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An adenovirus vector system used to express polyoma virus tumor antigens

(expression vector/DNA binding)

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ABSTRACT **We have used a generalized adenovirus vector system to express the three polyoma tumor (T) antigen proteins under the control of the adenovirus major late promoter. One hybrid virus, Ad-PySVR498, expresses high levels of polyoma middle and small T antigens. A second hybrid virus, Ad-LTSVR545, which contains a cDNA copy of the polyoma A gene, overproduces large T antigen. The T antigens produced are indistinguishable from their authentic polyoma counterparts as determined by immunoprecipitation and partial cleavage by V8 protease. Analysis of polyoma mRNAs encoded by the recombinant viruses showed that they initiate from the adenovirus major late promoter and contain the tripartite leader at their 5′ ends. Large T antigen isolated from Ad-LTSVR545-infected cells by immunoaffinity was shown to bind selectively to polyoma DNA sequences that contain the origin of viral DNA replication as well as the sites for transcription initiation.**

The ability to define the biochemical properties of viral transforming proteins is a critical step in understanding their function. However, it has been difficult to study these proteins because of their low level of synthesis in lytically infected and transformed cells. To circumvent this problem, we have developed an adenovirus vector system designed to express foreign proteins at high levels (1–3). We have previously used this vector system to overproduce simian virus 40 (SV40) large tumor (T) antigen (3). A variety of studies showed that the maximum amount of protein was produced when SV40 T-antigen coding sequences were transcribed from the adenovirus major late promoter, and the hybrid T mRNAs carried almost the entire adenovirus tripartite leader at their 5′ ends. Overproduction of SV40 large T antigen by these hybrid viruses has allowed us to purify large quantities of this transforming protein and has facilitated studies of its specific DNA binding properties, transcriptional regulatory functions, and enzymatic activities (4–6). It has also been shown that adenovirus E1a proteins are overproduced when their corresponding mRNAs are transcribed from the major late promoter and carry almost all the tripartite leader (7). These results have encouraged us to make use of similar adenovirus vectors to overproduce the polyoma tumor antigens so their properties could be studied and compared to those of SV40 large T antigen.

The early region of polyoma virus encodes three proteins of 100, 55, and 22 kDa, known as large, middle, and small tumor antigens, respectively. Polyoma large T antigen, a nuclear phosphoprotein like SV40 large T antigen, is important in both the lytic and transforming cycles of the virus. It is required for the initiation of viral DNA replication, stimulation of host-cell DNA synthesis, and repression of early viral transcription (for review, see refs. 8 and 9). Polyoma large T antigen can also immortalize primary rat embryo fibroblasts, cooperate with other oncogenes to transform these cells, and lower the serum requirement of rodent cell lines (10, 11). Middle T antigen, a protein found in the plasma membrane, has an associated protein kinase activity (8, 9) and is capable of morphologically transforming established rodent cell lines (12). Very little is known about the biochemical properties of polyoma small T antigen.

To express the polyoma virus T antigens, we have generalized an adenovirus vector system that was previously used to overproduce SV40 large T antigen (3). Isolation of adenovirus–SV40 recombinant viruses depended on a strong biological selection for SV40 large T antigen, which has a helper function activity that allows human adenoviruses to grow efficiently on otherwise nonpermissive monkey cells (13–15). None of the polyoma T antigens has a similar selectable property. Therefore, to select for recombinant adenoviruses that carry polyoma sequences, we also included the SV40 T antigen gene with its own promoter in the virus constructions and grew the recombinant viruses on monkey cells. To obtain maximal levels of expression, the polyoma early coding sequences were placed under the control of the adenovirus major late promoter, within the third segment of the tripartite leader, using a combination of *in vitro* and *in vivo* recombination (2). We have expressed all three T antigens by using the entire polyoma virus early region or only large T antigen by using a modified early region encoding only large T-antigen sequences (16).

