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Sequence recognition protein for the 17-base-pair A+T-rich tract in the replication origin of simian virus 40 DNA

(DNA synthesis/HeLa cells/DNA polymerase α/multienzyme complex)

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ABSTRACT **A DNA-binding protein has been identified that recognizes runs of deoxyadenines and/or deoxythymines (dA/dT sequences) and purified from a chromatographic fraction containing the multiprotein DNA polymerase α–primase complex of HeLa cells by successive steps of chromatography on oligo(dT)-cellulose and Q-Sepharose. Polyacrylamide gel electrophoresis of the purified dA/dT sequence-binding protein in the presence of $NaDodSO_4$ showed a single protein band of 62 kDa. Nitrocellulose filter binding assays using homopolydeoxynucleotides indicated that the purified protein preferentially binds to dA/dT sequences in single-stranded or duplex DNAs. Gel mobility shift assays with a variety of DNAs showed that the purified protein specifically binds to a fragment of simian virus 40 DNA containing the minimal (core) origin for replication. The binding occurred in a protein-dependent manner and in the presence of a vast excess of competing DNAs lacking the simian virus replication origin. The origin binding was reduced, however, when DNA fragments from simian virus 40 deletion mutants containing deletions within the 17-base-pair A+T-rich tract in the core DNA replication origin were used in the assays. These results indicate that the dA/dT sequence-binding protein preferentially binds to the 17-base-pair A+T-rich tract and suggest a possible role for the protein in the initiation of DNA replication.**

The replication origins in DNA from prokaryotic and eukaryotic cells as well as DNA viruses have commonly been found to contain A+T-rich tracts (1, 2). These A+T-rich tracts are considered to play a role in the initiation of DNA replication *in vivo*. A 17-base-pair (bp) A+T-rich tract residing within the 64-bp minimal (core) origin for simian virus 40 (SV40) DNA replication has been shown to be essential for SV40 replication *in vivo* (3–5) and *in vitro* (3). This A+T-rich tract is proximal to the large tumor (T) antigen binding site II and represents a bending locus (6). There has been speculation that this A+T-rich tract may function *in vivo* as a signal region for the binding of cellular proteins involved in the initiation of SV40 DNA replication (3–6).

In prokaryotes, site-specific DNA-binding proteins have been shown to function in the initiation of DNA replication (2, 7–11). The mechanisms involved in the initiation of DNA replication in eukaryotes are not as well delineated. However, highly purified forms of DNA polymerase α–primase from a variety of eukaryotic cells have now been shown to exist as multiprotein complexes that may function in the process (12). A 640-kDa multiprotein form of the DNA polymerase α–primase complex from HeLa cells (13) interacts with other enzymes and proteins for DNA synthesis to form a sedimentable multienzyme complex (18–20 S). The multienzyme complex is capable of complete replication of SV40 DNA *in vitro* in the presence of T antigen (14). In this report we describe the purification to apparent homogeneity of a DNA-binding protein, isolated from the multienzyme complex, that recognizes runs of deoxyadenines and/or deoxythymines (dA/dT sequences), and show that it preferentially binds to the 17-bp A+T-rich tract in the minimal origin of SV40 DNA.

MATERIALS AND METHODS

Materials. ^3H- and ^{32}P-labeled nucleotides were purchased from New England Nuclear and Amersham. Oligo(dT)-cellulose was obtained from Collaborative Research and Q-Sepharose was obtained from Pharmacia. [^3H]Poly(dT) and [^3H]poly(dA) were prepared as described (15). The $pSV0^+$ plasmid derived from pBR322 and containing an SV40 DNA insert of the replication origin and plasmids $pSV0^-$, pSV0dl5, pSV0dl9, pS1dl2, pS1dl5, and pS1d18 containing deletions in the SV40 origin DNA sequences were generously provided by Bruce Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and their construction has been described elsewhere (3). The SV40 DNA insert was isolated from plasmids $pSV0^+$, $pSV0^-$, pSV0dl5, and pSV0dl9 after digestion with *Hin*dIII and *Sph* I restriction endonucleases and from the plasmids pS1d12, pS1d15, and pS1d18 after digestion with *Bgl* II and *Taq* I. The DNA fragments were isolated by agarose gel electrophoresis as described (16). The complimentary, synthetic, octadecameric oligonucleotides (17) were kindly furnished by Paul Zamecnik (Worcester Foundation for Experimental Biology, Shrewsbury, MA). All other reagents used were as described by this laboratory (13–15, 18).

