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Polyomavirus Origin for DNA Replication Comprises Multiple Genetic Elements

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To define the minimal *cis*-acting sequences required for polyomavirus DNA replication (*ori*), we constructed a number of polyomavirus-plasmid recombinants and measured their replicative capacity after transfection of a permissive mouse cell line capable of providing polyomavirus large T antigen in *trans* (MOP cells). Recombinant plasmids containing a 251-base-pair fragment of noncoding viral DNA replicate efficiently in MOP cells. Mutational analyses of these viral sequences revealed that they can be physically separated into two genetic elements. One of these elements, termed the core, contains an adenine-thymine-rich area, a 32-base-pair guanine-cytosine-rich palindrome, and a large T antigen binding site, and likely includes the site from which bidirectional DNA replication initiates. The other, termed β, is located adjacent to the core near the late region and is devoid of outstanding sequence features. Surprisingly, another sequence element named α, located adjacent to β but outside the borders of the 251-base-pair fragment, can functionally substitute for β. This sequence too contains no readily recognized sequence features and possesses no obvious homology to the β element. The three elements together occupy a contiguous noncoding stretch of DNA no more than 345 base pairs in length in the order α, β, and core. These results indicate that the polyomavirus origin for DNA replication comprises multiple genetic elements.

Polyomavirus, one of the papovaviruses, has served as a model to study mechanisms of transcription and DNA replication in mammalian cells. The utility of the virus derives from the small size of its genome and its almost total reliance on cellular proteins for replication. The elucidation of the primary structure of the viral DNA, the identification of the viral proteins, and the construction of physical maps of the viral mRNAs (9, 15, 22, 38) have heightened interest in the virus not only as a tool to study cellular processes, but also as a vector for the introduction and expression of foreign DNA into mammalian cells (18, 32). Although much information has been accumulated about the coding sequences of the virus and the proteins they specify, much less is known about regulatory regions within the viral DNA and their interaction with viral and cellular proteins. We have begun to define the sequences required in *cis* for viral DNA replication and transcription as a prelude to help understand the mechanisms of gene expression, DNA replication, and their regulation in mammalian cells. Here we report our findings concerning those noncoding sequences required in *cis* for polyomavirus DNA replication. For convenience of expression we refer to these *cis*-acting sequences as *ori* throughout the text.

Genetic and biochemical analyses of polyomavirus DNA replication have revealed that efficient viral replication requires a permissive host cell, a viral gene product (large T antigen), and *cis*-acting viral sequences. Polyomavirus DNA replicates efficiently in cells of murine origin, but poorly or not at all in cells derived from other species (6). Restricted replication of polyomavirus DNA in nonmurine cells is due to the absence of permissive factors rather than the presence of inhibitors (1). The nature of these permissive factors has not yet been elucidated, although cell-cell hybridization studies suggest that they are encoded by the genome of the permissive cell (2). Another essential ingredient for efficient DNA replication is large T antigen. This protein is encoded by the viral genome and is synthesized early during infection before viral DNA replication commences. Genetic studies have revealed that large T antigen is required for the initiation of each new round of viral DNA synthesis (8). The latter likely requires the physical interaction of large T antigen with *ori*. Two large T antigen binding sites have been mapped on the viral genome (34), and one of these lies

near sequences from which DNA replication initiates. Finally, the *cis*-acting sequences that comprise *ori* are required for the initiation of viral DNA replication. The boundaries of *ori* have not been precisely defined. However, studies of evolutionary variants of polyomavirus (16, 24) and viable deletion mutants (3, 17, 27, 47) have shown that *ori* resides within noncoding sequences that include the site of initiation of DNA synthesis. To more precisely locate the limits of *ori* we have employed a strategy that measures the replicational capacity of polyomavirus recombinant plasmids in a line of mouse cells (MOP cells) that synthesize the viral large T antigen. MOP cell lines were established by the transformation of NIH 3T3 cells with a hybrid transcription unit composed of the simian virus 40 (SV40) early promoter and the polyomavirus early region. These cells produce all of the polyomavirus early proteins, large, middle, and small T antigen, in a permissive cellular environment (W. Muller, M. Naujokas, and J. A. Hassell, data not shown). We measured the replicational capacity of a series of virus-plasmid recombinants and deletion derivatives in one such MOP cell line. Our analyses revealed that *ori* is composed of three sets of sequences. Two of these sequence elements, termed alpha (α) and beta (β), are functionally redundant and exert their effect in conjunction with a third element called the core region. Although these elements lie close together in the viral genome, deletion analyses revealed that they are physically distinct.

MATERIALS AND METHODS

Mammalian cells. MOP-8 cells were grown on plastic dishes, with Dulbecco modified Eagle medium (DMEM) supplemented with 10% (vol/vol) calf serum and antibiotics, and maintained at 37°C in a humidified CO_2 atmosphere. Cells were passaged by trypsinization after reaching confluence and were replated at approximately 10^4 cells per cm^2.

Preparation of DNA and its modification. Recombinant plasmid DNAs were isolated from bacteria and purified by CsCl density gradient centrifugation (19). Digestions of these DNAs with restriction endonucleases were performed in accordance with the conditions specified by their manufacturers.

Reaction of DNA with the Klenow fragment of DNA polymerase I was performed by incubating 1 μg of DNA in a volume of 50 μl with 10 mM Tris-hydrochloride (pH 7.6), 5 mM $MgCl_2$, 1 mM dithiothreitol, the appropriate deoxynucleotide triphosphates (0.1 to 1.0 mM), and 5 U of the Klenow fragment of *Escherichia coli* DNA polymerase I for 1 h at 15°C. The reaction was terminated by sequential phenol and chloroform-isoamyl alcohol (24:1, vol/vol) extraction, and the DNA was isolated by ethanol precipitation. Digestion of DNA with the *Bal* 31 nuclease and ligation with T4 ligase were carried out as described previously (30).

Construction of recombinant plasmids. The recombinant plasmids that served as substrate for mutagenesis or replication were derived from molecules whose construction has been described previously (19, 30). We utilize the abbreviation pd (plasmid deleted) to denote those recombinant molecules whose plasmid sequences are derived from pML-2 DNA (25). Wherever possible we have tried to name recombinant plasmids according to their composition. For example, pPBR2 refers to plasmid (p), polyomavirus (P), *Bam*HI (B) to *Eco*RI (R), isolate 2. The parent plasmid for all the mutants described here was pPBR2, which is composed of the 2,220-base-pair (bp) polyomavirus *Bam*HI (nucleotide 4632)-to-*Eco*RI (nucleotide 1560) fragment and the large *Bam*HI-to-*Eco*RI DNA fragment of the plasmid pMK16.1 (30). pPBR2 was modified by the insertion of a *Hin*dIII linker at the unique *Bgl*I site within polyomavirus sequences (nucleotide 87) to create the plasmid pPin7. This was accomplished by linearizing pPBR2 DNA with *Bgl*I, removing the 3-bp 3′ projections with the exonucleolytic activity of the Klenow fragment of *E. coli* DNA polymerase I, and ligating a synthetic *Hin*dIII linker to the blunted ends. After *Hin*dIII cleavage, ligation, and transformation of *E. coli*, the plasmid DNAs from individual colonies were screened, and one that contained a *Hin*dIII site in place of the *Bgl*I site was isolated and named pPin7. This mutant DNA was sequenced across the site of insertion of the linker by the chemical method (28). A 3-bp stretch of viral DNA (nucleotides 91, 92, and 93) has been removed and replaced with 10 bp of linker DNA. Both pPBR2 and pPin7 served as substrates for the isolation of viral DNA fragments that were cloned within pML-2 DNA.