MATERIALS AND METHODS

Cells and Viruses. Monkey CV1 cells and human HeLa and 293 cells were maintained as described (1). Mouse 3T6 cells, the wild-type A2 strain of polyoma virus (9, 17), as well as the ts25E mutant (18) were obtained from W. Eckhart. The recombinant Ad-SVR284, as well as the method for the construction and amplification of Ad-PySVR498 and Ad-LTSVR545, have been described previously (1, 3).

Plasmid Constructions. pAdPySV and pAdLTSV were constructed using standard cloning procedures (19). The vector in each case is pBR322 (19), and the viral DNA insert is at the *Bam*HI site. pAdPySVP contains adenovirus DNA sequences (20) from the *Bal* I site at nucleotide 7748 (converted to *Bam*HI with linkers) to the *Xho* I site at nucleotide 9686 [converted to *Hin*dIII with linkers (3)]. A short *Hin*dIII to *Bgl* II polylinker DNA fragment derived from plink322 (19) was used to join the adenovirus DNA fragment to polyoma A3 strain (21) early region DNA (obtained from M. Botchan). The polyoma DNA fragment extends from a *Bam*HI linker at nucleotide 187 to the *Hinc*II site at nucleotide 2984. The polyoma DNA was joined to a SV40 DNA fragment (9) that extends from the *Pvu* II site at nucleotide

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Abbreviations: T antigen, tumor antigen; SV40, simian virus 40.

270 to the *Bam*HI site at nucleotide 2533 and contains the origin and early coding region.

pAdLTSV contains the adenovirus sequences described above except that the *Xho* I site at nucleotide 9686 was not altered. The adenovirus DNA was joined to a polyoma A2 strain (9) large T cDNA fragment that extends from an *Xho* I linker at nucleotide 154 to a *Bam*HI linker (filled in using Klenow polymerase) at nucleotide 2962. This polyoma large T cDNA fragment was excised from the plasmid pspLT5 (13) (generously provided by R. Kamen). As in pAdPySV, the polyoma large T cDNA sequence was joined to the SV40 origin and early region DNA fragment that extends from *Pvu* II to *Bam*HI.

Immunoprecipitation of T Antigens. The procedure for immunoprecipitation of T antigens from infected cell extracts has been described (22). Anti-polyoma tumor antiserum (23) was a gift of W. Eckhart, and the monoclonal antibody PAb419, directed against SV40 large T, was obtained from E. Harlow (24).

Primer Extension Analysis. CV1 cells infected with 25 plaque-forming units of each hybrid virus per cell were harvested 28 hr after infection. 3T6 cells were infected with polyoma ts25E virus as described by Favaloro *et al.* (25). Cytoplasmic RNA was isolated from the infected cells and subjected to oligo-dT cellulose chromatography as described (26, 27). An oligonucleotide primer containing polyoma sequence from nucleotides 173 to 196 (3′ to 5′, A2 strain numbering system; ref. 9) was synthesized using an Applied Biosystems machine. The 5′-labeled primer was annealed to mRNA for 1 hr at 60°C and was extended with reverse transcriptase as described (28).

Preparation of Nuclear Extracts. One-half liter of HeLa spinner cells (4–5 × 10^5 cells per ml) was infected with 25 plaque-forming units of Ad-LTSVR545 virus per cell and incubated at 37°C for 48 hr. The nuclei obtained after hypotonic lysis of the infected cells were extracted with 5 ml of 10 mM Tris·HCl, pH 8.0/1 mM EDTA/400 mM LiCl as described (29). Nuclear extracts containing wild-type polyoma large T antigen were similarly prepared from polyoma-infected 3T6 cells.

