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Construction and analysis of simian virus 40 origins defective in tumor antigen binding and DNA replication

(recombinant plasmids/mutagenesis *in vitro*/D2 protein)

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ABSTRACT **We have inserted a 311-base pair DNA fragment containing the simian virus 40 (SV40) origin of DNA replication, the early promoter, and the tumor (T) antigen binding sites into a bacterial plasmid and cloned it. This recombinant plasmid, pSV01, binds to a purified T antigen *in vitro* and replicates in monkey cells when supplied with large T antigen. A series of deletion mutations was generated in the origin sequences of pSV01 DNA by mutagenesis *in vitro*. The replication of these mutant DNAs in monkey cells was compared with their ability to bind to purified D2 protein. Mutant DNAs deficient in binding to D2 protein also exhibit reduced levels of replication in monkey cells. These findings provide biochemical evidence that the initiation of SV40 DNA synthesis may involve a direct interaction of T antigen with sequences at the origin of replication.**

The double-stranded circular genome of simian virus 40 (SV40) provides a relatively simple model system for studying the processes involved in eukaryotic DNA synthesis. Replication of the 5243-base pair (bp) genome of SV40 begins at a unique site and proceeds bidirectionally in a manner analogous to the replication of chromosomes of higher animals (1–3). However, unlike cells replicating their DNA, SV40 can reinitiate many rounds of DNA synthesis during S phase so long as the *A* gene product of SV40 is expressed (4). Genetic evidence indicates that the product of the *A* gene, tumor (T) antigen, is required for the initiation of viral DNA synthesis (5). Biochemical studies indicate that SV40 large T antigen is a 96,000-dalton (Dal) phosphoprotein that is expressed in high levels early after infection and is found predominantly in the nucleus of cells infected and transformed by SV40 (4). We recently demonstrated that T antigen interacts specifically with three tandem binding sites on SV40 DNA in a region centered at the origin of replication (6). Both the 96,000-Dal T antigen and the D2 hybrid protein isolated from an adenovirus–SV40 hybrid virus display specific binding to the origin sequences (7). In addition, binding of T antigen to the three recognition sites was shown to occur sequentially.

The fact that T antigen is required for the initiation of viral DNA synthesis and binds to the origin region suggests that a direct interaction between the protein and DNA may be necessary for viral replication. Recently Shortle *et al.* (8) provided strong genetic evidence in support of this idea. Viruses with substitution mutations in one of the T antigen binding sites were shown to have reduced rates of DNA synthesis at low temperatures. The mutations in these cold-sensitive origin mutants are overcome by second-site mutations mapping in the gene for T antigen.

It is generally difficult to obtain mutations in regions of DNA that contain regulatory information, such as origins of replication, transcription promoters, and translation signals, because they are often lethal to the organism. Recently, conditional mutations and genomes containing duplicate regulatory sequences have been successfully applied towards selecting mutations in the origin sequences of SV40 (9, 10). We have been able to generate a large collection of deletion mutations in the SV40 origin region by inserting a small fragment of SV40 DNA containing the viral origin of replication into a bacterial plasmid and cloning it. In order to study the biological activity of cloned origin sequences, a functional assay was devised that directly tests for the replication of these recombinant plasmids in monkey cells. In addition, a rapid and sensitive assay was developed to measure the binding of the D2 protein to mutant and wild-type origin DNAs.

MATERIALS AND METHODS

Enzymes. *Eco*RI and *Hin*dIII restriction endonucleases were gifts from D. Roulland-Dussoix and M. J. Chamberlin. Other restriction enzymes and BAL 31 nuclease were purchased from Bethesda Research Laboratories, Rockville, MD.

Cells. Monolayers of CV1L and Cos monkey cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum (Irvine Scientific, Santa Ana, CA), streptomycin (100 μg/ml), and penicillin (100 μg/ml). Cos cells were a generous gift from Y. Gluzman.