pdPBR2 was constructed by transfer of the polyomavirus sequences from pPBR2 to pML-2. This was accomplished by cleavage of both species with *Bam*HI and *Eco*RI and ligation of the viral sequences to the pML-2 vector sequences. The resulting recombinant plasmid, pdPBR2, contains the viral sequences from nucleotide 4632 to 1560 cloned between the *Eco*RI and *Bam*HI sites of pML-2 DNA.

pdPBBg(H) was constructed by ligating the viral *Bam*HI-to-*Hin*dIII fragment from pPin7 to pML-2 DNA digested with these same enzymes. pdPBBg(H) contains viral sequences from nucleotide 4632 to 90. pdPP1(B)Bg(H) was derived from pdPBBg(H) by cleavage with *Bam*HI and extension of the 3′-recessed termini by incubation with the Klenow fragment of *E. coli* DNA polymerase I and the four deoxynucleotide triphosphates. These blunt-ended molecules were then partially digested with *Pvu*II to remove the viral sequence between nucleotide numbers 4632 (the *Bam*HI site) and 5130 (a *Pvu*II site). Circularization of the blunt ends by ligation with T4 ligase led to the isolation of pdPP1(B)Bg(H). This procedure resulted in the placement of a *Bam*HI site at nucleotide 5131 of the viral insert. Sequencing of the virus-plasmid junctions with pdPP1(B)Bg(H) confirmed that the viral sequences between nucleotides 5131 and 90 are contained within this recombinant as a *Bam*HI-to-*Hin*dIII fragment.

pdPP1(B)R1 was constructed by ligation of the viral *Hin*dIII-to-*Eco*RI sequences from pPin7 (the sequences between nucleotides 90 and 1560) between the *Hin*dIII and *Eco*RI sites of pdPP1(B)Bg(H) DNA. pdPP1(B)R contains those polyomavirus sequences

between nucleotides 5131 and 1560 and a 10-bp *Hin*dIII linker between nucleotides 90 and 94.

pdPBP1 was derived from pdPBR2 by digestion with *Bam*HI and extension of the 3'-recessed ends after incubation with the Klenow fragment of *E. coli* DNA polymerase I and the four deoxynucleotide triphosphates. These molecules were then cleaved with *Pvu*II to generate partial products, circularized with T4 ligase, and used to transform *E. coli*. After screening a number of colonies, we identified several that contained a recombinant plasmid from which viral sequences had been deleted between the *Bam*HI site (nucleotide 4632) and the *Pvu*II site at nucleotide position 5262. A *Bam*HI site was created at the new junction between viral and plasmid sequences to yield pdPBP1, which carries viral sequences between nucleotides 5265 (a *Bam*HI site) and 1560 (an *Eco*RI site) within pML-2 DNA.

The recombinant plasmid pdPBHp contains the viral *Bam*HI (nucleotide 4632)-to-*Hph*I (nucleotide 152) DNA fragment between the pML-2 *Bam*HI and *Hin*dIII sites. The *Bam*HI site was restored, but the *Hph*I and *Hin*dIII sites were destroyed during the construction of pdPBHp. In short, pPBR2 DNA was digested with *Hph*I, and the 3' projections were removed with the Klenow fragment of *E. coli* DNA polymerase I. The DNA fragments were then reacted with *Bam*HI, and the fragment containing viral sequences enclosed by the *Bam*HI site (nucleotide 4632) and the *Hph*I site (nucleotide 152) was isolated by agarose gel electrophoresis. This viral fragment was then ligated to pML-2 DNA that had been cleaved sequentially with *Hin*dIII and *Bam*HI. Before cleavage with *Bam*HI, the vector DNA was digested with *Hin*dIII and the 3'-recessed ends were extended by reaction with the Klenow fragment of *E. coli* DNA polymerase I and the four deoxynucleotide triphosphates. Ligation of the vector and viral sequences to each other resulted in the formation of pdPBHp. The predicted sequences about the virus-plasmid joints were confirmed by sequencing them.

Isolation of mutant recombinant plasmids. The mutant series that carry unidirectional deletions from the *Bam*HI site in pdPBR2 DNA were, with two exceptions, constructed by *Bal* 31 nuclease digestion of *Bam*HI-linearized pdPBR2 DNA. After reaction with the nuclease, a synthetic *Bam*HI linker was ligated to the ends of the molecules, and they were then cleaved with *Bam*HI and *Eco*RI. The *Bam*HI- and *Eco*RI-terminated viral sequences were cloned between these same sites in pML-2 DNA. Each mutant DNA was sequenced by the chemical method to determine the endpoint of the deletion (28). Two mutants in this series, pdPP1(B)R1 and pdPBP1 (see Fig. 1), were constructed as described previously.

The mutant DNAs that bear deletions within viral sequences from the *Eco*RI site in pdPBR2 DNA were constructed as described previously, with one exception. pdPBS1 was isolated after cleavage of pdPBR2 DNA with *Sst*I and *Eco*RI. The *Sst*I 3' projections and the *Eco*RI 3'-recessed ends were removed and extended with the Klenow fragment of DNA polymerase I in the presence of dATP, dGTP, and TTP. Intramolecular ligation across these ends resulted in the creation of an *Eco*RI site at the junction. pdPBS1 contains those viral sequences between nucleotides 4632 and 569 as a *Bam*HI-to-*Eco*RI fragment within the large *Bam*HI-to-*Eco*RI segment of pML-2 DNA.

Mutants with deletions internal to viral sequences were constructed as described below. pdPdl1-8 was isolated after deletion of sequences about the unique *Bgl*I site in pPBR2 with *Bal* 31 nuclease (18). The *Bam*HI-*Eco*RI viral DNA fragment bearing the deletion was subsequently cloned between the *Bam*HI and *Eco*RI sites of pML-2 to yield pdPdl1-8. Sequences between nucleotides 1 and 148 have been deleted from the viral insert.

pdPdl300 was derived from pdPBR2 by partial cleavage with *Pvu*II followed by intramolecular ligation with T4 ligase. Among the bacteria transformed with this DNA were several that contained a recombinant plasmid with a deletion of the *Pvu*II fragment between nucleotides 5130 and 5265. pdPdl304 and pdPdl326 were isolated after partial cleavage of pdPdl300 with *Pvu*II, followed by digestion with *Bal* 31 nuclease. The eroded, linear DNA was then circularized with T4 ligase and used to transform *E. coli*. After screening a number of recombinant plasmids from independent colonies, we identified several with deletions about the *Pvu*II site at the deletion junction (5130/5265) in pdPdl300 DNA. Sequencing across the deletions of two mutants revealed that pdPdl304 is deleted between nucleotide positions 5130 and 5277, whereas pdPdl326 is deleted between nucleotides 5126 and 5265.

The pdPB500dl300 mutants (see Fig. 3) were assembled by digesting pdPBBg(H) DNA (see Fig. 2) to completion with *Bam*HI and *Pvu*II. The largest DNA fragment, composed of pML-2 DNA and viral sequences between nucleotide positions 5265 (a *Pvu*II site) and 90 (a former *Bgl*I site, now a *Hin*dIII site), was purified by agarose electrophoresis and ligated to the gel-purified *Bam*HI-to-*Pvu*II fragments of viral DNA from the pdPB500 mutant series (Fig. 1).