Purification of the dA/dT Sequence Recognition Protein. The dA/dT sequence recognition protein was purified from the 0.15 M KCl DEAE-Bio-Gel fraction (8 mg of protein) containing the 640-kDa multiprotein DNA polymerase α–primase complex (13) that was purified from a homogenate of HeLa cells (33 g) (14). The DEAE-Bio-Gel 0.15 M KCl eluate was dialyzed against TDEG buffer [50 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/1 mM EDTA/10% (vol/vol) glycerol] containing 0.2 M KCl and loaded onto a 3-cm^3 column of oligo(dT)-cellulose that had been equilibrated with 0.2 M KCl/TDEG buffer. After washing with 10 column volumes of 0.2 M KCl/TDEG buffer, material was eluted with a continuous gradient of 0.2 M–0.5 M KCl in TDEG buffer. Fractions containing the peak [^3H]poly(dA)- and [^3H]poly(dT)-binding activity were pooled, dialyzed against TDEG buffer containing 0.05 M KCl, and loaded onto a 0.5-cm^3 column of Q-Sepharose that was equilibrated with 50 mM KCl/TDEG buffer. After washing with 10 column volumes of 50 mM KCl/TDEG buffer, material was eluted by a continuous gradient of 0.05–1 M KCl in TDEG buffer. The peak fractions of binding activity to [^3H]poly(dA) and [^3H]poly(dT) were

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Abbreviations: dA/dT sequence, runs of deoxyadenines or deoxythymines; SV40, simian virus 40; T, tumor.

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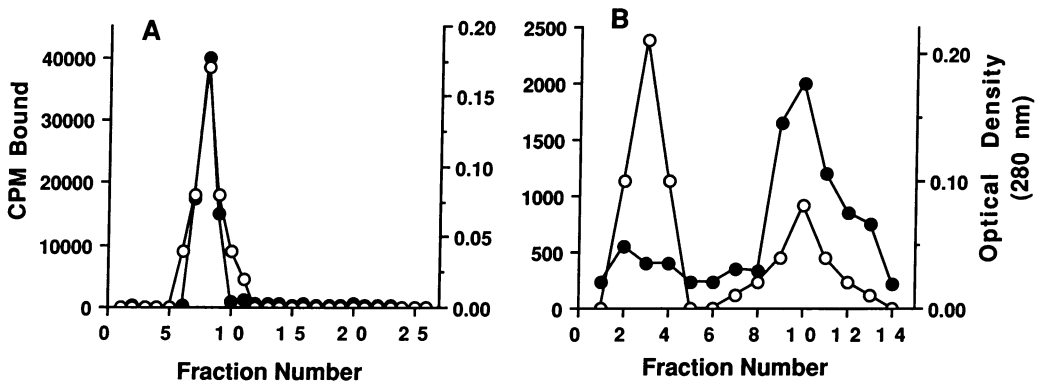


FIG. 1. Elution profiles of the poly(dA) and poly(dT) sequence-binding activities during chromatography on oligo(dT)-cellulose and Q-Sepharose. (*A*) The 0.15 M KCl eluate from DEAE-Bio-Gel containing the DNA polymerase α_2 activity (13) was chromatographed on an oligo(dT)-cellulose column. Fractions (1 ml) were collected and assayed for [^{3}H]poly(dT) and [^{3}H]poly(dA) (●) binding and OD_{280} (○). (*B*) The fractions from the oligo(dT)-cellulose containing the peak [^{3}H]poly(dT) and [^{3}H]poly(dA)-binding activities were pooled and chromatographed on a Q-Sepharose column. Fractions (1 ml) were collected for measurement of [^{3}H]poly(dT) and [^{3}H]poly(dA) binding (●) and OD_{280} (○). Approximately equal amounts of binding to [^{3}H]poly(dT), [^{3}H]poly(dA), and oligomers of these homopolymers were observed. However, no binding to poly(dC) or poly(dG) was observed.

pooled (0.21 mg of protein), dialyzed against TDEG buffer containing 0.2 M KCl, and stored at −70°C.

