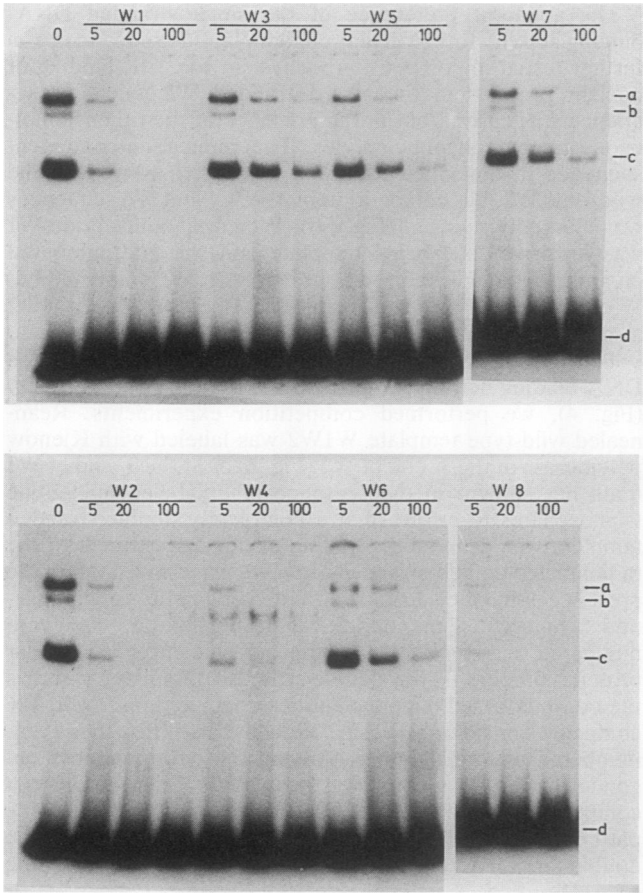


FIG. 6. Oligonucleotides W1W2 were annealed and labeled with Klenow polymerase. Binding of 10 fmol of labeled double-stranded W1W2 to TC7 nuclear extract was assayed without the addition of unlabeled oligonucleotide (lane 0) or in the presence of a 5-, 20-, or 100-fold molar amount of unlabeled single-stranded oligonucleotide, as indicated.

activity observed with single-stranded templates (Fig. 5). This similarity in template specificity suggests that the single-stranded binding activity is related to the double-stranded binding activity detected in Fig. 4.

Replication of mutant SV40 origin DNA in vivo. To facilitate the correlation of protein-binding studies with in vivo replication activity of the mutant SV40 origin DNAs, the W3W4, W5W6, and W7W8 mutations were also created in a plasmid bearing the core origin of replication by oligonucleotide-directed mutagenesis. The resulting plasmids were assayed for replication activity by transfection into COS1 monkey cells, which express T antigen constitutively. Two days after transfection, plasmid DNA was isolated, incubated with *Dpn*I to digest methylated input DNA, and linearized with *Eco*RI. Newly replicated DNA was analyzed by gel electrophoresis, hybridization to radiolabeled pUC18 vector DNA, and autoradiography. Figure 7 shows one band of newly replicated wild-type SV40 origin plasmid of the expected size. Mutant W7W8 replicated to the level of wild-type DNA, whereas replication of mutant W3W4 was weak and that of W5W6 was not detectable (Fig. 7). The mutants W3W4 and W5W6 bear lesions within the most conserved 7-bp sequence in this domain of the core origin, whereas the lesions in mutant W7W8 are localized outside the conserved region. Thus, the results indicate that the conserved sequence is required for SV40 replication in vivo and confirm a recent report from Deb et al. (8).

Interactions of SV40 origin DNA with nuclear proteins from various cell lines. To confirm the results obtained with the SV40 origin restriction fragment (Fig. 2), nuclear proteins prepared from several mammalian cell lines were assayed for specific binding to SV40 oligonucleotides. In Fig. 8A, 5′-end-labeled double-stranded wild-type W1W2 and mutant W3W4 DNAs formed complexes of different intensities and different mobilities with monkey and rat nuclear proteins. The complexes formed with monkey proteins were more intense, and complex c clearly migrated faster than the corresponding complex with rat protein. Since no difference in mobility was detected when the restriction fragment was used as a template (Fig. 2), the higher resolution provided by the oligonucleotide templates is probably required to distinguish this difference. Competition assays carried out with unlabeled double-stranded oligonucleotides demonstrated that the complexes formed with rat nuclear proteins are derived from a sequence-specific interaction (data not shown). The addition of rat nuclear proteins to monkey proteins before the binding reaction did not reduce the level of complex formation with the monkey protein (data not shown). The results suggest that a lower binding affinity or lower abundance of the rat proteins in nuclear extracts is probably responsible for the lower level of binding.

