DNA binding activity of polyoma virus large tumor antigen

(monoclonal antibodies/immunoaffinity chromatography/DNase I protection/DNA replication)

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ABSTRACT   Polyoma virus large tumor antigen from pro-
ductively infected mouse cells has been purified to >50% ho-
mogeneity by a simple immunoaffinity procedure using mono-
clonal antibodies. A radioimmunoreaction was devised for as-
saying purity. The purified large tumor antigen retained its
antigenicity and its ability to bind DNA specifically. The re-
gions on the polyoma virus genome recognized by the protein
were characterized. Three binding regions were localized
within the portion of the genome between the viral origin of
DNA replication and the protein coding sequence, overlapping
the early promoter and the sites of initiation of mRNAs that
specify the viral tumor antigens. The binding regions each con-
tain direct repeats of the pentanucleotide sequence G-R-G-G-
C.

The genome of the small tumor virus polyoma is a double-
stranded circular DNA that encodes six known proteins.
Three of these are expressed early during the lytic infection
cycle and have been designated the large, middle-sized, and
small tumor antigens (T-Ags) (1–3). The large T-Ag has an
apparent M_r of about 100,000 as measured by gel electropho-
resis. It is heavily phosphorylated in vivo and is found pre-
dominantly within the nucleus. This protein has been impli-
cated in a number of processes that occur subsequent to vi-
rus infection, among which are the initiation of viral DNA
synthesis, the stimulation of host cell DNA synthesis, the
repression of early gene expression, and the events that lead
to integration, excision, or amplification of viral DNA within
the host chromosome (3, 4). Stable expression of the protein
has been shown to reduce the serum growth factor require-
ment of certain normal or transformed established rodent
cell lines (5) and to "immortalize" embryo fibroblasts (6).
Only two biochemical activities have as yet been demon-
strated to be associated with the large T-Ag. These are an
ATP-phosphohydrolase activity (7) and an ability to bind to
DNA (8), with particularly high affinity for polyoma virus
DNA in the region of the origin of viral DNA replication (9,
10). All function studies have used either slightly purified
protein preparations or indirect techniques. The absence of
purified material has impeded further progress. By contrast,
the large T-Ag expressed by another papova virus, simian
virus 40 (SV40), has been purified and is available for study
in more detail (see refs. 3 and 11 for reviews). As the two
viruses are closely related (12, 13), comparisons between the
two large T-Ag will be of considerable interest. Some differ-
ences are already known. Notable among these is the fact
that there appears to be no close association between a host
protein and the polyoma virus large T-Ag, such as been ob-
served between the host cell p53 protein and SV40 large T-
Ag (3).

That the large T-Ag of polyoma virus has not hitherto been
purified may stem from the fact that there was no convenient
assay for it and that it is present in both lytically infected and
transformed cells in extremely limited quantities that have

defied conventional purification techniques. The isolation of
hybrid cell lines that secrete antibodies specific for large T-
Ag (14) now provides the means to overcome both of these
difficulties. The specificity and purity of the monoclonal
antibody probes have enabled a sensitive radioimmunoassay
(RIA) procedure to be devised to detect the protein, and the
production of these antibodies in large quantities has allowed
an immunoaffinity purification protocol to be used for the
isolation of the antigen. Because of the known heterogeneity
of large T-Ag (3, 14) the latter method was moreover consid-
ered to have advantages over conventional purification tech-
niques. The protocol adopted yielded highly enriched pro-
tein, sufficiently pure for DNA binding properties of large T-
Ag to be examined directly. Purified polyoma virus large T-
Ag was observed to bind to DNA in a sequence-specific
manner, strongly suggesting that the procedure developed
yields native protein that can be used for future detailed bio-
chemical definition of its activities.

MATERIALS AND METHODS

Cells and Virus Stocks. All cells were grown in Dulbecco's
modified Eagle's medium (DME medium) supplemented
with 5% fetal calf serum (hereafter referred to as serum)
(GIBCO). Virus stocks were grown on whole mouse embryo
cells at 32°C, by infection with 0.1 plaque-forming unit (pfu)
of virus per cell.