The inversion mutants (see Fig. 4) were constructed by ligation of the viral *Bam*HI (4632)-to-*Pvu*II (5130) fragment of pPBR2 DNA to *Bam*HI-digested pdPBP1 DNA (Fig. 1). After the *Bam*HI cohesive ends of both species were joined, the molecules were reacted with the Klenow fragment of DNA polymerase I and the four deoxynucleotide triphosphates to fill in the remaining *Bam*HI ends. The DNA was then circularized by ligation across the filled-in *Bam*HI and *Pvu*II termini with T4 ligase and used to transform *E. coli*. After screening and characterizing the plasmid DNA from a number of colonies, we used two, pdPBP5-1 and pdPBP1-5, for the experiments reported here. An identical strategy enabled us to clone the viral *Bam*HI (4632)-to-*Pvu*II (5130) fragment in both orientations at the *Bam*HI site of pdPP1(B)Bg(H) (Fig. 2) to generate the recombinants plasmids pdPP1P5-1 and pdPP1P1-5 (Fig. 4).

DNA transfection. CsCl gradient-purified, supercoiled plasmid DNAs were transfected into MOP-8 cells by a modification (39) of the DEAE-dextran transfection technique (29). A 60-mm dish containing 7.5×10^5 MOP-8 cells was washed with 5 ml of serum-free DMEM, and the cells were then incubated with 1 ml of DMEM supplemented with 250 μg of DEAE-dextran (molecular weight, 500,000) per ml and 2 μg of plasmid DNA at 37°C for 4 h in a humdified CO_2 incubator. The cells were then washed twice with 5 ml of serum-free DMEM and maintained in 5 ml of DMEM containing 10% calf serum.

In separate experiments we have varied the amount of DNA used for transfection between 0.02 and 2.0 μg

per dish and found that the phenotype of the mutant plasmids does not change. We decided to routinely employ 2.0 μg of DNA per dish to insure that we could detect replication of recombinant plasmids that were severely impeded in this capacity.

DNA replication assay. At 72 h posttransfection, low-molecular-weight plasmid DNA was isolated from MOP-8 cells by using the Hirt (21) extraction procedure. After the sedimentation of high-molecular-weight DNA, 0.5 ml of the cleared lysate was diluted in 4.5 ml of TE buffer (10 mM Tris-hydrochloride, pH 8.0, 1 mM EDTA) and extracted once with buffer-saturated phenol and once with chloroform-isoamyl alcohol (24:1). Nucleic acids were precipitated by the addition of 2.5 volumes of cold ethanol and storage overnight at −20°C. Precipitates were collected by centrifugation and suspended in 50 μl of TE buffer. A 15-μl portion of the DNA sample was sequentially digested with *Bam*HI and *Dpn*I restriction endonucleases.

The digested plasmid DNAs were subjected to electrophoresis through 1.0% (wt/vol) agarose gels, and the DNA fragments were transferred to nitrocellulose filters by the Southern (40) technique. The nitrocellulose filters were hybridized to ^{32}P-labeled, nick-translated pdPBR2 DNA (1×10^8 to 5×10^8 cpm/μg) by employing dextran-sulfate (46). After washing, the filters were dried and autoradiographed for 3 to 12 h with Kodak XAR-5 film and Du Pont Lightning-Plus intensifying screens. We estimate that by 72 h posttransfection there are from 500 to 2,000 replicated plasmid molecules per cell. However, not all of these cells take up and replicate the recombinant plasmid DNA, and therefore the number of copies of replicated DNA per transfected cell is likely to be higher than this. Each recombinant DNA species was assayed in duplicate on at least three separate occasions, and the duplicate samples were processed separately. We detected only minor variations in the recovery of replicated DNA between the duplicates, and the phenotypes of the mutants did not vary significantly among the various experiments.

RESULTS

Limits of the polyomavirus sequences required in *cis* for DNA replication. To define the borders of *ori* we constructed a number of polyomavirus DNA-plasmid recombinants and measured their replicational capacity in a MOP cell line (MOP-8; W. Muller, M. Naujokas, and J. A. Hassell, data not shown). We employed pML-2 as a vector in these experiments because, like Lusky and Botchan (25), we observed that *cis*-acting sequences within pBR322 DNA inhibited the replication of polyomavirus-pBR322 DNA molecules in MOP-8 cells (data not shown). These "poison" sequences have been deleted from pML-2 DNA (25). To assess the replicative capacity of the various plasmids, we employed an assay that allows viral recombinant plasmid DNA propagated in mammalian cells to be distinguished from plasmid DNA that has been propagated in *E. coli* (33). This assay takes advantage of the fact that plasmid DNA isolated from a DNA-adenosine methylase-positive (*dam*$^+$) bacterial host is no longer methylated after multiple rounds of replication within a mammalian cell. By using the restriction endonuclease *Dpn*I, which cleaves only methylated DNA, and another appropriate one-cut restriction endonuclease, the recovered, low-molecular-weight, replicated DNA can be visualized as a *Dpn*I-resistant linear species above the unreplicated, *Dpn*I-cleaved, input DNA after Southern blot transfer and hybridization to a suitable viral DNA probe. Application of this method to measure the replication of various polyomavirus–pML-2 recombinant molecules in MOP-8 cells revealed that all of the *cis*-acting sequences necessary for replication resided within the small *Bam*HI (nucleotide 4632)-to-*Eco*RI (nucleotide 1560) fragment of polyomavirus DNA (pdPBR2; Fig. 1A). We employ the nucleotide numbering scheme proposed by Soeda et al. (38) and report the boundaries of DNA fragments from the clockwise direction on the circular polyomavirus genome. This 2,220-bp fragment includes the region from which DNA replication initiates.

To more precisely delineate the boundaries of the polyomavirus *ori* we constructed a series of mutants that contain progressive deletions within viral DNA sequences originating from either the *Bam*HI site (nucleotide 4632) or the *Eco*RI site (nucleotide 1560) in pdPBR2 DNA and measured their capacity to replicate in MOP-8 cells as described above. The structure and replicational competence of the various DNAs are shown in Fig. 1. Polyomavirus recombinant plasmids possessing deletion endpoints up to nucleotide number 5131 were found to replicate within the MOP-8 cell line at levels comparable to those of the parent plasmid pdPBR2. However, further deletions extending to nucleotides 5182, 5190, and 5265 rendered these recombinant plasmids replication defective (Fig. 1). On the basis of these results, the leftward limit of the polyomavirus *ori* can be positioned between nucleotides 5131 and 5182.

To establish the rightward boundary of *ori*, deletions were created from the *Eco*RI site towards the *Bam*HI site through viral sequences in pdPBR2 DNA (Fig. 2). The structure and replicative capacity of these recombinant plasmids are illustrated in Fig. 2. All of the recombinant plasmids, including one [pdPBBg(H)] with a 1,470-bp deletion from the *Eco*RI site, were capable of autonomous replication in MOP-8 cells (Fig. 2). These data demonstrate that the rightward boundary of *ori* is located to the left of nucleotide 90 in polyomavirus DNA. To position this boundary more precisely we employed a derivative of pdPBR2, termed pdPdl1-8, which bears a deletion between nucleotides 1 and 148 (18). Measurement of the capacity of this mutant