A survey of other cell lines was conducted to determine the best sources of the SV40 origin-specific DNA-binding activity. Figure 8B shows an example of the complexes formed between Klenow-labeled wild-type W1W2 template and nuclear proteins from several primate and rodent cell lines. In general, intense complexes were observed with primate nuclear extracts. Strong complexes observed with extracts of several human lymphoid cell lines fell into the same pattern observed with TC7 and HeLa extracts (Fig. 2 and data not shown). The complexes formed in the presence and absence of SV40 T antigen were identical (data not shown), in keeping with the inability of T antigen to bind single pentanucleotides at 150 mM salt (38). Weaker complex formation was observed with all rodent extracts tested. Furthermore, the mobility of rodent band c′ was consistently slower than that of primate band c. No complexes were

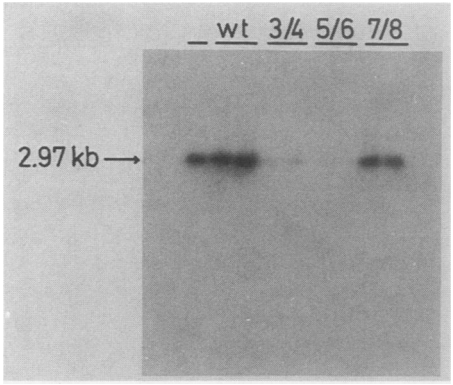


FIG. 7. DNA replication of SV40 origin-containing plasmids in COS1 cells. Plasmid DNAs carrying the wild-type origin or mutant origins 3/4, 5/6, or 7/8 were transfected in duplicate into COS1 cells. After 48 h, low-molecular-weight DNA was extracted, digested with *Eco*RI and *Dpn*I, and analyzed by agarose gel electrophoresis and Southern blot hybridization with labeled pUC18 vector DNA. The marker was *Eco*RI-linearized pUC18-HSwt DNA. kb, Kilobases.

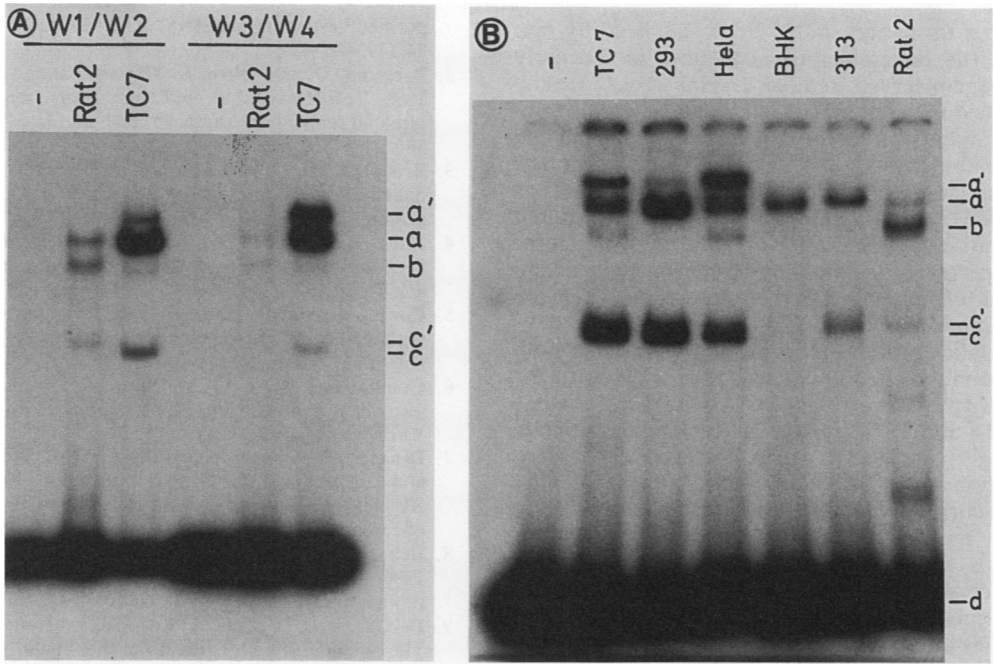


FIG. 8. SV40 oligonucleotide-binding activity of rodent and primate cell extracts. (A) Binding of 10 fmol of 5'-end-labeled reannealed W1W2 or W3W4 to proteins in 6 μg of nuclear extract from TC7 or Rat 2 cells was assayed by gel retention. (B) Binding of 10 fmol of reannealed W1W2 labeled with Klenow polymerase to 6 μg of nuclear proteins from various cell lines was assayed by gel retention. The left lane contained oligonucleotide but no nuclear extracts. Some binding of proteolytic products can be detected in the Rat 2 lane above band d.

detected with nuclear extracts of pig liver or *Xenopus* oocytes (data not shown).

DISCUSSION

We have identified proteins from monkey cells that specifically recognize a sequence located in the early domain of the SV40 core origin of DNA replication. Several bands of DNA-protein complexes were detected which appear to arise from sequence-specific interactions. The present data do not distinguish whether the three major complexes contain the same protein, complexed with homologous or heterologous proteins in different oligomeric forms, or whether different proteins bind to the same sequence. However, preliminary results indicate that the protein that forms complex c can be separated by ion-exchange chromatography from the factors that form complexes a and b (P. Alliger, W. Traut, and E. Fanning, unpublished results).