Assay of the dA/dT Sequence Recognition Protein. The dA/dT sequence-binding protein was routinely monitored during the purification for binding to [^{3}H]poly(dT) and [^{3}H]poly(dA) by a filter binding assay. The incubation system (50 μl) contained 20 mM Tris·HCl, pH 7.5/150 mM KCl/1 mM dithiothreitol/1 mM EDTA/10% (vol/vol) glycerol/bovine serum albumin at 0.1 mg/ml, 10 ng of [^{3}H]poly(dA) (2000 cpm/ng) or [^{3}H]poly(dT) (2000 cpm/ng) or in some cases 1 ng of SV40 5′ [^{32}P]DNA fragments (85,000 cpm/ng) containing the replication origin. Incubation was for 30 min at room temperature followed by filtration through a nitrocellulose membrane filter (0.45 μm) at a flow rate of 25 ml/hr. The filters were washed with 2.5 ml of 20 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/1 mM EDTA/10% (vol/vol) glycerol and air-dried, and the radioactivity retained on the filter was measured by liquid scintillation counting.

The dA/dT sequence recognition protein purified through the oligo(dT)-cellulose and Q-Sepharose steps was also assayed by a gel mobility shift (gel retention) assay (19). The purified protein (0.2–2.5 μg) was incubated with 0.5–1 ng of SV40 5′ [^{32}P]DNA fragments containing the replication origin in the incubation system described above that also included 1 μg of pBR322 DNA or poly[(dI-dC)·(dI-dC)] and 100 μg of bovine serum albumin to reduce nonspecific binding and adsorption. Incubation was at 25°C for 30 min followed by electrophoresis on a 4% polyacrylamide gel at 12.5 V/cm of gel for 2 hr (16). The gel was dried under vacuum and exposed to Kodak XAR-5 film at −80°C.

Other Assays. Protein concentrations were determined by the procedure of Bradford (20) with bovine serum albumin as the standard.

RESULTS

Purification of the dA/dT Sequence Recognition Protein. The dA/dT sequence recognition protein was isolated with a sedimentable multienzyme complex (18–20 S) for DNA synthesis in HeLa cells (14) and cofractionated with the 640-kDa multiprotein DNA polymerase α complex (13) from HeLa cell homogenates (data not shown). We have not shown, however, by immunoaffinity purifications or by gel transfer experiments whether the protein is actually a component of the multiprotein DNA polymerase α complex. The sequence recognition protein can be separated from DNA primase and polymerase α activities by affinity chromatography on oligo(dT)-cellulose. The dA/dT sequence-binding activity in the polymerase α fraction bound to oligo(dT)-cellulose in 0.2 M KCl, whereas the DNA polymerase and primase activities did not and appeared in the column flow-through fraction (Fig. 1*A* and data not shown). The poly(dA) and poly(dT) sequence-binding activities eluted coincidentally as a single peak from oligo(dT)-cellulose at ≈0.30 M KCl (Fig. 1*A*). The poly(dA) and poly(dT) sequence-binding activities also eluted coincidentally as a single peak by 0.6 M KCl during chromatography on Q-Sepharose (Fig. 1*B*). The specificity of binding by the sequence recognition protein purified through the Q-Sepharose stage of the purification was the same as that observed for the protein purified through the oligo(dT)-cellulose stage. The protein bound with equal affinity to single-stranded poly(dA), poly(dT), and the duplex poly(dA)·poly(dT) but not to poly(dC), poly(dG), or poly(dC)·poly(dG) (Fig. 1, see Fig. 5 and data not shown).

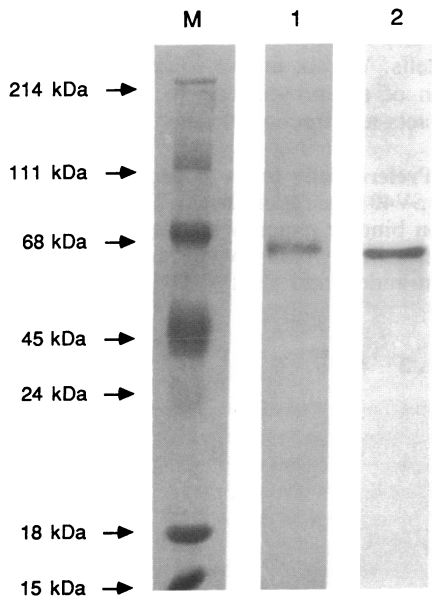


FIG. 2. Polyacrylamide gel electrophoresis of the dA/dT sequence recognition protein eluted from Q-Sepharose. Aliquots (50 μl) of the pooled peak of poly(dA)- and poly(dT)-binding activities eluted from the Q-Sepharose column and containing 0.5 μg of protein were electrophoresed on a 10% $NaDodSO_4$/polyacrylamide slab gel (13). Lanes: M, molecular mass markers; 1, Coomassie blue staining; 2, silver staining.