Immunoaffinity Assay. Aliquots of nuclear extracts containing polyoma large T antigen were subjected to immunoprecipitation with anti-polyoma tumor antiserum or with PAb419 as described (22). The antigen–antibody complexes bound to *Staphylococcus aureus* cells were resuspended in 0.1 ml of binding buffer (20 mM sodium phosphate, pH 7.0/2 mM dithiothreitol/0.01% (wt/vol) bovine serum albumin/0.1 mM EDTA/0.5% (vol/vol) Nonidet P-40/3% (vol/vol) dimethyl sulfoxide; ref. 30) containing 150 mM NaCl/10 μg of calf thymus DNA per ml/25 ng of end-labeled DNA fragments. After 1 hr at room temperature, the unbound DNA fragments were removed by washing the immune complexes with binding buffer containing 150 mM NaCl. The bound DNA fragments were eluted with 2% $NaDodSO_4$/10 mM EDTA and were fractionated on a 1.5% agarose gel (31). The gel was dried and subjected to autoradiography.

RESULTS

Construction of Recombinant Viruses. Two plasmids carrying polyoma early coding sequences were constructed for insertion into the adenoviral genome. pAdPySV contains the entire polyoma early region encoding all three tumor antigens. pAdLTSV carries a cDNA copy of the polyoma large T mRNA (Fig. 1*A*). In each plasmid, the polyoma sequences were fused to DNA sequences within the third segment of the adenovirus tripartite leader. The plasmids also contain the SV40 early promoter and SV40 large T-antigen coding sequences. The plasmid inserts were positioned within the adenoviral genome using *in vitro* and *in vivo* recombination

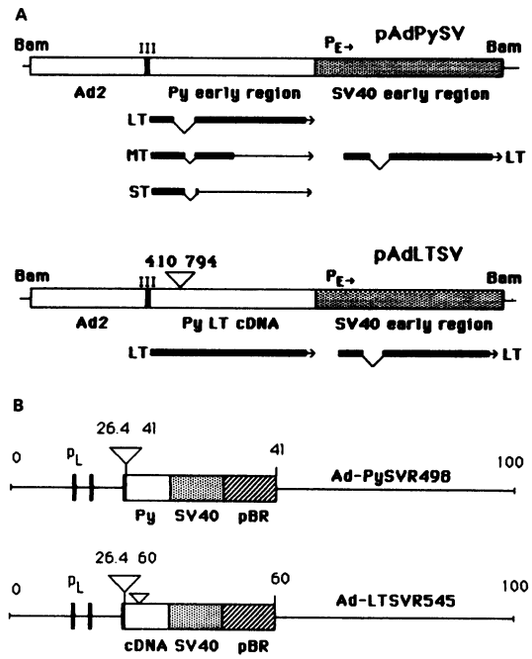

FIG. 1. (*A*) Structure of recombinant plasmids used to construct Ad-PySVR498 and Ad-LTSVR595. Both plasmids contain a fragment of the polyoma early coding region fused to the third leader segment (III) of the adenovirus genome. The SV40 early promoter (P_E) and T-antigen gene are fused downstream of the polyoma sequences. The triangle above the polyoma sequences in pAdLTSV indicates the nucleotides that were deleted from polyoma genomic DNA to construct an early region that encodes only large T antigen (16, 32). Beneath each plasmid are depicted the expected structures of major polyoma and SV40 mRNAs. Dark boxes show the region of each mRNA that is translated. LT, MT, and ST, large, middle, and small T antigens, respectively. (*B*) Structure of recombinant viruses Ad-PySVR498 and Ad-LTSVR545. Relative positions of adenovirus late promoter (P_L), late leader segments (dark boxes), and deleted adenovirus DNA (large triangles) are shown. Open boxes represent foreign DNA sequences incorporated into the adenovirus genome by recombination. The polyoma large T-antigen intron deletion in Ad-LTSVR545 is indicated by a small triangle. Numbers designate adenoviral map units (1 map unit = 360 base pairs).