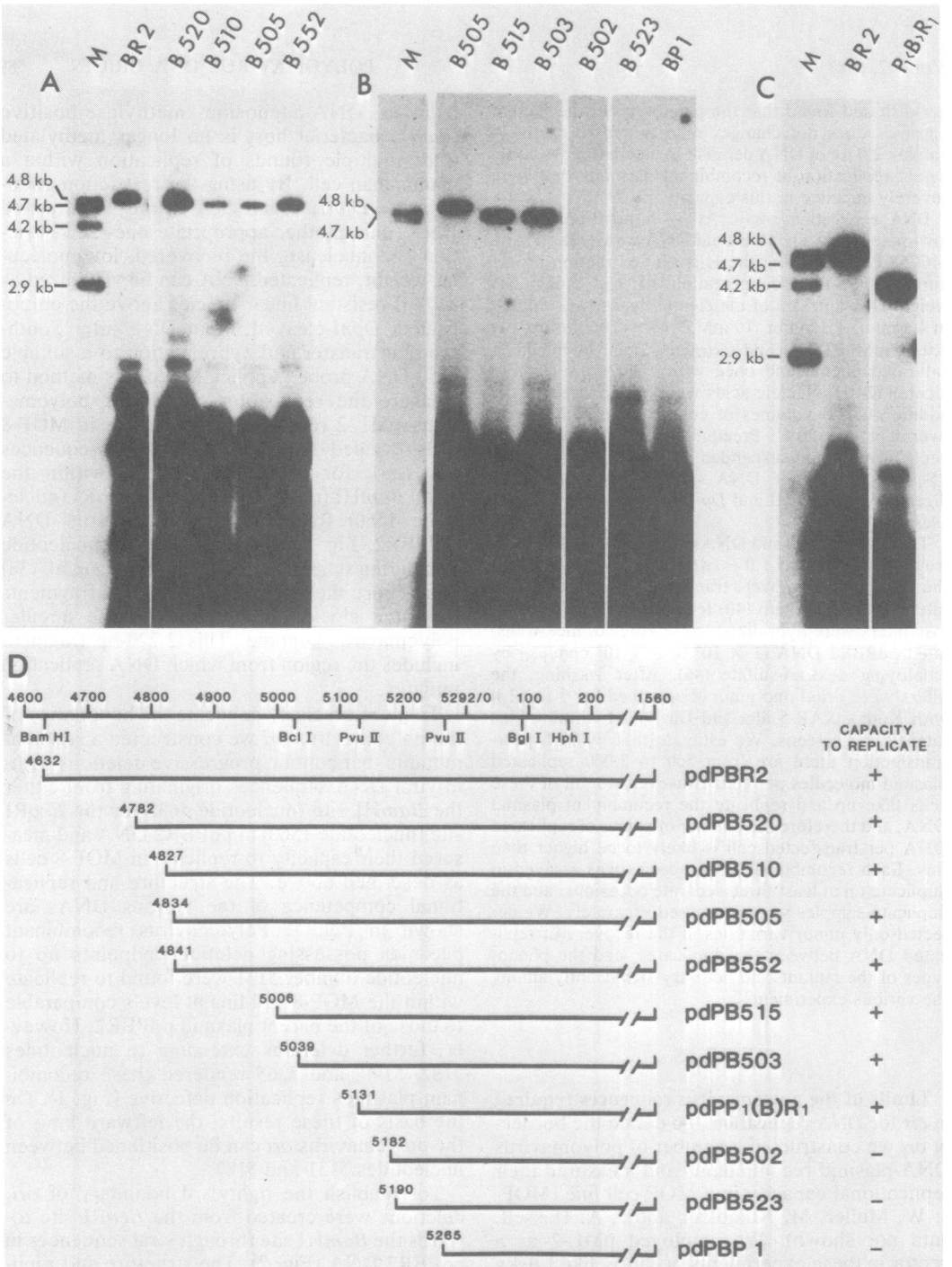

FIG. 1. Deletion analysis of the leftward boundary of the polyomavirus *ori*. (A, B, and C) A 2-μg sample of polyomavirus recombinant plasmid DNA was transfected into MOP-8 cells, and replication was assayed 72 h posttransfection as described in the text. After simultaneous digestion with *Bam*HI and *Dpn*I, the plasmid DNAs were subjected to electrophoresis through a 1% agarose gel and transferred to nitrocellulose filters. Replicating recombinant plasmid DNA can be visualized as a *Dpn*I-resistant band comigrating with the linearized parental plasmid after hybridization and autoradiography. The smear that appears at the bottom of the autoradiogram represents the closely spaced fragments of *Dpn*I-cleaved recombinant plasmid DNA that has not replicated. Because large quantities of DNA were used to transfect the cells, only a small fraction of the total input DNA is replicated in these MOP-8 cells. The autoradiograms shown in each panel are taken from the same film, which was clipped to allow comparisons to be made between adjacent lanes. The lanes labeled M contain DNA fragments of known sequence. The size of each fragment is shown adjacent to its position in the autoradiogram. (D) Schematic illustration of the structures of deletion mutants relative to known restriction endonuclease cleavage sites on the polyomavirus genome. The nucleotide numbering is according to Soeda et al. (38). Because no attempt was made to quantitate the precise level of DNA replication, the replicational phenotypes are designated as either + or −.