Containment Conditions. Experiments involving recombinant DNA were conducted under P2–EK1 conditions as described in the National Institutes of Health guidelines.

Mutagenesis Using Nuclease BAL 31. Deletion mutations were made in the SV40 origin of replication by using BAL 31 nuclease, an enzyme from *Pseudomonas* BAL 31 that has a single-stranded endonucleolytic activity and a processive exonucleolytic activity for double-stranded DNA (11). One microgram of linearized plasmid DNA at 10–20 μg/ml was incubated with 0.001 unit of nuclease BAL 31 for 20–40 min at 30°C (1 unit is the amount of enzyme that releases 1 μg of acid-soluble nucleotide in 1 min at 30°C).The enzyme was removed by phenol extraction and the DNA was concentrated by precipitation with ethanol.

Transfection of Monkey Cells with Plasmid and Viral DNA. Monolayers of CV1L or Cos monkey cells were grown to 40–50% confluency on 6-cm plastic dishes. The cells were washed with phosphate-buffered saline (P_i/NaCl) and Tris-buffered saline (Tris/NaCl), and 200 μl of a solution containing 50 ng of plasmid DNA or 100 ng of SV40 DNA and DEAE-dextran (Sigma) at 500 μg/ml in Tris/NaCl was layered over the cells. After incubation at 37°C for 25 min, the DNA solution was removed and the cells were washed with Tris/NaCl and

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Abbreviations: SV40, simian virus 40; T antigen, tumor antigen; Dal, dalton(s); P_i/NaCl, phosphate-buffered saline; Tris/NaCl, Tris-buffered saline; bp, base pair(s).

P_i/NaCl. Five milliliters of medium was added and the cells were incubated at 37°C for various times.

Purification of D2 Protein. We have used HeLa cells infected with a defective adenovirus–SV40 hybrid virus, $Ad2^+D2$, as the source of T antigen for the binding studies reported here. This hybrid virus encodes a 107,000-Dal polypeptide that is functionally equivalent to SV40 large T antigen by all criteria applied thus far (12). The D2 protein is composed of approximately 10,000 Dal of an adenovirus protein at its amino terminus and 90,000 Dal of SV40 large T antigen at its carboxy terminus. Purification of the D2 protein to homogeneity was as described (6).

Filter Binding Assay. Plasmid DNAs were digested with *Eco*RI restriction enzyme and labeled with ^{32}P at the 5′ ends by using phage T4 polynucleotide kinase (13). Reaction mixtures containing end-labeled DNA (2 μg/ml) and D2 protein (1–25 μg/ml) were incubated at room temperature for 15 min in 25 mM 1,4-piperazinediethanesulfonic acid (Pipes) (pH 6.8)/150 mM NaCl/0.2 mM dithiothreitol. The reaction mixtures were filtered through 18-mm nitrocellulose membranes (0.45 μm pore size) and washed with a buffer containing 20 mM sodium phosphate (pH 7.0), 1 mM EDTA, and 3% (vol/vol) 2-mercaptoethanol. Cherenkov radiation from the dried filters was quantitated in a Beckman counter.

RESULTS

Construction of plasmids with wild-type and mutant SV40 origin sequences

We have inserted a small region of the SV40 genome containing the origin of DNA replication into a bacterial plasmid and cloned it in order to facilitate the construction of mutations in this regulatory region and to provide a ready source of DNA for replication, transcription, and T antigen binding studies. The restriction endonuclease *Eco*RII was used to cleave the SV40 genome into 16 fragments. The 311-bp *Eco*RII-G fragment containing the viral origin of DNA replication and the three tandem binding sites for SV40 large T antigen were purified by agarose gel electrophoresis and treated with nuclease S1 to remove the single-stranded ends (Fig. 1). *Eco*RI linker sequences were ligated to the flush-ended *Eco*RII G fragment and the fragment was inserted into the *Eco*RI site of the *Escherichia coli* plasmid pBR322. This recombinant plasmid, pSV01, contains a wild-type SV40 origin of DNA replication, the early promoter, and T antigen binding sites.