(1–3). Briefly, the plasmid inserts were excised with *Bam*HI, ligated to restricted adenoviral DNA, mixed with uncut viral helper DNA, and transfected into 293 human cells. The resulting virus lysate was passaged in the presence of wild-type helper virus on CV1 monkey cells to select for those viruses that express SV40 large T antigen. After plaque purification and growth on CV1 cells, individual recombinant stocks were selected for analysis. Viral DNA was extracted from cells infected with Ad-PySVR498 and Ad-LTSVR545 (33) and was subjected to restriction enzyme digestion followed by Southern blot hybridization (34). The results showed that the plasmid insert had recombined with homologous sequences in the adenovirus vector, resulting in the genome structures shown in Fig. 1*B*. It should be noted that both viruses acquired some pBR322 sequences during the initial ligation that are located 3′ to the viral DNA insert. Each recombinant virus constitutes <5% of the total viral population.

Expression of Polyoma T Antigens in Recombinant Virus-Infected Cells. CV1 cells infected with Ad-PySVR498 and Ad-LTSVR545 showed bright nuclear fluorescence, as assayed by indirect immunofluorescence using anti-polyoma tumor antiserum (data not shown). To determine which T antigens were expressed, extracts prepared from CV1 cells infected with Ad-PySVR498 or Ad-LTSVR545 and labeled with [^{35}S]methionine were subjected to immunoprecipitation with anti-polyoma tumor antiserum or with a monoclonal

antibody directed against SV40 large T antigen. Proteins that comigrated with wild-type polyoma large, middle, and small T antigens were precipitated from Ad-PySVR498 extracts by anti-polyoma tumor antiserum (Fig. 2). Analysis of ^{35}S-labeled immunoprecipitated proteins shows that this virus expresses ≈5-fold more middle T antigen and 20-fold more small T antigen than does wild-type polyoma virus. In fact, small T antigen is visible in the total labeled extract of Ad-PySVR498-infected cells. The proteins in the 30- to 40-kDa range could be degradation products of middle or large T antigen. The level of these truncated proteins is reduced in extracts prepared from HeLa cells, which are less prone to *in vitro* proteolysis. The level of polyoma large T antigen expressed by Ad-PySVR498 is relatively low, and two forms of the protein can be seen.

A protein that comigrated with wild-type polyoma large T antigen was precipitated from extracts of Ad-LTSVR545-infected cells by anti-polyoma tumor antiserum (Fig. 2). As expected, no middle or small T antigens were detected. Analysis of Coomassie blue-stained (data not shown) and ^{35}S-labeled immunoprecipitated proteins shows that Ad-LTSVR545 produces 5-fold more large T antigen than do wild-type polyoma-infected mouse cells. Thus, ≈250 μg of large T antigen could be obtained from 1 liter of Ad-LTSVR545-infected HeLa spinner cells. Small amounts of SV40 large T antigen were detected in extracts of both hybrid virus-infected cells using a monoclonal antibody directed against SV40 large T antigen (Fig. 2). This low level of SV40 T-antigen expression could be due in part to autoregulation of the SV40 early promoter present in the hybrid viruses.

Structure of Hybrid Virus-Encoded Polyoma Antigens. Partial V8 protease digestion of immunoprecipitated, gel-purified middle and large T antigens was used to compare the primary structure of T antigens produced by the recombinant viruses with that of their wild-type counterparts. Comparison of Ad-LTSVR545 large T antigen with polyoma large T antigen (Fig. 3*A*) showed that the two proteins give almost