Viral DNA. The A2 strain of polyoma virus DNA (form I)
was prepared by standard procedures (12). Cloned polyoma
virus DNA was prepared from recombinant p37.3.A2, in
which viral DNA cleaved at the BamHI restriction enzyme
site was inserted into the homologous site of vector pAT 153
(15).

RIA. Labeled αPyLT1 (PAb 711) antibody was isolated by
incubating the antibody-secreting cell line with 250 μCi (1 Ci
= 37 GBq) of [³⁵S]methionine (Radiochemical Centre;
Amersham) per 10⁷ cells for 16 hr at 37°C in DME medium
containing 10% of the normal concentration of methionine
and 5% serum. The cells were then removed by centrifuga-
tion (1000 × g) for 10 min, and the proteins present in the
supernatant were precipitated by the addition of ammonium
sulfate (BDH, Aristar Grade) to 50% saturation. The precipi-
tate was collected by centrifugation and, dissolved in a small
volume of phosphate-buffered saline (Pᵢ/NaCl).

The RIA utilized the property of the antibody to bind to
the protein A molecule of Staphylococcus aureus Cowan I
(SAC) (16) only when complexed with its antigen (14). A
constant amount of ³⁵S-labeled αPyLT1 antibody with an ex-
cess of unlabeled αPyLT4 (PAb 712) antibody was incubated
with varying quantities of the protein solution to be assayed.
After 1 hr at 0°C, 10 μl of a 20% suspension of fixed S. aure-
us was added and the mixture was left for 30 min at 0°C. The

Abbreviations: T-Ag, tumor antigen; SV40, simian virus 40; pfu,
plaque-forming unit(s); SAC, Staphylococcus aureus Cowan I.
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SAC was then pelleted by centrifugation in an Eppendorf Microfuge and washed once by resuspension in Tris-buffered saline [50 mM Tris·HCl, pH 7.4/150 mM NaCl (Tris/ NaCl)], and the bound ^{35}S was assayed by scintillation spectroscopy. Samples were quantitated by reference to a standard curve.

Preparation of Antibody Linked to Sepharose 4B. Monoclonal antibody containing ascites fluid was prepared in persistant primed nude mice. Antibody was purified by chromatography on DEAE-Sephacel (Pharmacia) and reacted with CNBr-activated Sepharose 4B (Pharmacia) in 50 mM citrate buffer, pH 6.0/300 mM NaCl by adding ≈10 mg of antibody in coupling buffer to a 1-ml packed volume of washed, activated Sepharose. Coupling was allowed to proceed for 45 min at room temperature, and any remaining active groups were inactivated by addition of 100 mM ethanolamine·HCl (10 ml), pH 8.5 and incubation for 30 min at room temperature. The Sepharose was washed free of unbound protein and stored at 4°C in P_i/NaCl. Typically, >90% of the antibody was linked to the column.

Large T-Ag Purification. Approximately 10^9 3T6 mouse cells were infected with >50 pfu per cell of A2 strain wildtype polyoma virus and incubated for 42 hr at 32°C in DME medium/5% serum. The cells were then washed with ice-cold Tris/NaCl and lysed on the dish by the addition of 10 ml of ice-cold lysis buffer [100 mM NaCl/100 mM Tris·HCl, pH 8.8/0.3 trypsin inhibitor unit of aprotinin (Sigma) per ml] per 10^8 cells, followed by incubation at 0°C for 30 min with gentle agitation. The lysate was then scraped into a centrifuge tube and pelleted at 10,000 rpm for 20 min at 4°C in a SS-34 rotor. The supernatant was removed and sufficient antibody–Sepharose was added to bind >90% of the large T-Ag present, as determined by RIA. The suspension was incubated at 0°C with constant mixing for 18 hr. The antibody–Sepharose complex was then isolated by centrifugation (500 × *g*) for 5 min, washed twice with Tris/NaCl containing 0.05% Noni-

det P-40 and twice with Tris/NaCl, without detergent, then packed into a small column, and again washed with Tris/ NaCl. The adsorbed proteins were eluted with a solution of 3.5 M $MgCl_2$/20 mM Tris·HCl, pH 7.4. Fractions that contained large T-Ag, as determined by RIA, were pooled, dialyzed against 50 mM NaCl/20 mM Tris·HCl pH 7.4/10% glycerol, and subsequently concentrated by dialysis against dry Sephadex G-50.