Site-directed mutagenesis of T antigen binding sites I and II was achieved by cutting pSV01 DNA with *Hin*dIII and then treating the linearized plasmid DNA with BAL 31 nuclease to remove small sequences of DNA. The blunt-ended DNAs in the resulting mixture were recircularized with T4 DNA ligase and used to transform *E. coli* cells. By using this procedure a collection of plasmid DNAs was obtained that lacked sequences in binding site I and in binding sites I and II (Fig. 2*B*).

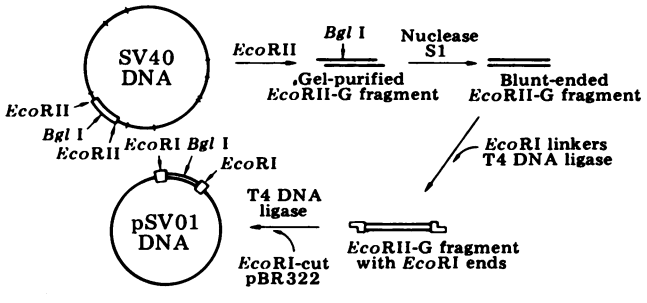

FIG. 1. Construction of pSV01.

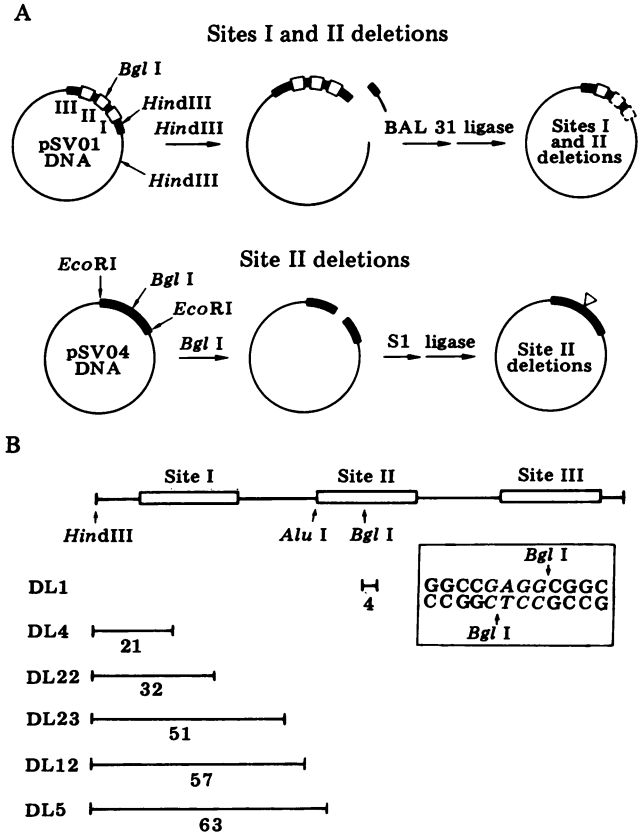

FIG. 2. Construction of mutant origin clones. (*A*) Techniques used to generate deletions in T antigen binding sites I and II. (*B*) Map of deletions obtained in cloned origin sequences. Upper drawing represents 140 bp of the wild-type origin region. Below are depicted deletions in six different clones; the numbers below the lines are the sizes of the deletions in bp. The DNA sequence around the *Bgl* I cleavage site is shown in the inset, and the 4 bp lacking in DL1 DNA are shown in italic letters.