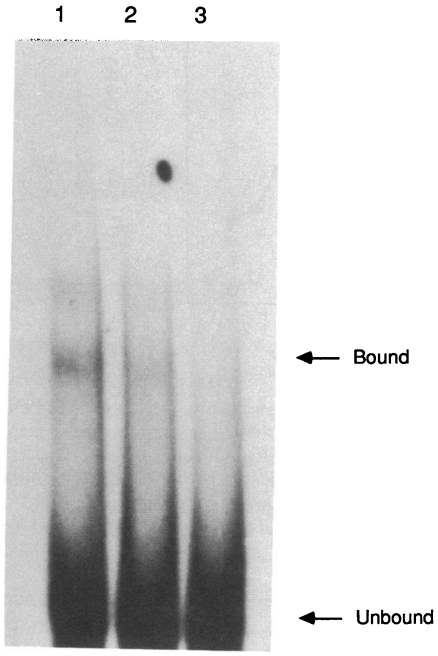


FIG. 3. Binding of dA/dT sequence recognition protein to SV40 origin-containing DNA fragment. The SV40 origin-containing DNA insert was isolated from pSV0$^+$ plasmid and the 5′ terminus was labeled with [γ-^{32}P]ATP (16, 18). The SV40 origin 5′ [^{32}P]DNA fragment (0.5 ng, 85,000 cpm/ng) was incubated with purified dA/dT sequence recognition protein (Q-Sepharose fraction, 0.2 μg of protein) in the absence or in the presence of the designated concentrations of unlabeled SV40 origin DNA fragment for 30 min at 25°C and electrophoresed for the gel retention assay (21). Lanes: 1–3, 0, 5, and 50 ng, respectively, of unlabeled SV40 origin DNA fragment were present during the incubation of the dA/dT sequence recognition protein with SV40 origin 5′ [^{32}P]DNA fragment.

Polyacrylamide gel electrophoresis of the peak of binding activity from Q-Sepharose showed a single 62-kDa protein band by both silver and Coomassie blue staining (Fig. 2).

The yield of purified protein obtained through the Q-Sepharose step in the purification was ≈6 μg/g (wet weight) of HeLa cells. We are unable to reliably estimate the fold purification of the protein due to competing reactions in crude extracts and fractions in the early stage of the purification.

Protein Preferentially Binds to dA/dT Sequences and Recognizes the SV40 Replication Origin. The results from the DNA competition binding experiments suggested that the binding protein preferentially binds to poly(dA) or poly(dT) sequences in single-stranded and duplex DNAs and recognizes the replication origin of SV40 DNA (Fig. 1, see Fig. 5 and data not shown). In gel retention assays for the binding of the purified protein to the 5′ [^{32}P]DNA fragment containing the SV40 origin, a single displaced band relative to the unbound DNA and representing the DNA–protein complex was observed even in the presence of a 2000-fold excess of poly[(dI-dC)·(dI-dC)] (Fig. 3, lane 1). Unlabeled SV40 origin DNA fragment, however, did compete with the SV40 origin 5′ [^{32}P]DNA fragment for binding the protein. A 100-fold excess of unlabeled SV40 DNA completely abolished the protein binding to the SV40 origin 5′ [^{32}P]DNA (Fig. 3, lane 3).

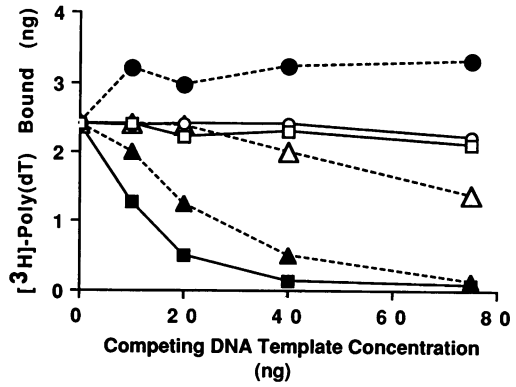


FIG. 5. Competition by various DNAs for the binding of the dA/dT sequence recognition protein to [^{3}H]poly(dT). The dA/dT sequence recognition protein [2 μg of the oligo(dT)-cellulose fraction] was incubated with [^{3}H]poly(dT) (10 ng, 2500 cpm/ng) in the presence of the designated amounts of unlabeled competitor DNAs at 25°C for 30 min and assayed for binding by the nitrocellulose filter assay. The competing DNAs included: plasmid DNAs, pBR322 (△) and pJL0 (■); a 200-bp *Hin*dIII–*Sph* I fragment of SV40 origin-containing DNA (▲); and the synthetic double-stranded octadecamer representing nucleotides 5193–5210 in SV40 DNA (●), $(dC)_{1000}$ (○), and $(dG)_{1000}$ (□).