DNase I Protection Experiments. DNase I protection studies were carried out by using a modification of the method of Galas and Schmitz (17). Five to ten nanogram equivalents of 5'-end labeled (18) DNA fragment were incubated with varying quantities of purified large T-Ag in 100 μl of 100 mM NaCl/20 mM Tris·HCl, pH 7.0/2 mM dithiothreitol/3% dimethyl sulfoxide/0.01% bovine serum albumin at 20°C for 30 min. The DNA was then partially digested by the addition of $MgCl_2$ to a final concentration of 5 mM, $CaCl_2$ to 1 mM, and DNase I (Worthington) to 10 ng/ml. After incubation for 5 min at 20°C, the DNase I was inactivated by the addition of an equal volume of 2 M ammonium acetate/100 mM EDTA/100 μg of sonicated calf thymus DNA per ml. The sample was then extracted with phenol and precipitated with ethanol. The air-dried pellets were dissolved in formamide dye mix (19), heated at 90°C for 3 min, and subjected to denaturing electrophoresis on 12% acrylamide gels in the presence of 8.3 M urea (20), and the DNA was detected by autoradiography.

RESULTS

Purification of Large T-Ag. The monoclonal antibodies αPyLT1 and α-PyLT4, with specificity for the large T-Ag of polyoma virus, have been shown to bind to protein A from SAC only when complexed with their antigen (14). This property has been used to establish a RIA technique by measuring the increased antibody binding in the presence of large T-Ag. Fig. 1 *Left* shows a standard curve obtained by
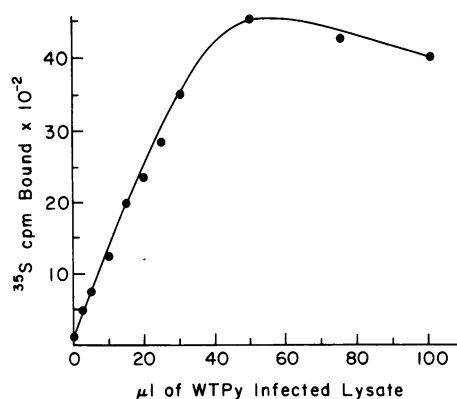

FIG. 1. (*Left*) Standard curve used for measuring the quantity of large T-Ag in the RIA. A constant amount of [^{35}S]methionine-labeled α-PyLT1 antibody together with a fixed amount of unlabeled α-PyLT4 antibody (14) were incubated with varying quantities of lysate from polyoma virus-infected 3T6 mouse cells and then adsorbed onto SAC. The bound ^{35}S is shown relative to the amount (microliters) of lysate used. In this experiment, 1 ml of lysate represents the proteins from 10^7 cells. (*Right*) NaDodSO$_4$/PAGE separation (21) of the partially purified large T-Ag after staining by the silver method (Bio-Rad). Marker proteins (lane M), with the appropriate molecular weights (shown as M_r × 10^{-3}) are indicated on the left. Two different quantities of protein were loaded to give a reflection of the amount of contamination present. Lanes 1 and 2 contain ≈0.1 μg and ≈1 μg, respectively, of total protein (as determined by the Bio-Rad protein assay method). The positions of the large T-Ag (LT), immunoglobulin heavy chain (IgG-H), and actin are indicated on the right.
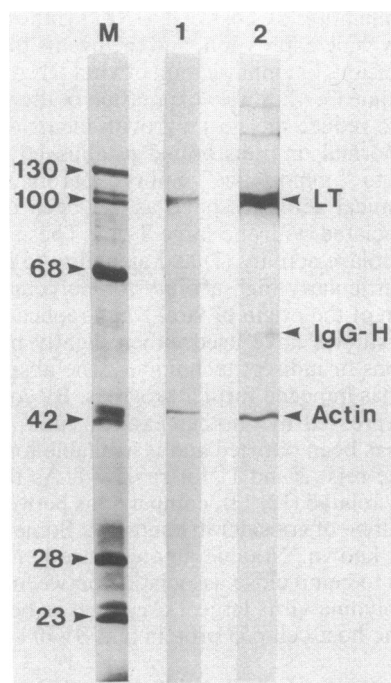
this procedure in the presence of increasing amounts of a lytically infected cell lysate. This assay can be seen to be linear, reaching a maximal response in the presence of lysate from ≈2 × 10⁵ lytically infected cells. This was sufficiently sensitive and quantitative to be used in assessing the purification of large T-Ag.