In addition, mutants lacking sequences within binding site II were obtained by removal of nucleotides at the *Bgl* I restriction endonuclease site in the SV40 origin sequence. To facilitate this step the 311-bp *Eco*RII-G fragment was excised from pSV01 with *Eco*RI, inserted into a plasmid (pNB1) containing no *Bgl* I cleavage sites, and recloned. The resulting plasmid, containing a single *Bgl* I cleavage site in the SV40 sequence, was designated pSV04. After cleavage of pSV04 with *Bgl* I, the single-stranded tails of the linearized molecules were removed with S1 nuclease (Fig. 2*A*). The blunt-ended molecules were circularized with T4 DNA ligase and introduced into *E. coli* cells. One of the cloned plasmid DNAs, DL1, was shown by Maxam and Gilbert nucleotide sequence determination (14) to lack four base pairs at the *Bgl* I site (Fig. 2*B*).

Replication of pSV01 DNA in monkey cells

In order to study the relationship between T antigen binding sites and SV40 DNA replication, we needed to devise a functional assay that would allow us to test the biological activity of the cloned SV40 origin sequence. As a first step toward this goal, we determined whether pSV01 DNA would replicate in CV1L monkey cells when the *A* gene product was supplied by coinfection with SV40 DNA. A mixture of supercoiled pSV01 DNA and SV40 DNA was used to transfect monolayers of CV1Ls in the presence of DEAE-dextran. After incubation of the cells at 37°C for various times, the small supercoiled viral and plasmid DNAs were isolated from the cells by using the Hirt lysis procedure (15). The DNAs were subjected to agarose gel electrophoresis and transferred to nitrocellulose (16). In

order to visualize pSV01 DNA, the nitrocellulose filter was probed with ^{32}P-labeled pBR322 DNA and subjected to autoradiography. Immediately after transfection of the cells with a mixture of pSV01 and SV40 DNA, forms I (supercoiled DNA), II (relaxed circular DNA), and III (linear DNA) of the plasmid were observed (Fig. 3A). At 12 hr after transfection, only forms II and III persisted. After 50 hr of incubation, there was a marked increase in the amount of supercoiled plasmid DNA relative to the level of form I seen at time zero and at 12 hr after transfection. This observation provides direct evidence that pSV01 DNA is capable of replicating in the monkey cells. No replication of plasmid DNA was seen when a similar experiment was performed in the absence of coinfection with SV40 DNA, indicating that the replication of pSV01 DNA is T antigen dependent. Furthermore, the plasmid pBR322, lacking the origin sequences of SV40 DNA, does not replicate in monkey cells either in the presence or in the absence of T antigen.

A different monkey cell line, Cos, was also used to study the replication of cloned origin DNAs. This cell line contains integrated copies of SV40 DNA and expresses a high level of T antigen that is capable of complementing temperature-sensitive A mutants (17). By using the experimental approach described above, replication of pSV01 DNA in this cell line was found to occur in the absence of coinfection with SV40 DNA (Fig. 3B). In fact, the efficiency of pSV01 DNA replication in Cos cells is 5- to 10-fold higher than in CV1L cells transfected with pSV01 and SV40 DNA. In the case of Cos, every cell that takes up a pSV01 DNA molecule can replicate the plasmid DNA, because every cell contains T antigen. However, in CV1Ls, only those cells that absorb both pSV01 and SV40 DNA molecules are able to initiate replication at the SV40 origin in the plasmid.