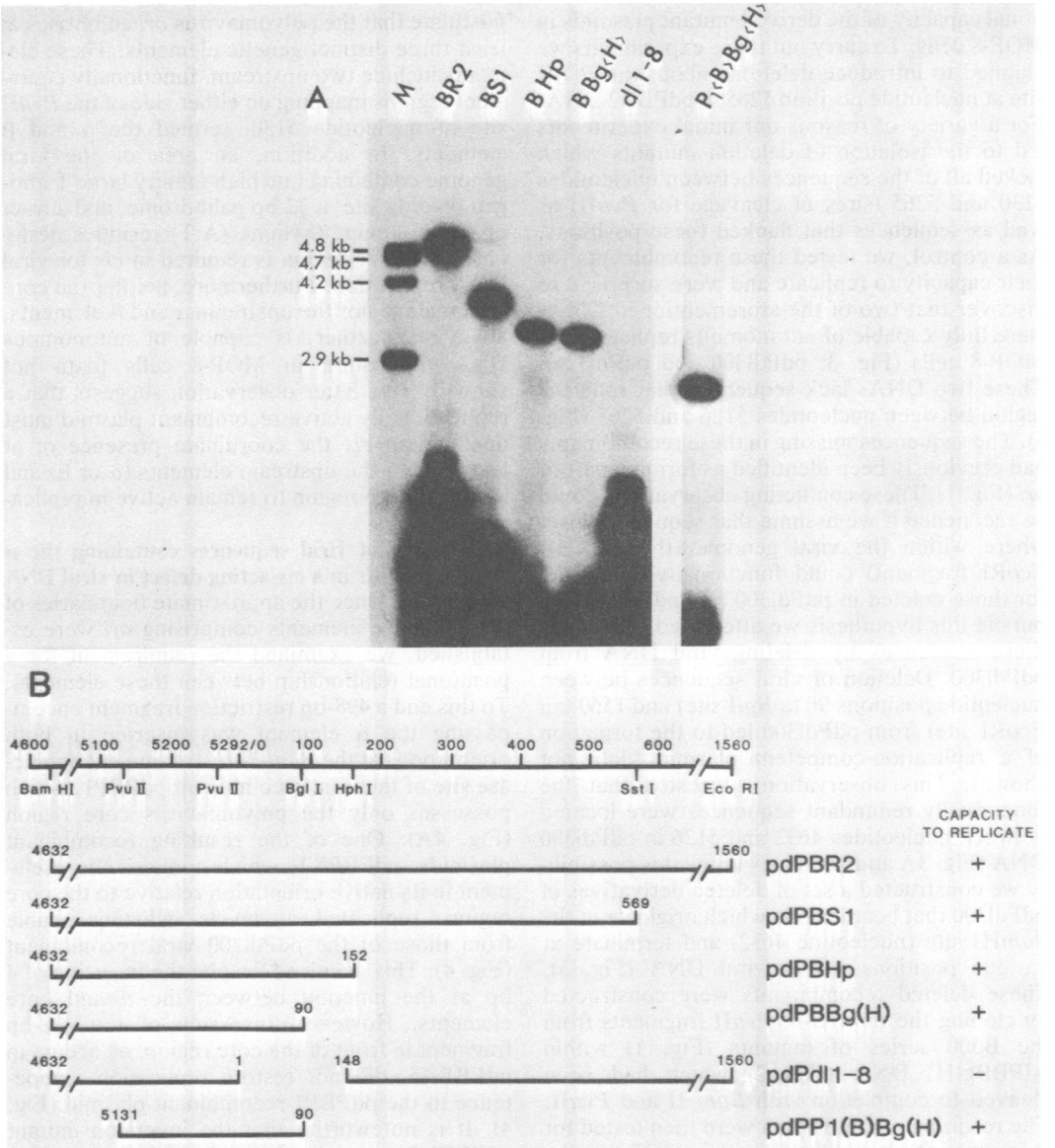

FIG. 2. Deletion analysis of the rightward boundary of the polyomavirus *ori*. (A) The replicative capacities of the polyomavirus recombinant plasmids were determined as described for Fig. 1. (B) The physical maps and replication phenotypes of the viral deletion mutants are illustrated as described for Fig. 1.

DNA to replicate revealed that it was defective (Fig. 2). This observation and previous results permit us to define the rightward boundary of *ori* between nucleotides 1 and 90. On the basis of these measurements we predict that one boundary of *ori* is between nucleotide positions 5131 and 5182 and that the other boundary is located between nucleotide positions 1 and 90. This prediction was tested by cloning a 251-bp fragment of the polyomavirus genome between the *Pvu*II site at nucleotide 5131 and the *Bgl*I site at nucleotide 90 as a *Bam*HI-*Hin*dIII fragment (see above) within pML-2 DNA and measuring its replicational potential in MOP-8 cells. The result (Fig. 2) demonstrates that this viral recombinant plasmid, pdPP1(B)Bg(H), is capable of autonomous DNA replication in MOP-8 cells. Therefore, the 251-bp viral DNA segment situated between nucleotides 5131 and 90 contains all the necessary genetic information required in *cis* for polyomavirus DNA replication.

Polyomavirus *ori* comprises multiple genetic elements. To determine whether the viral *ori* is a single contiguous element or is composed of multiple sequence motifs, we constructed deletions within *ori* and then measured the replica-

tional capacity of the derived mutant plasmids in MOP-8 cells. To carry out these experiments we planned to introduce deletions about the *Pvu*II site at nucleotide position 5265 in pdPBR2 DNA. For a variety of reasons our initial experiments led to the isolation of deletion mutants which lacked all of the sequences between nucleotides 5130 and 5265 (sites of cleavage for *Pvu*II) as well as sequences that flanked these positions. As a control, we tested these recombinants for their capacity to replicate and were surprised to discover that two of the aforementioned DNAs were fully capable of autonomous replication in MOP-8 cells (Fig. 3: pdPdl300 and pdPdl326). These two DNAs lack sequences that span the region between nucleotides 5126 and 5265 (Fig. 3). The sequences missing in these recombinants had previously been identified as forming part of *ori* (Fig. 1). These conflicting observations could be reconciled if we assume that sequences elsewhere within the viral genome (the *Bam*HI-*Eco*RI fragment) could functionally substitute for those deleted in pdPdl300 and pdPdl326. To pursue this hypothesis we attempted to identify these sequences by deleting viral DNA from pdPdl300. Deletion of viral sequences between nucleotide positions 90 (a *Bgl*I site) and 1560 (an *Eco*RI site) from pdPdl300 led to the formation of a replication-competent plasmid (data not shown). This observation suggested that the functionally redundant sequences were located between nucleotides 4632 and 5126 in pdPdl300 DNA (Fig. 3A and D). To examine this possibility we constructed a set of deleted derivatives of pdPdl300 that bear lesions which originate at the *Bam*HI site (nucleotide 4632) and terminate at various positions within viral DNA (Fig. 3). These deleted recombinants were constructed by cloning the *Bam*HI-to-*Pvu*II fragments from the B500 series of mutants (Fig. 1) within pdPBBg(H) DNA (Fig. 2) which had been cleaved to completion with *Bam*HI and *Pvu*II. The resulting recombinants were then tested for their capacity to replicate within MOP-8 cells. The results are presented in Fig. 3B and C and summarized in Fig. 3D. They demonstrate that viral sequences between nucleotides 5039 and 5130 can functionally substitute for those located between nucleotide positions 5131 and 5265. We have named these *cis*-acting sequences the α and β elements, respectively. The α element and the β element together are incapable of forming a functional origin (Fig. 2; pdPdl1-8). Yet another sequence motif located between nucleotides 5265 and 90 is required to form *ori*. This conclusion is supported by the isolation of a mutant, pdPdl304, which carries a deletion of 12 bp between nucleotides 5265 and 5277 and which is replication defective (Fig. 3A and D).

On the basis of these deletion studies we postulate that the polyomavirus *ori* comprises at least three distinct genetic elements. These elements include two upstream, functionally equivalent regions mapping on either side of the *Pvu*II site at nucleotide 5130, termed the α and β elements. In addition, an area of the viral genome containing one high-affinity large T antigen binding site, a 32-bp palindrome, and a tract of eight adenine-thymine (A:T) residues designated the core region is required in *cis* for viral DNA replication. Furthermore, neither the core region alone nor the upstream α and β elements, alone or together, is capable of autonomous DNA replication in MOP-8 cells (data not shown). This latter observation suggests that a replicationally active recombinant plasmid must maintain in *cis* the coordinate presence of at least one of the upstream elements (α or β) and an intact core region to remain active in replication.

Inversion of viral sequences containing the α element results in a *cis*-acting defect in viral DNA replication. Once the approximate boundaries of the sequence elements comprising *ori* were established, we examined the requirement for a positional relationship between these elements. To this end a 498-bp restriction fragment encompassing the α element was inserted in both orientations at the *Bam*HI restriction endonuclease site of the viral recombinant pdPBP1, which possesses only the polyomavirus core region (Fig. 4A). One of the resulting recombinant plasmids, pdPBP5-1, which contains the α element in its native orientation relative to the core region, replicated at levels indistinguishable from those of the pdPdl300 viral recombinant (Fig. 4). This occurred despite the insertion of 4 bp at the junction between the α and core elements. However, inversion of the 498-bp fragment in front of the core region, as occurs in pdPBP1-5, did not restore replication competence to the pdPBP1 recombinant plasmid (Fig. 4). It is noteworthy that the inversion mutant pdPBP1-5 not only alters the orientation of α relative to the core region, but also effectively places 400 bp between the α element and the core region (Fig. 4). To ensure that the phenotype of the inversion mutant was not due to the inadvertent generation of sequences inhibitory to DNA replication, the same 498-bp restriction fragment bearing α was inserted in both orientations at the *Bam*HI site of a replication-competent plasmid, pdPP1(B)Bg(H), possessing both the β and core regions. The replicative capacities of the resulting recombinants, pdPP1P5-1 and pdPP1P1-5, were unaltered regardless of the orientation of the upstream α element (Fig. 4). From these results it is apparent that the inversion of α has no detrimental effect on the replicational capacity of a viral recombinant possessing