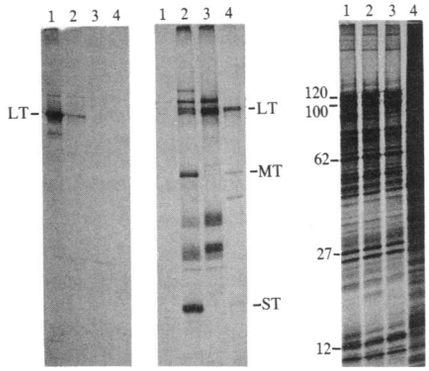

FIG. 2. Immunoprecipitation of hybrid virus-encoded T antigens. CV-1 cells (1×10^6) were infected with either Ad-SVR284 (3) (lanes 1), Ad-PySVR498 (lanes 2), or Ad-LTSVR545 (lanes 3) and 3T6 cells (1×10^6) were infected with polyoma virus (lanes 4). The amount of virus used was sufficient to cause all of the cells to become T-antigen positive in an immunofluorescence assay. Infected cells were pulse-labeled for 2.5 hr with 100 μCi of [^{35}S]methionine (1 Ci = 37 GBq) 28 hr after infection. The cells were harvested, and aliquots containing 1×10^7 cpm were subjected separately to immunoprecipitation with an anti-SV40 monoclonal (PAb419, *Left*) and with anti-polyoma tumor antiserum (*Center*). The immunoprecipitated proteins were fractionated on a 7%–15% gradient of polyacrylamide containing $NaDodSO_4$ (35). Aliquots containing 1×10^5 cpm of total labeled extract were fractionated on the same gel (*Right*). SV40 large T antigen (LT) is identified (*Left*). Polyoma large, middle (MT), and small T (ST) antigens are identified (*Center*). Numbers indicate size in kDa of some prominent adenovirus late proteins (9). (*Right*) The portion of the gel shown was exposed 5 times longer than the rest of the gel.

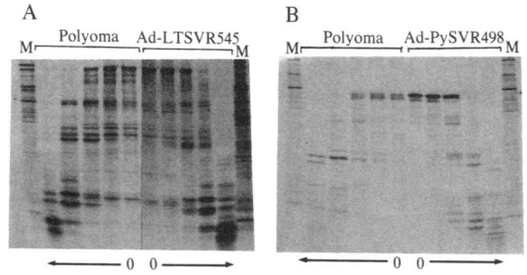

FIG. 3. Structural analysis of T antigens encoded by Ad-LTSVR545 and Ad-PySVR498. [^{35}S]Methionine-labeled large or middle T antigen was purified by immunoprecipitation and $NaDodSO_4$/polyacrylamide gel electrophoresis. Gel slices containing antigen were homogenized, aliquoted, and treated with increasing amounts (0.01, 0.1, 1.0, and 10 μg) of *S. aureus* V8 protease as described by Cleveland *et al.* (36). Central lanes indicated with 0 show the purified large T antigens encoded by Ad-LTSVR545 and polyoma virus (*A*) or middle T antigens encoded by Ad-PySVR498 and polyoma virus (*B*) fractionated on a 7%–18% polyacrylamide gradient containing $NaDodSO_4$ with no addition of V8 protease. Arrows indicate treatment of antigens with increasing amounts of protease. Outer lanes (M) contain [^{35}S]methionine-labeled proteins from Ad-LTSVR545-infected HeLa cells.

indistinguishable patterns. A similar analysis of Ad-PySVR498 large T antigen (data not shown) showed a pattern nearly identical to wild-type large T antigen. In addition, the protease digestion products of Ad-PySVR498 middle T antigen comigrate with those of wild-type polyoma middle T antigen (Fig. 3*B*). Thus, the primary structure of the hybrid virus-encoded middle and large T antigen appears to be wild type.

Since polyoma large T antigen is known to be a phosphorylated protein (37, 38), we wanted to determine whether the hybrid virus-encoded polyoma large T antigen was similarly modified. Monkey cells infected with either hybrid virus were labeled with [^{32}P]orthophosphate and subjected to immunoprecipitation with anti-polyoma tumor antiserum. A protein comigrating with authentic ^{35}S-labeled or ^{32}P-labeled polyoma large T antigen was detected (data not shown). Partial V8 protease digestion of this ^{32}P-labeled gel-purified large T antigen from Ad-LTSVR545-infected cells gave a pattern identical to that of authentic ^{32}P-labeled polyoma T antigen (data not shown). This suggests that the hybrid virus-encoded polyoma large T antigen is phosphorylated in the same manner as the wild-type protein.