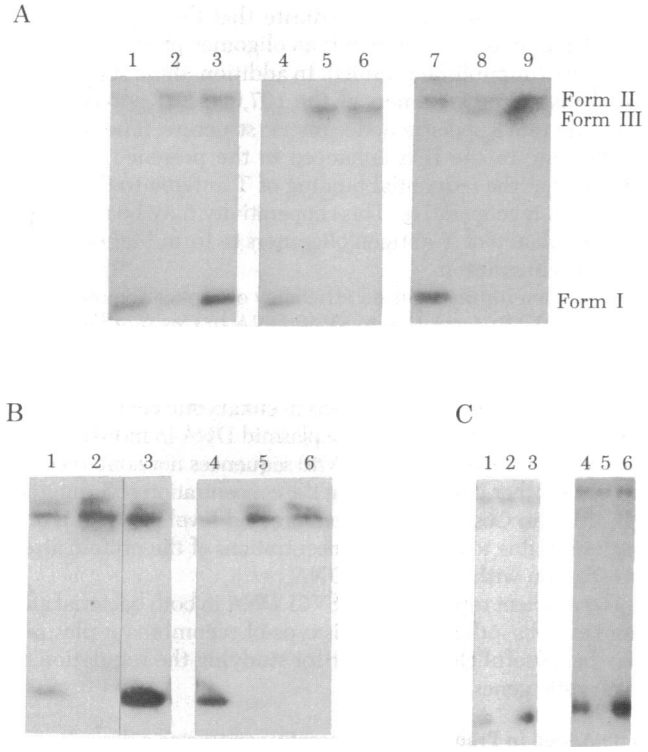

FIG. 3. Gel electrophoresis of supercoiled and relaxed forms of plasmid and viral DNAs isolated from monkey cells. Supercoiled DNA was introduced into monkey cells and analyzed by Southern blot hybridization. (A) DNA from Hirt lysates of CV1L cells at 0 hr (lanes 1, 4, and 7), 12 hr (lanes 2, 5, and 8), and 50 hr (lanes 3, 6, and 9) after transfection with pSV01 and SV40 DNA (lanes 1–3), pSV01 DNA alone (lanes 4–6), and pBR322 DNA (lanes 7–9). (B) DNA from Cos cells transfected with pSV01 DNA (lanes 1–3) and pBR322 DNA (lanes 4–6) at 0, 12, and 50 hr after transfection. (C) Relative efficiency of replication of SV01 DNA (lanes 1–3) and SV40 DNA (lanes 4–6) in Cos cells at 0 hr (lanes 1 and 4), 12 hr (lanes 2 and 5), and 50 hr (lanes 3 and 6) after transfection.

Our findings with both CV1L and Cos monkey cells indicate that the 311-bp *Eco*RII-G fragment of SV40 DNA is sufficient to direct the replication of a recombinant plasmid in monkey cells, provided that functional T antigen is supplied in sufficiently high amounts. Despite the relative efficiency of pSV01 replication in Cos cells, we observed that the plasmid DNA replicates 1/5 to 1/10 as efficiently as SV40 DNA (Fig. 3C).

Introduction of mutant origin plasmids into Cos monkey cells

Having determined that a plasmid containing a wild-type SV40 origin can replicate efficiently in monkey cells, we then used mutant origin clones to determine which deletions render the origin of viral replication nonfunctional. Plasmid DNAs from mutant origin clones were tested for their ability to replicate in Cos monkey cells by using the procedure described above. No replication of plasmid DNA is seen when Cos cells are transfected with DL1 DNA, a plasmid that is missing 4 bp in the T antigen binding site II (Fig. 4, lane 4). However, a wild-type level of replication occurs when a mutant DNA (DL4) missing approximately one-third of binding site I is introduced into Cos cells (Fig. 4, lane 5). When DNA lacking 20 of the 26 nucleotides in binding site I (DL22) is introduced into Cos monkey cells, replication occurs at a level 1/2 to 1/5 of that in wild-type (Fig. 4, lane 6). No replication of plasmid DNA occurs when DNA lacking binding site I and approximately two-thirds of the region between sites I and II (DL23) is used to transfect Cos cells (Fig. 4, lane 7). Similarly, DL12 and DL5 DNA, which lack site I and part of site II, do not replicate in Cos cells (data not shown). These findings confirm that sequences in site II are required for DNA replication and in addition suggest that sequences between binding sites I and II are necessary for a functional origin. Moreover, it appears that sequences in binding site I may also play a role in viral DNA replication.