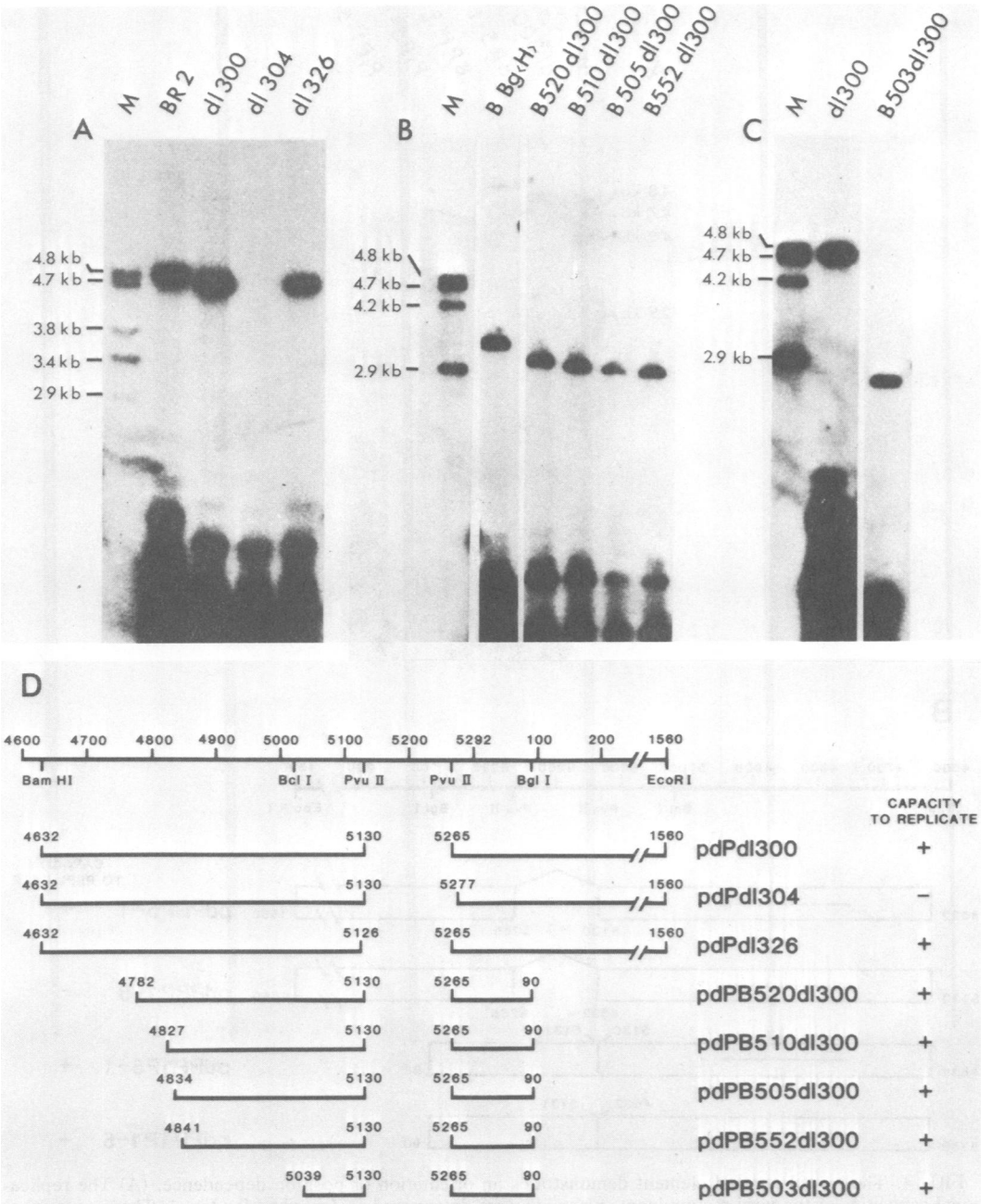

FIG. 3. Internal deletion analysis of the polyoma virus *ori*. (A, B, and C) The replicative capacities of the viral deletion mutants were assessed as described for Fig. 1. The recombinant plasmids pdPBR2, pdPBBg(H), and pdPdl300 were included in (A), (B), and (C), respectively, for comparative purposes. (D) The structures of the deletion mutants and their associated replicational properties are illustrated as described for Fig. 1.

an intact β and core region. It is therefore unlikely that the inversion of the α element in front of the core region leads to the chance creation of an inhibitory sequence detrimental to DNA replication. Instead it seems more likely that either the orientation or the spacing (or both) of the α and core regions relative to each other is critical to create a functional origin.

DISCUSSION

The polyomavirus origin for DNA replication comprises several genetic elements. These in-