Analysis of Hybrid Polyoma mRNAs. The polyoma T-antigen coding sequences that were positioned behind the adenovirus major late promoter and tripartite leader sequences should give rise to hybrid mRNAs. We wanted to ascertain that the polyoma mRNAs in hybrid virus-infected cells in fact contained the tripartite leader at their 5′ ends. A synthetic oligonucleotide primer complementary to the first 24 bases of the polyoma early coding region was hybridized to mRNA isolated from CV1 cells infected with Ad-PySVR498 or Ad-LTSVR545 and then extended with reverse transcriptase. The cDNA products were fractionated on a sequencing gel along with the extension products obtained when the same primer was annealed to early mRNA isolated from polyoma-infected 3T6 cells. The expected 211-nucleotide cDNA was observed with Ad-PySVR498 mRNA, and the expected 217-nucleotide cDNA was observed with Ad-LTSVR545 mRNA (Fig. 4); this maps the initiation site of these mRNAs to the adenovirus late promoter and shows that the tripartite leader is indeed attached to their 5′ ends. In contrast, primer extension of polyoma early mRNA yields several shorter cDNAs that map to the known start sites of the polyoma early promoter (39, 40). The primer extension products were excised from

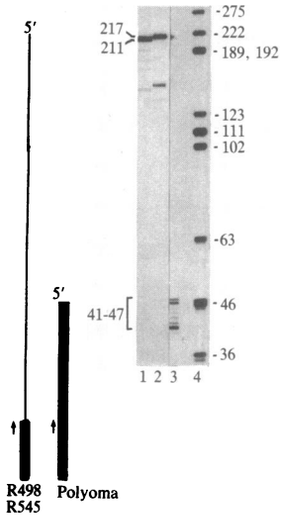

FIG. 4. Structural analysis of the 5′ ends of hybrid mRNAs encoded by Ad-PySVR498 and Ad-LTSVR545. A synthetic polyoma primer was annealed to mRNAs derived from Ad-PySVR498 (1 μg) (lane 1), Ad-LTSVR545 (1 μg) (lane 2), and polyoma ts25E (2 μg) (lane 3). The products of reverse transcription were displayed on an 8% urea/polyacrylamide gel, shown here as a composite of two exposures. The lengths of the major primer extension products derived from Ad-PySVR498, Ad-LTSVR545, and polyoma ts25E mRNAs are 211 nucleotides, 217 nucleotides, and 41–47 nucleotides, respectively. The difference in length between the Ad-PySVR498 and Ad-LTSVR545 cDNAs is due to a difference in the junction between the adenovirus and polyoma DNA sequences generated in the construction of the hybrid viruses. The lengths of ^{32}P-labeled DNA standards are indicated in the marker lane (lane 4). (*Left*) Schematic depiction of the hybrid virus (R498, R545) and polyoma mRNAs that hybridize to the synthetic primer. Dark boxes indicate polyoma sequences and line represents adenovirus leader sequences. Primer is indicated by an arrow with the head at its 3′ end.