Immunoaffinity chromatography using monoclonal antibodies directed against polyoma virus large T-Ag has been used to purify the small amount of this protein away from the bulk of other proteins in cells. The antibody chosen for this purpose was αPyLT1 (PAb 711) (14), which has been well-characterized, recognizing specifically large T-Ag and binding to >95% of this protein in lytically infected cells. Use of this antibody minimizes the risk of selectively enriching for subpopulations of molecules. The antibody was purified from ascites fluid and linked to CNBr-activated Sepharose 4B. This adsorbant could be shown to bind, after overnight incubation, >90% of the large T-Ag present in a crude, pH 8.8, non-ionic detergent lysate of wild-type polyoma virus lytically infected 3T6 mouse cells.

To assess the activity of the antigen after elution by various procedures from the antibody–Sepharose adsorbent, two assays were conducted—the ability of the protein to be detected in the RIA, hence assaying its antigenic state, and the ability to bind specifically to DNA at or near the viral origin of DNA replication. Elution with 3.5 M MgCl₂ proved to be the procedure of choice. After dialysis and concentration in the presence of 10% glycerol, the large T-Ag eluted in this manner typically retained >50% of the DNA binding activity present in the starting lysate and >75% of the antigenic activity. To assess the purity of the isolated protein, a fraction of it was separated by NaDodSO₄/PAGE (21) and the polypeptides were detected by silver staining (Fig. 1 *Right*). The major polypeptide species was found to comigrate with large T-Ag at M_r 100,000. Quantitation of the protein bands by densitometry tracing of the stained gel indicated that >50% of the total protein present was found in this M_r 100,000 polypeptide. On average, 1 μg of this polypeptide could be isolated from 10⁷ lytically infected cells. Two of the major contaminating polypeptides are actin and antibody heavy chain, which "leaches" off the column.

The ability of the protein preparation to bind specifically to the origin region of polyoma virus DNA is shown in Fig. 2. Here we used a method involving immunoprecipitation of DNA–protein complexes (22) to identify labeled restriction fragments that contain regions with high binding affinity for the large T-Ag. Previous experiments using this technique and crude lysates from infected cells as the source of large T-Ag (ref. 10; A. Scheller and C. Prives, personal communication; unpublished data) identified two independent binding regions at either side of the single *Bgl* I site in polyoma virus DNA, which lie halfway between the viral origin of DNA replication and the early mRNA cap site (Fig. 2). With crude lysate no more than 5% of the radioactivity in any DNA fragment could be immunoprecipitated, and fragment "a," containing the origin binding region, was recovered in three to five times the molar yield of fragment "b," the latter containing the cap site binding region. The a/b fragment ratio may reflect a difference in binding affinity. The purified preparation of large T-Ag specifically immunoprecipitated the same two restriction fragments. With small amounts of protein the a/b fragment ratio was similar to that obtained with crude lysate, but with larger amounts equimolar quantities of fragments a and b were immunoprecipitated. This presumably reflects saturation of the binding regions with excess protein.

Large T-Ag DNA Binding Sites. Previous investigations of the regions of polyoma virus DNA to which the large T-Ag binds have mainly used indirect assays that are incapable of analyzing the interaction in detail. Having available a highly
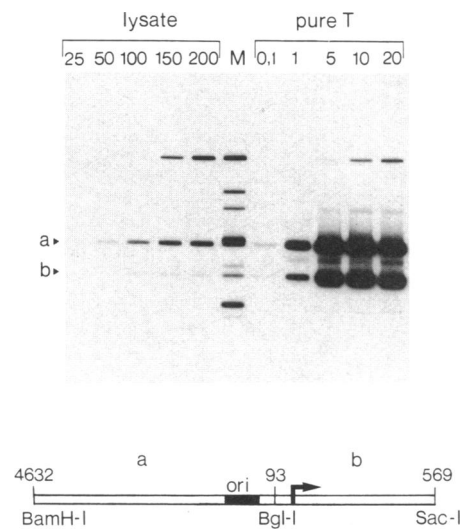