Binding of purified D2 hybrid protein to origin DNA sequences

In an effort to understand the relationship between the binding of T antigen to the SV40 origin of DNA replication and the role of T antigen in DNA replication, we developed a rapid and quantitative filter binding assay to measure the specific binding of D2 protein to origin sequences. End-labeled DNAs containing wild-type or mutant origin sequences were incubated with the D2 protein (1–25 μg/ml) and filtered through a ni-

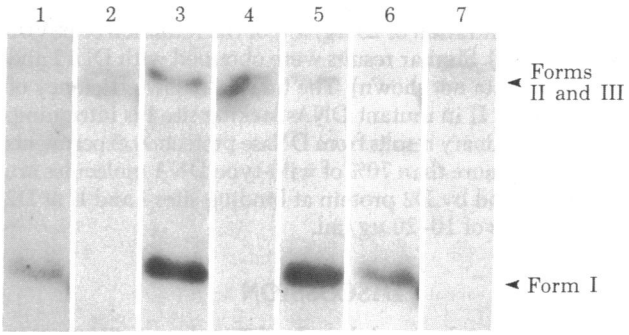

FIG. 4. Gel electrophoresis of mutant origin DNAs introduced into Cos monkey cells. pSV01 DNA in Hirt lysates at 0 hr (lane 1), 12 hr (lane 2), and 50 hr (lane 3) after transfection. Lanes 4–7 are from 50-hr lysates of DL1, DL4, DL22, and DL23, respectively.

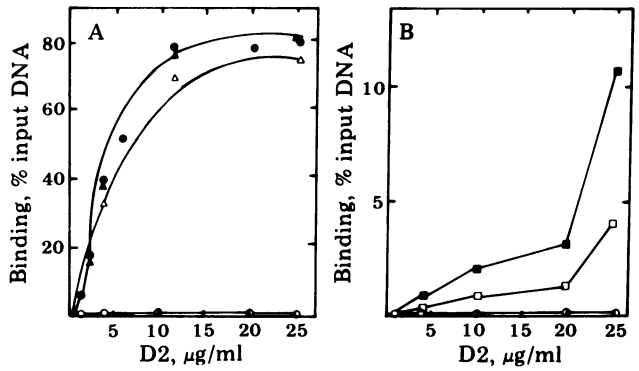

FIG. 5. Binding of D2 protein to wild-type and mutant origin clones. DNA labeled at the 5′ ends was incubated with various concentrations of D2 protein and filtered through nitrocellulose. The degree of binding was calculated as the percent of the input DNA molecules retained on the filter by the protein. (*A*) pSV01 (●), DL1 (▲), DL4 (△), and pBR322 (○) DNA. (*B*) DL22 (■), DL23 (□), and pBR322 (○) DNA.

trocellulose membrane. Under the conditions of the assay, greater than 70% of the input pSV01 DNA is retained on the filter (Fig. 5*A*). Similarly, end-labeled SV40 DNA is efficiently bound by the D2 protein under the assay conditions. By contrast, less than 0.2% of input pBR322 DNA (lacking SV40 origin sequences) is retained on the filter by the D2 protein. These results indicate that it is possible to develop a sensitive and quantitative filter binding assay potentially capable of detecting small differences in the binding interaction between the D2 protein and mutant DNA sequences at the SV40 origin of replication.

We have used this assay to measure the efficiency of the interaction between D2 protein and DNA from various deletion mutants lacking sequences in the T antigen binding sites. It was predicted that any mutant DNA containing binding site I would be retained on a nitrocellulose filter by D2 protein to the same extent as pSV01 DNA, because site I is the strongest binding site. Indeed, DNA from mutant DL1, which lacks only 4 bp in T antigen binding site II (at the *Bgl* I cleavage site), is retained on the filter by the D2 protein as efficiently as the wild-type sequence of pSV01 DNA (Fig. 5*A*). Likewise, mutant DNA lacking a small portion of the T antigen binding site I (DL4) also exhibits a normal level of binding to the D2 protein. By contrast, DNA from mutant DL22, which lacks 75% of the sequences for binding site I, is retained on the filter approximately 1/20 to 1/30 as efficiently as pSV01 DNA at D2 protein concentrations of 1–20 μg/ml (Fig. 5*B*). At a D2 protein concentration of 25 μg/ml, DL22 DNA is retained on the filter 1/8 as efficiently as wild-type DNA. The retention of DL23 DNA on the filter is 1/40 to 1/80 (at D2 concentrations of 1–20 μg/ml) and 1/20 (at a D2 concentration of 25 μg/ml) of the retention of pSV01 DNA (Fig. 5*B*). Similar results were obtained with DL12 and DL5 DNA (data not shown). The unusually low efficiency of binding to site II in mutant DNAs lacking site I is intriguing, because preliminary results from DNase protection experiments indicate that more than 70% of wild-type DNA molecules are normally bound by D2 protein at binding sites I and II at D2 concentrations of 10–20 μg/ml.