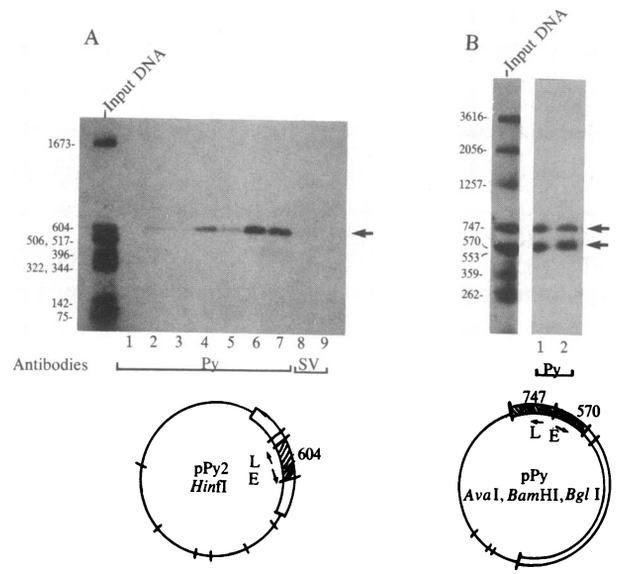

FIG. 5. DNA binding activity of Ad-LTSVR545 polyoma large T antigen. (*A*) Polyoma large T antigen purified from Ad-LTSVR545-infected cells was incubated with fragments of pPy2 DNA generated by *Hin*fI digestion. pPy2 contains the *Ava* I to *Bam*HI fragment of polyoma A3 DNA (open boxes) that includes the origin of viral DNA replication and transcription control region (hatched box). Plasmid vector is indicated by a thin line and *Hin*fI sites are shown by slashes. L and E show directions of late and early transcription, respectively. DNA fragments used in the binding experiment are displayed in the lane marked input DNA. The following extracts were used: lane 1, Ad-LTSVR545 cytoplasm; lanes 2, 4, 6, and 8, Ad-LTSVR545 nuclear extract; lanes 3, 5, 7, and 9, polyoma nuclear extract. Py indicates anti-polyoma tumor antiserum. SV represents anti-SV40 monoclonal antibody. Amounts of extract used are as follows: lane 1, 80 μl; lanes 2 and 3, 20 μl; lanes 4 and 5, 40 μl; lanes 6–9, 80 μl. Arrow shows position of specifically bound fragment. (*B*) Immunoprecipitated large T antigen was incubated with DNA from pPy that had been digested with *Ava* I, *Bam*HI and *Bgl* I. pPy contains the entire polyoma A3 genome cloned into pBR322. Polyoma sequences are indicated by an open box and slashes represent sites of digestion by the restriction enzymes. DNA fragments specifically bound by large T antigen are shown as hatched boxes. All other symbols are the same as for *A*. DNA fragments used in the binding experiment are displayed in the lane marked input DNA. Lane 1, 100 μl of Ad-LTSVR545 extract. Lane 2, 100 μl of polyoma extract. Sizes of input DNA fragments are shown in base pairs.

the gel, and the content of ^{32}P showed that the hybrid virus mRNAs are ≈40-fold overproduced relative to polyoma early mRNAs.

S1 nuclease analysis of Ad-PySVR498-encoded polyoma mRNAs using polyoma probes revealed the presence of T-antigen exons of the expected sizes (data not shown; see ref. 32). The amount of the large T-antigen 5′ exon was considerably lower than the amounts of the middle and small T-antigen 5′ exons combined. Thus, the levels of the three polyoma tumor antigens expressed by this virus reflect the levels of their mRNAs.

Binding of Ad-SVLTR545 Polyoma Large T Antigen to Polyoma Virus DNA. To determine whether Ad-LTSVR545 polyoma large T antigen possesses biochemical activities characteristic of the wild-type polyoma protein, we examined its ability to bind to specific sequences of polyoma DNA using a DNA fragment immunoaffinity assay. Polyoma large T antigen in a nuclear extract prepared from Ad-LTSVR545-infected HeLa cells or from polyoma-infected 3T6 cells was purified by immunoprecipitation with anti-polyoma tumor antiserum. After subjecting the antigen–antibody complexes to extensive washing to remove nonspecifically bound protein, the purified large T antigen was incubated with a collection of ^{32}P-labeled DNA fragments generated from a plasmid containing the polyoma origin region (*Bam*HI to *Ava* I). DNA fragments that do not contain a high-affinity binding site for polyoma large T antigen were removed by washing the immune complexes. The fragments that bound were eluted with $NaDodSO_4$, fractionated by agarose gel electrophoresis, and subjected to autoradiography. Increasing amounts of large T antigen purified from both hybrid virus- and polyoma virus-infected cells were shown to bind selectively to increasing amounts of a single 604-base-pair fragment that contains the polyoma origin of replication and promoter region (Fig. 5*A*). By contrast, antibody directed against the small amounts of SV40 large T antigen present in the Ad-LTSVR545 extract did not precipitate the polyoma origin fragment.