FIG. 2. Comparison of the DNA binding ability of the partially purified large T-Ag preparation with that observed with antigen derived from a crude lysate, as detected by DNA immunoprecipitation procedures (22). p37.3A2 DNA (10 ng), digested with *Bam*HI, *Bgl* I, and *Sac* I (New England BioLabs) labeled at their 3' ends with T4 DNA polymerase (P-L Biochemicals), was incubated for 1 hr at room temperature in either 1 ml of binding buffer with varying amounts (microliters) of the crude lysate or in 100 μl of binding buffer with increasing quantities of the purified large T-Ag, as indicated. After immunoprecipitation (ref. 22; unpublished data), DNA was eluted, separated by electrophoresis on a 2% agarose gel, and detected by autoradiography. Lane M shows the separation of 0.2 ng of labeled material. The two DNA fragments (a and b) that are specifically precipitated are noted on the left and their positions on the polyoma virus genome are indicated diagrammatically. Fragment a contains the putative origin of replication (designated "ori"; see Fig. 4); fragment b includes the "TATA" box and the principal cap site for early transcription (indicated by the arrow). The nucleotide numbering system used throughout is that of Soeda *et al.* (23).

enriched preparation of large T-Ag we were able to use the more powerful DNase I protection technique introduced by Galas and Schmitz (17). In this method, protein is allowed to interact with an end-labeled fragment of DNA, which is then cleaved to a limited extent with a relatively nonspecific nuclease, and the resulting fragments are separated by electrophoresis on polyacrylamide gel. The results of one such experiment, using a fragment of polyoma virus DNA that encompasses most of the noncoding region surrounding the origin of DNA replication, are shown in Fig. 3A. The DNA was protected by the protein from DNase I cleavage in three regions, designated A, B, and C, the locations of which are illustrated in Fig. 4. DNase I protection was examined in the presence of increasing amounts of large T-Ag (Fig. 3 B and C); no obvious evidence for progressive binding was observed. All three sites appeared to be occupied by protein to similar extents at the different protein levels tested.

DISCUSSION

Previous attempts to purify the large T-Ag expressed by polyoma virus have resulted only in the isolation of small quantities of material of limited purity (3, 8). Monoclonal antibodies directed against the protein (14) have now been used to develop a sensitive RIA and a powerful immunoaffinity purification. By means of the latter technique, large T-Ag was purified in good yield from lytically infected mouse cell lysates to 50% homogeneity (Fig. 1 *Right*). It retained most of its antigenic activity and bound with high affinity to polyoma DNA in the region near the viral origin of DNA replication (Fig. 2). Thus, native large T-Ag has been purified to a degree that enables at least some of its activities to
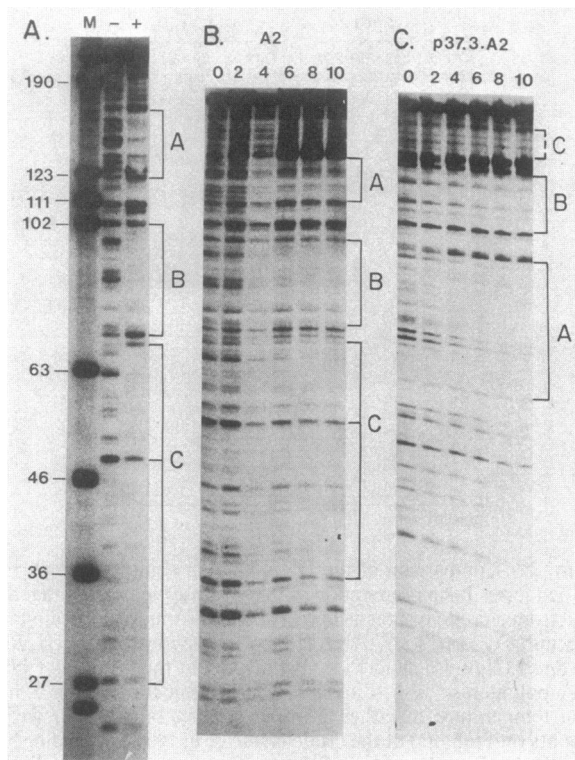
FIG. 3. Partial DNase I digestion of fragments of polyoma virus DNA; DNA fragments were reacted with varying amounts of purified large T-Ag before DNase I digestion. The quantity (microliters) of antigen added is indicated above each lane. The resulting fragments were separated by electrophoresis on a 12% polyacrylamide/8.3 M urea denaturing gel and detected by autoradiography. In each case, the regions protected by large T-Ag are indicated on the right. (A) Polyoma virus A2 DNA cleaved and 5' end labeled at the *Dde* I site (nucleotide 188) and recut with *Pvu* II nucleotide 5267) after incubation with either no (−) large T-Ag or 10 μl (+) of large T-Ag. (B) The same fragment of A2 strain polyoma virus DNA used in A. (C) A fragment of plasmid p37.3.A2 DNA labeled at the 5' end of the *Pvu* II site (nucleotide 5267) and cleaved with *Dde* I (nucleotide 188) (22). Use of this reverse orientation allows site A to be mapped more precisely but, due to insufficient resolution at the top of the gel, does not allow site C to be mapped.