The binding of the dA/dT sequence recognition protein to the SV40 origin DNA fragment occurred in a protein-concentration-dependent manner that resulted in a single displaced band in the gel retention assays even at the highest protein concentrations used (Fig. 4). This also occurred in the presence of a 2000-fold excess of poly[(dI-dC)·(dI-dC) (Fig. 4*A*) or linearized pBR322 DNA (Fig. 4*B*).

Further evidence that the binding protein recognizes dA/dT sequences within the SV40 replication origin was obtained from nitrocellulose filter binding competition assays. As shown in Fig. 5, pJL0 plasmid DNA (an identical construct to pSV0$^+$ and containing the SV40 origin DNA insert) competed in the binding of the protein to [^{3}H]poly(dT) or [^{3}H]poly(dA) to about the same extent as did the isolated

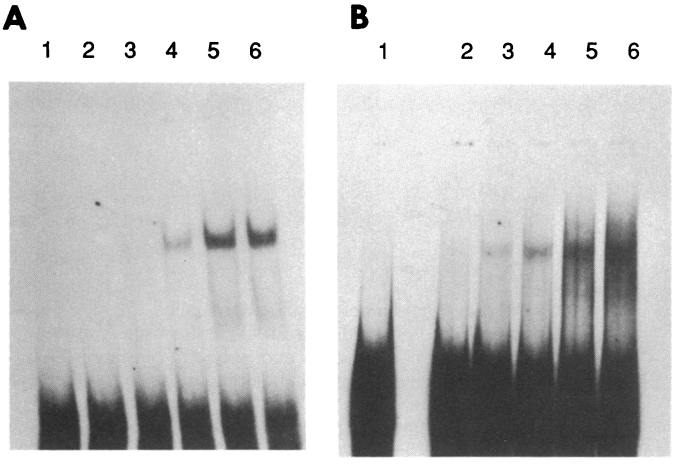
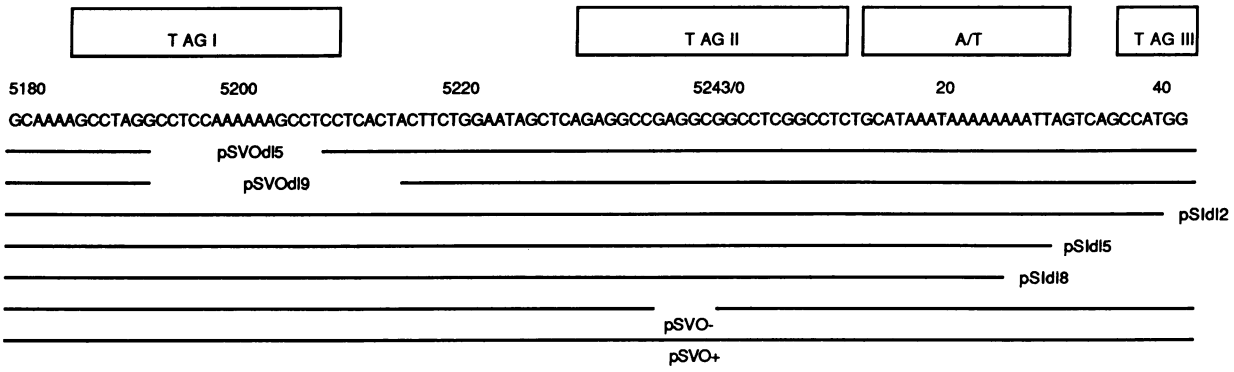


FIG. 4. Protein-concentration-dependent binding of dA/dT sequence recognition protein to SV40 origin 5′ [^{32}P]DNA in the presence of poly[(dI-dC)·(dI-dC)] or pBR322 plasmid DNA as competing DNAs. The dA/dT sequence recognition protein, oligo(dT)-cellulose fraction, was incubated at various protein concentrations with 0.5 ng (85,000 cpm/ng) of SV40 origin 5′ [^{32}P]DNA fragment in the presence of a 2000-fold excess of competing DNAs for gel retention assays. (*A*) Assayed in the presence of 1 μg of poly[(dI-dC)·(dI-dC)]. (*B*) Assayed in the presence of 1 μg of pBR322 DNA. Lanes: 1–6, 0, 0.24, 0.50, 1.25, 2, and 2.5 μg, respectively, of dA/dT sequence recognition protein.