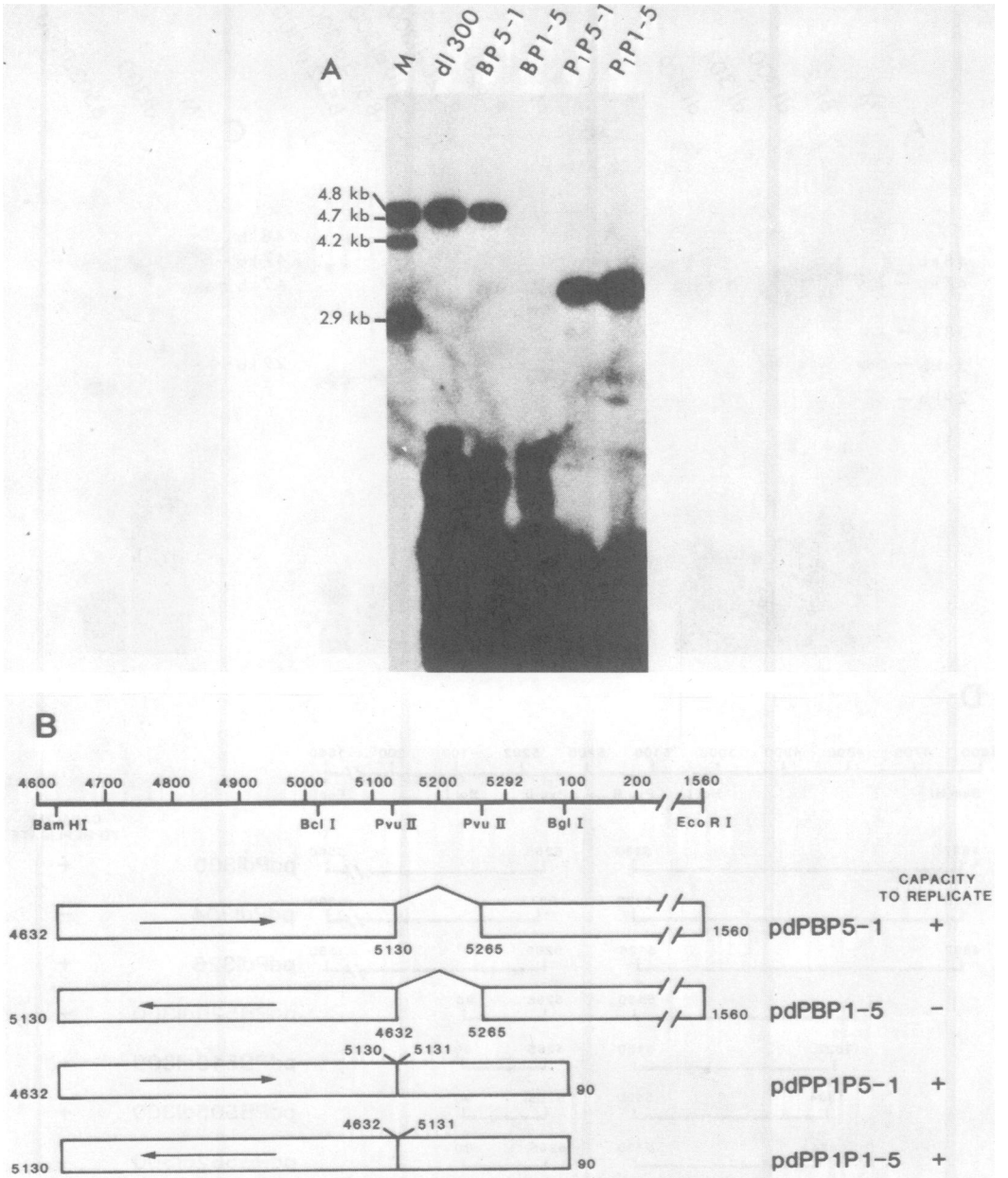

FIG. 4. The α replicational element demonstrates an orientation or position dependence. (A) The replicational capacities of the viral recombinants plasmids were determined as described for Fig. 1. The recombinant plasmid pdPdl300 is included for comparative purposes. (B) The structures of polyomavirus recombinant plasmids and their associated replication phenotypes are illustrated as described for Fig. 1.

clude two functionally equivalent but unique sets of viral sequences, designated α and β, that are situated next to a third element termed the core. The simultaneous presence of either α or β and an intact core region is required to form *ori*. All three elements together occupy a contiguous, noncoding stretch of the viral genome no more than 345 bp in length, between nucleotides 5039 and 90. The sequences that comprise *ori* and other features resident in this area of the viral genome are summarized in Fig. 5.

The core region is composed of no more than 117 bp situated between nucleotides 5265 and 90. Located within these borders are several unusual sequence features. They include an 8-bp stretch of A:T residues positioned between nu-

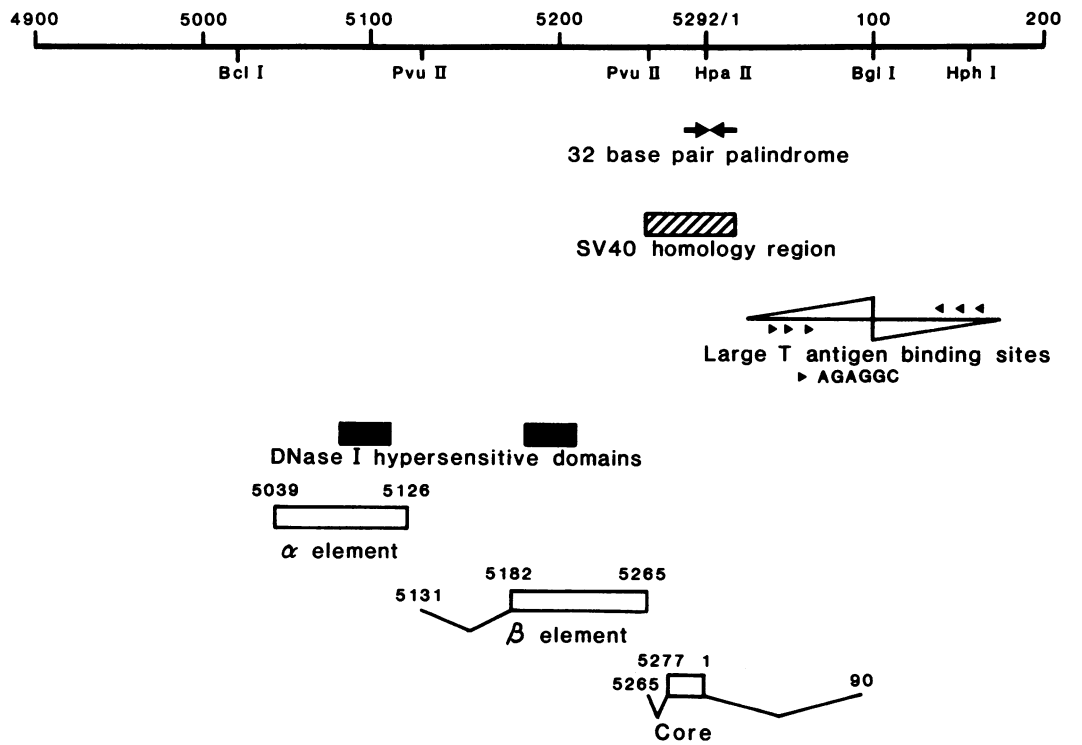

FIG. 5. Physical features near the polyomavirus *ori*. The viral large T antigen binding sites are positioned according to Pomerantz et al. (34). Repeats of the nucleotide sequence –AGAGGC– are designated by arrowheads. The 32-bp region of dyad symmetry is indicated by bold arrows. Domains of DNase I hypersensitivity are shown according to Herbomel et al. (20). The various replicational elements are shown at the bottom of the figure. The borders of the elements are denoted by a V-shaped line, where they are known.

cleotides 5268 and 5275, a guanine-cytosine (G:C)-rich 32-bp palindrome (between nucleotides 5281 and 20), and a large T antigen binding site that resides within the borders of nucleotides 5292 and 90 (binding site II; 12, 34). Removal of the entire tract of eight consecutive A:T bp, as occurs in pdPdl304 (Fig. 3), or four of the eight A:T residues (26), renders the DNA replication defective. These observations suggest that the A:T tract is an essential component of the core region. Interestingly, this sequence feature is conserved in the DNA of two other papovaviruses, SV40 and BK virus, and it is known to be a component of the SV40 origin for DNA replication (5). There is also evidence to support the contention that the polyomavirus 32-bp palindrome is required for *ori* function. Deletion of 9 bp between nucleotides 5277 and 5287 from polyomavirus DNA renders that DNA incapable of replicating (26). This deletion removes 6 bp that constitute one border of the 32-bp palindrome. However, removal of 2 contiguous bp within the palindrome does not alter the replicational properties of the viral DNA, and such molecules can serve as the genetic material of viable viruses (26). A 27-bp palindrome that is related in sequence to the polyomavirus inverted repeat is found within the origin of SV40. Moreover, genetic analyses have demonstrated that the 27-bp palindrome is an essential element of the SV40 origin (37). Located adjacent to the 32-bp palindrome in polyomavirus DNA are sequences which serve as a binding site for large T antigen (Fig. 5). This large T antigen binding site is composed of sequences between nucleotide positions 5292 and 90 (12, 34). Because the rightward border of the core region has been mapped between nucleotides 1 and 90, it is presently not possible to know whether this binding site constitutes part of the core region. Additional mutants with lesions in this area will be required to determine unambiguously whether large T antigen binding site II is part of the core region or not. Nonetheless, it is probably not coincidental that all viable deletion mutants of polyomavirus isolated to date retain sequences that include large T antigen binding site II (3, 4, 17, 26, 27, 47). The minimal *cis*-acting sequences required for SV40 DNA replication also include sequences that serve as binding sites for large T antigen (5, 14, 30, 42), and there is a wealth of genetic evidence

that supports the contention that SV40 large T antigen must interact with *ori* sequences to initiate DNA replication (14, 36, 37). Taken together, these observations suggest that an A:T stretch, a G:C-rich region of dyad symmetry, and a large T antigen binding site are features that not only are shared among papovaviruses but also are functionally important for the initiation of viral DNA replication and constitute domains of the core regions of these viruses.