FIG. 4. (A) The DNA sequence around the replication origin region of polyoma virus (23). Above the sequence are indicated the approximate locations of nucleotides protected by the binding of polyoma virus large T-Ag. Also indicated are the positions of the pentanucleotide sequences G-R-G-G-C (solid arrows). (B) Schematic representation of the genomes of polyoma virus (Py) and SV40 around the replication origin regions. The numbering system of Buchman, Burnett and Berg (1) is used for SV40. The genomes are aligned so that early transcription occurs from left to right and the regions of maximal DNA sequence homology are superimposed (25). The approximate positions of the genetically defined replication origins are indicated ("ORI") (taken from refs. 26–28 for SV40 and refs. 29–34 for polyoma virus), together with the 5' ends of the respective early RNA molecules (18, 35, 36) and TATA sequences. Also indicated are the respective large T-Ag binding sites (ref. 9 and Fig. 3).

be studied in detail and unknown activities to be probed. The monoclonal antibody used is known to react with most (if not all) of the detectable large T-Ag in infected cells, and thus the purified protein probably includes all of the forms already identified (3, 14). There is no evidence for selective enrichment for subpopulations having different biochemical activities as observed in the case of SV40 large T-Ag (3, 11, 24).

The advantages of purified large T-Ag have been illustrated by an investigation of its ability to bind specific regions of polyoma virus DNA. Three regions near the replication origin, designated A, B, and C, were protected from DNase I digestion by bound large T-Ag (Fig. 3). The positions of these regions in the polyoma virus DNA sequence are illustrated in Fig. 4, together with a comparison of the results of similar studies on SV40. Region A corresponds to the high affinity site first localized by Gaudray *et al.* (9). Region C is the second site identified in DNA immunoprecipitation studies (ref. 10; A. Scheller and C. Prives, personal communication; unpublished data). However, binding to region B was hitherto undetected.