Binding Of The Sequence Recognition Protein To SV40 Origin DNA And Deletion Fragments

	[32P] - DNA	[32P] - DNA RETAINED ON NITROCELLULOSE FILTER
		PICOGRAMS
pSVO+		430
pSVO-		440
pSVOdl5		500
pSVOdl9		460
pSIdl2		500
pSIdl5		270
pSIdl8		200
pBR322		80
640 bp pBR322 fragment		80

FIG. 6. Comparison of the binding of dA/dT sequence recognition protein to SV40 origin DNA and to deletion-containing SV40 origin DNAs and to pBR322 DNA. The dA/dT sequence recognition protein [2.5 μg of oligo(dT)-cellulose purified fraction] was incubated in the standard incubation system that included 0.5 ng of one of the following 5′-^{32}P-end-labeled DNAs: SV40 origin DNA inserts from plasmids pSV0$^+$ (10,392 cpm/ng), pSV0$^-$ (8957 cpm/ng), pSV0dl5 (9972 cpm/ng), pSV0dl9 (11,627 cpm/ng), pS1d12 (5894 cpm/ng), pS1d15 (1308 cpm/ng), or pS1d18 (6530 cpm/ng); or pBR322 DNA (2000 cpm/ng); or a 640-bp fragment of pBR322 DNA (10,315 cpm/ng). The 5′-^{32}P-end-labeling was performed as described in the legend to Fig. 3 and binding was measured by the nitrocellulose filter assay. TAG, T-antigen binding site.

SV40 DNA insert (i.e., SV0$^+$). In contrast, the pBR322 plasmid did not compete with [^{3}H]poly(dT) for binding the protein except at high concentrations of the plasmid where nonspecific interactions begin to occur (Figs. 2 and 3). Two major A+T-rich tracts reside within the SV40 replication origin, in T-antigen binding site I between nucleotides 5198 and 5203 and in the 17-bp A+T tract between nucleotides 15 and 31. Interestingly, the double-stranded synthetic octadecamer polymer (17),

5′-d(G–G–A–G–G–C–T–T–T–T–T–T–G–G–A–G–G–C)-3′
3′-(C–C–T–C–C–G–A–A–A–A–A–A–C–C–T–C–C–G)d–5′,

which represents the nucleotide sequences from residues 5193 through 5210 in the T-antigen binding site I of SV40 DNA and contains a 6-bp A+T tract, did not compete with [^{3}H]poly(dT) or [^{3}H]poly(dA) for binding by the dA/dT sequence recognition protein (Fig. 5 and data not shown). Actually, it enhanced the binding of the protein to poly(dT). At this time we do not know the basis for the enhancement.

Origin Recognition Protein Binds to a Specific Locus in SV40 Origin. To ascertain if the origin recognition protein binds to a specific locus in the SV40 replication origin, filter binding assays were performed with deletion-containing SV40 origin DNA fragments (3). As shown in Fig. 6, the purified protein did bind with equal affinity to the complete origin-containing DNA fragment pSV0$^+$ and the DNA fragment pSV0$^-$ that contains 4 bp within the T-antigen binding site II. The binding protein also showed a high affinity for the SV40 origin DNA fragments pSV0dl9 and pSV0dl5 that contain deletions on the early side of the replication origin that include deletion of the A+T tract within T-antigen binding site I (Fig. 6). The binding was reduced, however, to the SV40 origin DNA fragments pS1d15 and pS1d18 that contain deletions on the late side of the core origin for replication. These deletions extend into the 17-bp A+T-rich tract within the core origin DNA domain (Fig. 6).