cis-acting viral sequences in addition to the core region are needed to form a functional polyomavirus *ori*. One such element that, together with the core region, can form *ori* has been termed β. The β element is located within the polyomavirus *Pvu*II-4 DNA fragment and is no larger than 134 bp. One of its borders maps between nucleotides 5131 and 5182, and the other is situated near nucleotide 5265 (Fig. 5). We have recently discovered that either the insertion of a linker DNA at nucleotide number 5265 or the deletion of sequences between the β and the core regions has no effect on the replicational capacity of the resulting recombinants (data not shown). This result demonstrates that although the β element and core are contiguous within the viral genome they are nonetheless physically distinct genetic elements. There are few readily recognized sequence features within the borders of β. A region of potential secondary structure can be drawn that involves base pairing between 43 of the 82 to 134 nucleotides of one of the DNA strands that make up this element (45). It may be noteworthy that a replication-defective viral recombinant possessing a deletion that extends to nucleotide 5182 (pdPB502; Fig. 1) destroys this region of putative secondary structure, suggesting that it may be of regulatory significance. The β element resides within an area of the viral genome that is tandemly duplicated and mutated by base substitutions in variants of polyomavirus which, unlike the wild-type virus, are capable of productively infecting the F-9 embryonal carcinoma cell line (35). The observation that coinfection of F-9 embryonal carcinoma cells with wild-type and mutant strains of polyomavirus yields only mutant virus progeny, despite the presence of complementing large T antigen (in the infected cell), indicates that the sequence modifications in these mutants likely allow them to overcome a *cis*-acting defect in viral DNA replication present in wild-type DNA (11).

Although the β element is implicated in viral DNA replication, it can be deleted from the viral genome by *Pvu*II cleavage with little phenotypic effect on the replicational capacity of the resulting viral recombinant. An adjacent region of viral DNA between nucleotides 5039 and 5126, designated α, can functionally substitute for the β element. Recently, Tyndall et al. (44) have described the replicational properties of a virus-recombinant plasmid possessing an analogous 134-bp deletion between the *Pvu*II sites at nucleotides 5130 and 5265. They found that removal of these sequences had a pronounced inhibitory effect on the replicational capacity of the deleted recombinant plasmid DNA and rendered polyomavirus nonviable. We therefore measured the replicational capacity of the *Pvu*II-4 deletion mutant DNA (pdPdl300; Fig. 3) in competition with another recombinant plasmid DNA (pdPBS1; Fig. 2) which contains all three segments, namely, α, β, and core. The results revealed that both species replicated with equal efficiency (data not shown). Moreover, Luthman et al. (26) have recently described the construction of a viable deletion mutant of polyomavirus (*dl*1024) from which all but 8 bp of the 134-bp *Pvu*II-4 fragment has been deleted. Their results and ours suggest that this area of the viral genome (between nucleotides 5130 and 5265) is dispensable for viral gene expression and DNA replication (see also reference 18). The difference between these results and those of Tyndall et al. (44) may be due to as yet undetected differences in the virus strains or perhaps to mutations in the various cloned viral DNAs. In addition, the amounts of large T antigen within COP cells (44) and MOP-8 cells undoubtedly differ, and this parameter may influence the extent of replication of mutant viral genomes. Whatever the explanation, there is little doubt that the α element can functionally substitute for β, quantitative differences notwithstanding. It is interesting that Tyndall et al. (44) have isolated unidirectional and bidirectional mutants about the *Pvu*II site at nucleotide position 5130 (Fig. 5). Many of the bidirectional mutants fail to replicate, whereas the unidirectional mutants that are deleted toward either the early or late transcription units do replicate. These results are readily explained by the model we have presented. The bidirectional mutants were replicationally inactive because sequences that comprise α and β were deleted. By contrast, the unidirectional deletion mutant DNAs bear lesions that remove either α or β and are therefore replication competent.

Variants of polyomavirus have been described that replicate efficiently in PCC4 embryonal carcinoma cells. These variants possess common sequence rearrangements that result in the duplication of viral sequences between nucleotides 5072 and 5135 (the borders of α are between 5039 and 5126) and the deletion of those sequences between nucleotides 5185 and 5215 (the latter map within β; 23). These sequence modifications likely delete the α element while duplicating the β element. Because these virus

variants, capable of productive infection of PCC4 cells, retain the capacity to replicate in secondary cultures of mouse embryo cells, these results too suggest that α sequences can functionally substitute for the β element.

The activity of the α element is dependent on its position or orientation relative to the core sequences. Insertion of a 498-bp fragment that contains α in front of the core region in its native orientation resulted in a DNA molecule capable of autonomous replication in MOP cells. However, insertion of the 498-bp α-containing fragment in the opposite orientation relative to the core did not result in a replication-competent plasmid. Control experiments demonstrated that this was not due to the inadvertent creation of a sequence that retards replication like that which occurs in pBR322 DNA (25). Because the α and core sequences are also more distantly separated in the inversion mutant in comparison with their native state, it is not possible to state whether the orientation or spacing of α relative to the core is important for origin function. Whatever the nature of the defect, the inversion mutant illustrates that the mere presence of α and the core on the same DNA molecule is not sufficient for its replication in MOP cells.

The function of the α and β elements is not known. The viral sequences that span nucleotides 5021 to 5265 (α and β together map between nucleotides 5039 and 5265) have been reported to enhance transcription of the rabbit β-globin gene in human and mouse cells (7). It is tempting to suggest that α and β are composed of sequences that serve a dual function in both DNA replication and gene expression. For example, it is possible that DNA must be anchored to the nuclear matrix or some other cellular constituent to be efficiently expressed and replicated. If specific sequences are required to form such complexes, then these might be located within the α and β elements. However, until the sequences required for gene expression and those required for DNA replication are more precisely defined, such models should be considered speculative.

The α and β elements share little if any sequence homology, yet they apparently function in the same manner. This implies either that different sequences within each element are functionally important or that these regions can assume similar secondary or tertiary structures. Within lytically infected cells at late times, both the α and β elements are relatively free of nucleosomes and hypersensitive to DNase I digestion (Fig. 5; 20). It is possible that the α and β sequences serve as sites of recognition for cellular proteins required for DNA replication. It is tempting to speculate that cellular permissive factors interact with sequences present within these elements. Such a role is consistent with the sequence changes that occur within these regions when polyomavirus is adapted to replicate within F9 and PCC4 embryonal carcinoma cells.

The multielement organization of the polyomavirus *ori* resembles the structure of the SV40 *ori*. In SV40 DNA the functional origin is composed of a core segment analogous to that found in polyomavirus and an adjacent region consisting of two perfect and one imperfect G:C-rich, 21-bp repeats (5). The efficient replication of SV40-plasmid recombinant DNAs in COS cells (13) requires the core and at least one of these repeats (5). In contrast to the polyomavirus core region, the SV40 core, when linked to plasmid sequences, is capable of replicating autonomously in COS cells. The auxiliary region containing the 21-bp repeats has an enhancing effect on replication efficiency that is dependent on the number of copies of the 21-bp repeats (5, 10). Interestingly, an identical 21-bp sequence has been reported to reside within the inverted repeat termini of simian adenovirus 7 DNA (43). Moreover, sequences of related primary structure are conserved within the terminal, inverted repeats of the DNA of many different serotypes of human adenovirus (41). Although it is not known whether these adenoviral sequences are elements of the functional replication origin, their location near the sites of initiation of viral DNA replication is suggestive of a role in this process. Taken together, these observations suggest to us that a multielement organization may be a common feature of viral and, by analogy, cellular replication origins.

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