Monkey proteins also bound to single-stranded SV40 origin DNA. Rather unexpectedly, these proteins preferred certain single-stranded SV40 oligonucleotides over others. However, the fact that the complementary sequences W1 and W2 were both bound suggests that the single-strand binding activity is not truly sequence specific. Moreover, if the conserved sequence in W2 were bound specifically, the related sequence in the spacer region adjacent to T-antigen-binding site I would have to account for W1 binding. However, W3, W5, and W7 bear the same sequence as W1 in the spacer region and yet bind very poorly to the cellular proteins. Moreover, some unrelated single-stranded sequences from adenovirus or SV40 coding sequences are also bound by these proteins (W. Traut and E. Fanning, unpublished data). Thus, a sequence-specific single-strand binding activity must be ruled out. On the other hand, some single-stranded DNAs, such as the myc oligonucleotides, do not form complexes with these proteins (Fig. 5A), suggesting a preference for certain types of nucleotide sequences or composition.

The patterns of protein-DNA complexes formed with single- and double-stranded SV40 templates are remarkably similar. Thus, the possibility that the protein, in conjunction with other components of the nuclear extract, generates single-stranded DNA from the double-stranded input DNA must be considered. However, the addition of chelating agents to the binding assays, which would presumably affect an enzymatic unwinding reaction, did not influence the pattern of complexes observed in gel retention (unpublished results). Furthermore, given the stronger binding observed with even-numbered single-stranded oligonucleotides, this explanation would predict that the signal from complexes formed with double-stranded template labeled on the even-numbered strand using Klenow polymerase should be more intense than that from complexes formed with double-stranded template labeled on the other strand. However, DNA-protein complexes were formed equally well with both templates (unpublished results).

The results of competition experiments indicate that the same cellular protein could be responsible for both single-strand and double-strand binding activities (Fig. 6). On the other hand, other explanations could also account for these observations. The early domain of the core origin includes an imperfect palindrome sequence in SV40 (Fig. 1). Thus, the single-stranded oligonucleotides could possibly form hairpins that could be recognized by a double-strand-specific DNA-binding protein. However, this alternative seems unlikely for several reasons. First, to explain the data in Fig. 5 and 6, hairpins formed by the even-numbered oligonucleotides would have to be recognized more efficiently than those formed by odd-numbered oligonucleotides, which would carry essentially the same double-stranded DNA sequence.

Second, a hairpin formation should be destabilized by mutations in both the nonconserved sequences in the early domain and the conserved sequence. However, monkey proteins bind to double-stranded and single-stranded templates carrying mutations in the nonconserved half of the palindrome (unpublished results). Finally, the palindrome is not conserved among other primate papovavirus origins of replication (Fig. 1), nor is a palindromic sequence required for SV40 replication (8). Thus, the simplest explanation for the binding properties of the monkey proteins on single- and double-stranded templates is that the same factor is responsible for both activities.

Binding of monkey proteins to form bands a, b, and c with double-stranded mutant templates was correlated with the replication activity of core origin DNA carrying the same mutations (Fig. 4 and 7). The extent of binding to template W3W4 was slightly reduced, which corresponded to decreased replication activity. Binding to W5W6 was markedly reduced, which corresponded to a further drop in replication activity of the mutant origin. Mutant W7W8 bound to monkey proteins and replicated as well as the wild-type template. This correlation raises the question as to whether binding of the monkey protein to this origin sequence is involved in SV40 replication. In support of this interpretation, protein-DNA complexes of similar intensity and mobility were detected in extracts of other primate cells which support SV40 replication in vitro (30, 35, 44). Complexes of reduced intensity and, when oligonucleotide templates were used, different mobility were observed with extracts of rodent cells, which do not permit SV40 DNA replication in vivo or in vitro. Furthermore, cytoplasmic extracts prepared from monkey cells for in vitro replication assays (29) contain the same DNA-binding activity as the nuclear extracts (unpublished results).

In the context of DNA replication, the ability of the monkey cell factor to bind to certain types of single-stranded DNA is particularly intriguing. The even-numbered single-stranded oligonucleotide preferred by the factor is the template for continuous strand synthesis in the direction of the early genes in vivo (26). Priming and initiation of DNA synthesis in the early direction occurs first, followed later by priming and continuous strand synthesis in the late direction (10). If the monkey cell factor identified here were in fact involved in SV40 replication, it would be interesting to determine whether it plays a role in prepriming, assisting T antigen in unwinding at the origin for example (7, 43), or whether it might help to direct DNA polymerase α-primase to primer start sites on the early template. Clearly, purification of the monkey cell factor and in vitro SV40 replication assays will provide the means with which to answer these questions.

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