DISCUSSION

The dA/dT sequence recognition protein described here specifically binds to dA/dT sequences in single-stranded or duplex DNA and binds with high affinity to the minimal core origin for SV40 replication. The binding of the protein to pBR322 plasmid DNA lacking the SV40 origin was greatly reduced compared to binding of the protein to the SV40 origin containing pSV0$^+$ plasmid (Fig. 5). Moreover, a vast excess of pBR322 plasmid DNA in the gel retention assays did not

compete for the binding of the dA/dT sequence recognition protein to the isolated SV40 origin 5′ [^{32}P]DNA fragment (Fig. 4*B*). Since pBR322 DNA contains a number of A+T-rich tracts (22), these results suggest that the dA/dT sequence recognition protein does not merely bind indiscriminately to sequences containing adenines or thymines in DNA. The results from deletion and competition binding analysis indicate that the 6-bp A+T tract residing between nucleotides 5198 and 5203 outside of the minimal origin in SV40 DNA did not affect the binding of the protein to the isolated SV40 origin-containing DNA fragment (Figs. 5 and 6). However, deletions in the late gene region of SV40 DNA (e.g., pS1d15 and pS1d18 in Fig. 6) that extend into the 17-bp A+T tract reduced the binding to the SV40 minimal origin. These results indicate that the 17-bp A+T tract is the probable locus of binding of the protein to the SV40 origin.

We have been unable to define by DNase I footprinting specific sequences that are involved in the binding of the purified protein to the 17-bp A+T-rich tract in SV40 DNA. The 17-bp A+T-rich tract has been reported to assume a bent DNA conformation (6). The A+T-rich sequences in replication origins, including the 17-bp A+T tract in SV40 DNA, have been reported (23) to represent readily unwound and unpaired regions in supercoiled DNA. Also, it has been proposed that sequences flanking the A+T-rich sequences in DNA are essential for maintaining the bent DNA conformation (21). Thus, it is quite possible that the protein recognizes the unusual conformation in the 17-bp A+T-rich region of SV40 DNA rather than any specific sequences.

The dA/dT sequence recognition protein described here appears different from the D-protein that was reported to cofractionate with DNA polymerase α from mouse liver (24). The D-protein was reported to preferentially bind to single-stranded oligo(dT) but not to oligo(dA) or poly(dA)-poly(dT) (25). The D-protein was also found to stimulate a variety of DNA polymerase activities on poly(dT) templates (24). The sequence recognition protein that we report here binds to poly(dA) and poly(dA)·poly(dT) as well as to poly(dT). Also, we have not observed any stimulation of polymerase α activity by the protein by using activated or primed single-stranded [e.g., poly(dA)·$(dT)_4$ and poly(dT)·$(dA)_4$] DNA templates (L.H.M., unpublished results).

There is a growing body of evidence for the existence of viral-encoded and cellular proteins that specifically bind to the replication origins of DNA viruses and eukaryotic chromosomes. An 83-kDa herpes simplex virus 1-encoded origin-binding protein has been purified and characterized (26). A 135-kDa protein has been purified (27) from yeast cells and was shown to interact specifically with the chromosomal replicator autonomously replicating sequence 1 (ARS*1*). Also, monkey cell factors have been reported (28) to specifically recognize a conserved sequence in the early domain of the SV40 core replication origin. Our isolation of a dA/dT sequence recognition protein from a multienzyme complex that is involved in SV40 replication *in vitro* and the demonstration of its specific binding to the 17-bp A+T-rich tract in the SV40 core origin may relate to the speculated function of this A+T-rich tract in the initiation of replication (3–6, 29, 30). The elucidation of the actual functional role of this host cell dA/dT sequence recognition protein in SV40 and cellular DNA replication, however, awaits further studies.

Note Added in Proof. While this paper was in press a report appeared of induced bending of the SV40 replication origin by the specific binding of a partially purified, 3.5S HeLa cell protein to the region of the 17-bp dA/dT tract in SV40 origin containing DNA fragments (31). The 3.5S protein may be related to the purified 62-kDa protein reported here.

We thank Dr. Robert J. Hickey for helpful discussions, Dr. Bruce Stillman (Cold Spring Harbor Laboratory) for providing the plasmids containing deletions in the SV40 origin, Dr. Paul Zamecnik for providing the synthetic double-stranded octadecamer oligonucleotide, Matthew Potter for expert technical assistance, and Sandra Johnson for help in preparation of the manuscript. This work was supported by National Institutes of Health Grants P30-12708 and CA 15187. L.H.M. is the recipient of National Research Service Award CA 08173 from the National Institutes of Health.

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