DISCUSSION

In an effort to understand the role of T antigen in SV40 DNA synthesis, we have cloned the viral origin of replication in a bacterial plasmid and used this parent plasmid to generate a series of deletion mutations in the origin sequences. Mutant DNAs deficient in binding to the D2 protein *in vitro* are also deficient in replication in monkey cells. By contrast, some deletion mutants (in site II) that are unable to replicate retain the ability to bind T antigen at site I. These results suggest that the mechanism for the initiation of SV40 DNA replication involves a direct interaction of T antigen with origin sequences and that the binding of T antigen is necessary but not sufficient to trigger viral DNA synthesis. Analysis of additional origin mutants is necessary to confirm this hypothesis. We recently demonstrated that the binding of D2 protein to its regulatory sites selectively modulates the transcription of SV40 early genes in an *in vitro* RNA synthesizing system (18). Thus, it appears that the binding of T antigen to regulatory sequences in SV40 plays an important role in viral DNA synthesis and transcriptional autoregulation.

DNA sequences near the *Bgl* I recognition sequence in binding site II are necessary for viral DNA replication (9). Here we report that in addition to binding site II, DNA sequences in binding site I, the 18 nucleotide pairs between binding sites I and II, or both are also required for replication of viral DNA. A mutant DNA lacking three-fourths of binding site I (DL22) replicates less efficiently than wild-type DNA. A mutant DNA containing an intact binding site II but lacking site I and sequences between sites I and II (DL23) does not replicate in monkey cells. Furthermore, the binding of the D2 protein to site II in these mutant DNAs is 1/10 to 1/20 as efficient as binding to site II in wild-type DNA. This lowered binding to site II may be responsible for the defective replication of these mutant DNAs.

Electron microscopic data indicate that the predominant form of D2 protein in solution is an oligomer presumed to be a tetramer (unpublished results). In addition, these studies revealed that three tetramers of the 107,000-Dal polypeptide aggregate into a specific dodecameric structure. The finding that binding to site II is enhanced in the presence of site I suggests that the sequential binding of T antigen to these adjacent sites is cooperative. This cooperativity may be reflected in the tendency of T antigen oligomers to form higher-order structures in solution.

The reason for the reduced efficiency of replication observed with pSV01 DNA relative to SV40 DNA in Cos monkey cells is unknown. It is possible that specific plasmid sequences or modification of plasmid DNA in bacteria decrease the elongation rate during DNA synthesis in eukaryotic cells. Alternatively, optimal replication of the plasmid DNA in monkey cells may require some additional SV40 sequences not contained in pSV01. Another possibility is that the concentration of T antigen is limiting in Cos cells, and the increased level of SV40 DNA synthesis is due to increased concentrations of the protein after transfection with intact viral DNA.

The efficient replication of pSV01 DNA in both bacterial and monkey cells indicates that this type of recombinant plasmid may be a useful cloning vector for studying the regulation of eukaryotic genes.

Note Added in Proof. We have recently constructed a derivative of pSV01 that lacks pBR322 sequences from 0.00 to 0.56 on the conventional pBR322 map. This new plasmid, designated pSV05, should be more useful than pSV01 as a hybrid vector because it replicates 5–10 times more efficiently than pSV01 DNA in monkey cells.

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