Because the polyoma origin of DNA replication is separated from the early transcriptional initiation sites by ≈60 base pairs, we wanted to determine whether polyoma large T antigen could bind independently to these two regions of the viral DNA. Therefore, we subdivided the polyoma regulatory region by cleavage with *Bgl* I into two fragments, one containing the origin of DNA replication (747 base pairs) and one containing the early transcriptional initiation sites (570 base pairs). Using the fragment immunoaffinity assay, polyoma large T antigen from both the hybrid and wild-type viruses was shown to bind specifically to both of these DNA fragments.

DISCUSSION

We have obtained recombinant adenoviruses that contain and express polyoma T-antigen coding sequences. In addi-

tion to large amounts of T antigens synthesized in Ad-PySVR498- and Ad-LTSVR545-infected cells, adenovirus inhibits host protein synthesis late in the infectious cycle, making it easier to detect and purify viral-encoded proteins (41, 42). Therefore, cells infected with both of the recombinant viruses should constitute good starting material for the purification of polyoma T antigens. Because the hybrid viruses contain pBR322 sequences downstream of the polyoma insert and represent <5% of the viral population, it is difficult to assess the maximum amount of polyoma T antigens that could be produced. We expect that new hybrid viruses containing polyoma large T cDNA sequences and lacking plasmid sequences will constitute a higher proportion of the viral population.

The polyoma sequences in the recombinant viruses are maintained and expressed, even though we had no direct biological selection for their expression. This was accomplished by placing a selectable marker, the SV40 A gene, downstream of the polyoma sequences and growing hybrid viruses in monkey cells where the helper function encoded by SV40 large T antigen is required for efficient adenovirus propagation (13–15). In this way, recombinant viruses that carried polyoma DNA sequences were selected and maintained by virtue of their linkage to the SV40 helper function. This strategy is general and has been used to obtain viruses carrying other unselected genes. For example, M. Yamada has constructed adenoviruses that express the HSV-1 thymidine kinase gene at levels equivalent to late viral capsid proteins as well as hybrid viruses that produce human α-chorionic gonadotropin in a glycosylated form that is efficiently secreted from infected cells (personal communication).

It should be noted that, while all three polyoma T antigens are synthesized in Ad-PySVR498-infected human or monkey cells, the ratios between the proteins are different from those found in polyoma-infected mouse cells. However, these lower levels of large T antigen relative to levels of middle and small T antigens correspond to the amounts of the respective mRNAs produced by the hybrid virus. This suggests that the donor splice sites for large T antigen versus middle and small T antigen mRNAs are used with different efficiencies in adenovirus-infected cells. A similar situation is found with adenovirus–SV40 hybrid virus-infected monkey cells in which the donor splice sites for SV40 large and small T antigens are differentially utilized (1).

Having overproduced these various polyoma T-antigen proteins, it is important to determine whether they can carry out the biological functions that are associated with the wild-type protein. Here, we have shown that the origin binding activity of Ad-LTSVR545 polyoma large T antigen is fundamentally equivalent to the wild-type protein isolated from polyoma-infected 3T6 cells. It is able to bind independently to two distinct regions of the viral genome as has previously been reported for the wild-type protein (43, 44). The amount of protein we can now obtain from HeLa spinner cells infected with Ad-LTSVR545 (250 μg/liter) will allow us to purify large T antigen and analyze its enzymatic properties as well as the specific binding interactions at different sites on polyoma DNA. We also intend to assess the role of polyoma large T antigen in autoregulation and to compare its properties to those of SV40 large T antigen. Because the replication origin and large T-antigen binding sites of polyoma DNA are spatially distinct from those of SV40, the ability to carry out detailed comparative binding studies may help us to understand the regulatory interactions between these proteins and their target DNA sequences.

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