A number of similarities and differences between the polyoma and SV40 T-Ag binding sites are readily apparent. For example, region A on the polyoma virus genome and region I on SV40 DNA lie in regions that show considerable DNA
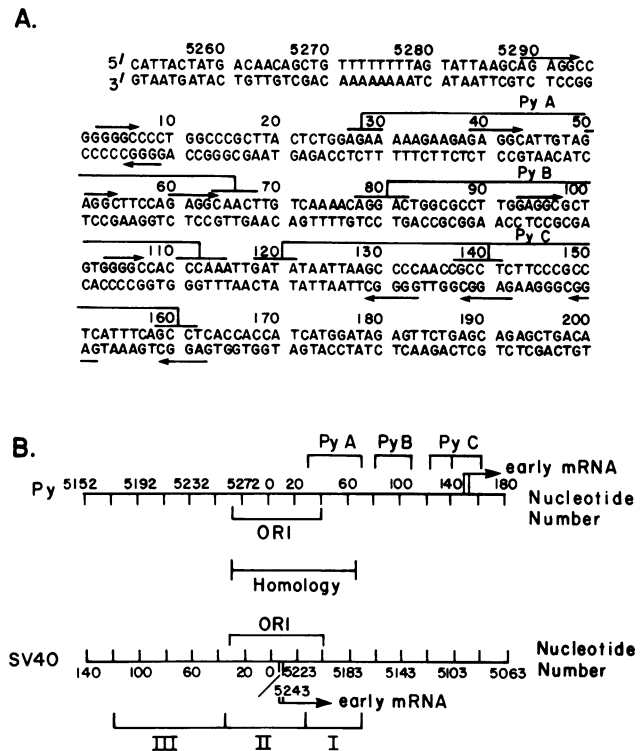
sequence homology (25) and are close to areas shown by mutant studies to be necessary for DNA replication in both viruses (28–34). However, the other regions do not correspond. Regions II and III for SV40 large T-Ag extend from region I across the replication origin towards the "late" region, whereas polyoma virus regions B and C extend away from its origin towards the "early" region. Therefore, for polyoma virus, only region A is close to the sequences involved in the initiation of viral DNA replication (34). In the case of SV40, it has been argued that region II, rather than region I, is probably more important in DNA replication (11, 27, 37, 38), because the DNA sequence of the latter can be removed, or altered, without significantly affecting replication (39). Similarly, substantially all of polyoma virus region A can be deleted without abolishing DNA replication (34). A binding region within the polyoma virus DNA analogous to SV40 region II has not been found in this study. Therefore, a polyoma virus large T-Ag–DNA interaction within polyoma virus origin sequences clearly important for DNA replication remains to be demonstrated directly.

Another difference between the present results and those previously obtained in the SV40 system is that polyoma virus large T-Ag binds to the three regions A, B, and C with apparently equivalent affinities (Fig. 3 B and C). SV40 large T-Ag binds its three regions with markedly different affini-

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ties; these decrease in the order: region I, region II, region III (3, 11).

Sequences outside the "origin region" shown in Fig. 4 appear to be involved in polyoma viral DNA replication (32). These lie in the late region about 130 base pairs away from the designated origin of replication and large T-Ag does not appear to bind to them. Therefore, binding of large T-Ag to viral DNA must not be the only process essential to the initiation of DNA synthesis of this virus.

The binding of large T-Ag to DNA also plays a role in transcriptional control of the virus. The 5' ends of SV40 early mRNA(s) and its TATA box (a putative RNA polymerase promoter; for review, see ref. 40) lie within the area of the origin of replication (35), at the SV40 large T-Ag binding region II. Large T-Ag has been suggested to be responsible for the repression of early gene synthesis in SV40, possibly by binding to sites I and II and thereby preventing initiation of early RNA synthesis (11, 41). The 5' ends of polyoma virus early mRNA(s) and the TATA box are found between the origin and the translation initiation codon (18, 36) and lie within the large T-Ag binding region C. If the regulation of transcription in papova viruses is accomplished by the binding of large T-Ag to the promoter regions for early RNA synthesis, thus preventing the binding of RNA polymerase, differences between the locations of the SV40 and polyoma virus binding regions are readily understood.

At the DNA sequence level, the pentanucleotide sequence G-R-G-G-C has been implicated in the binding of SV40 large T-Ag to its DNA (11, 28, 37), and a similar suggestion has been made for polyoma virus (9, 10). Fig. 4A shows that the three large T-Ag binding regions identified for polyoma virus DNA (Fig. 3) each have at least two direct repeats of this pentanucleotide. It appears that these repeats can exist on either strand (region C has the opposite polarity of regions A and B). The correlation between the sequences recognized by polyoma virus and SV40 large T-Ag, despite their differing locations, suggests that in both viruses the pentanucleotide sequence is involved in DNA recognition. Now that a convenient purification procedure for polyoma virus large T-Ag has been established, further studies using both wild-type and mutant viral DNAs should provide a detailed understanding of the interactions between this protein and the viral genome and its role in viral replication and expression. The apparent physical separation on the polyoma viral DNA of the regions involved in these functions may make these studies easier than in the case of SV40.

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