MOLECULAR AND CELLULAR BIOLOGY, Oct. 1987, p. 3694–3704
0270-7306/87/103694-11$02.00/0

Vol 7, No. 10

DNA Sequence Requirements for Replication of Polyomavirus DNA In Vivo and In Vitro

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Received 19 March 1987/Accepted 15 July 1987

Cell extracts of FM3A mouse cells replicate polyomavirus (Py) DNA in the presence of immunoaffinity-purified Py large T antigen, deoxynucleoside triphosphates, ATP, and an ATP-generating system. This system was used to examine the effects of mutations within or adjacent to the Py core origin (*ori*) region in vitro. The analysis of plasmid DNAs containing deletions within the early-gene side of the Py core *ori* indicated that sequences between nucleotides 41 and 57 define the early boundary of Py DNA replication in vitro. This is consistent with previously published studies on the early-region sequence requirements for Py replication in vivo. Deleting portions of the T-antigen high-affinity binding sites A and B (between nucleotides 57 and 146) on the early-gene side of the core *ori* led to increased levels of replication in vitro and to normal levels of replication in vivo. Point mutations within the core *ori* region that abolish Py DNA replication in vivo also reduced replication in vitro. A mutant with a reversed orientation of the Py core *ori* region replicated in vitro, but to a lesser extent than wild-type Py DNA. Plasmids with deletions on the late-gene side of the core *ori*, within the enhancer region, that either greatly reduced or virtually abolished Py DNA replication in vivo replicated to levels similar to those of wild-type Py DNA plasmids in vitro. Thus, as has been observed with simian virus 40, DNA sequences needed for Py replication in vivo are different from and more stringent than those required in vitro.

Replication of polyomavirus (Py) DNA proceeds bidirectionally from a fixed point within the noncoding region of the viral genome (for a review, see reference 16 and references therein). This replication origin (*ori*) region (Fig. 1) contains multiple T-antigen (T Ag)-binding sites within a G+C-rich 32-base-pair (bp) sequence of dyad symmetry adjacent to a region in which 13 of 14 residues form A · T pairs. It thus bears structural features similar to the replication *ori* of simian virus 40 (SV40). However, the relationship of the Py *ori* to other regulatory signals in the noncoding segment is somewhat different from that of SV40. The number and arrangement of large T Ag high-affinity binding sites on the early side of both viral *ori* regions differ. Also, the relative affinities of Py and SV40 T Ags for binding sites within their respective *ori* regions are apparently dissimilar (12, 14, 28, 47). Moreover, while both viruses encode enhancer elements, the Py enhancer is almost directly adjacent to the late boundary of the *ori* region and consists of a series of discrete elements that bear homologies to other enhancer elements including those of SV40, adenovirus E1a, and mouse immunoglobulin genes (2, 22, 23, 26, 31, 38, 46, 51, 53). By contrast, the SV40 enhancer, a 72-bp repeated sequence (see reference 22 and references therein), is separated from the core *ori* by a series of G+C-rich sequences (the 21-bp repeats) that form a distinct transcriptional control element to which the cellular transcription factor, Sp1, binds (17) and that is lacking in the Py genome.

The roles that these different sequence elements play in DNA replication are important for understanding the processes of both viral and cellular DNA replication. The analysis of deletion mutants has led to the identification of the early (13, 30) and late (35, 39, 53) boundaries required for minimum (core) *ori* function in vivo. Certain base substitution mutations within the core *ori* have been shown to abolish replication (36, 52). Moreover, noncontiguous sequences on the late side of the core *ori* that lie within the enhancer region are required for Py DNA replication in vivo (15, 23, 39, 51, 54). This appears to relate to enhancer function per se since replication can be restored by reinsertion of either the SV40 or the immunoglobulin enhancer region in either orientation (15, 23, 54).

The process of papovavirus DNA replication can be examined carefully owing to the development of a system that replicates SV40 DNA in vitro (33, 49, 56, 58). This has made possible experiments in which some of the factors and DNA sequences required for SV40 replication in vitro have been analyzed. More recently, similar strategies have led to the development of a system that replicates Py DNA in vitro (40). To determine the sequences required for Py DNA replication in vitro, we utilized several deletion, inversion, and base substitution mutants that alter sequences within and adjacent to the Py core *ori*. Our results indicate both similarities and marked differences in the in vivo and in vitro sequence requirements for Py DNA replication.

MATERIALS AND METHODS

Materials, cells, and viruses. Hybridoma mouse cells producing F-4 monoclonal antibody (42) were obtained from C. Schley and E. Harlow. The helper-dependent adenovirus vector Ad-SVR587 (37) was kindly provided by S. Mansour and R. Tjian. CV-1 cells were obtained from T. Grodziker. Goat anti-mouse immunoglobulin G coupled to agarose was

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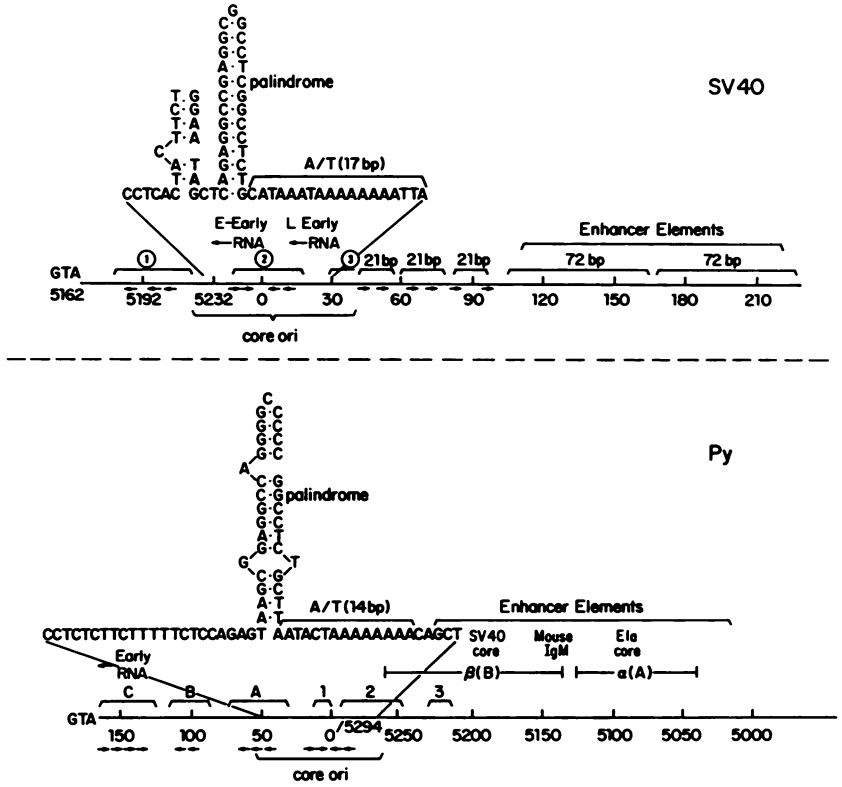

FIG. 1. Comparison of origin-containing regions of SV40 and Py. Origin regions of SV40 (upper panel) and Py (lower panel) show, in each case (above the line), an expanded region of the core *ori* sequence, the positions of early RNA start sites, T Ag-binding sites (SV40, sites 1, 2, and 3; Py, strong sites A, B, and C and weak sites 1, 2, and 3), and late-gene-side transcriptional regulatory elements (in SV40, 21-bp repeats and 72-bp repeated enhancer elements; in Py, enhancer elements). Py enhancer regions α (A) and β (B) and regions of homology to SV40, mouse immunoglobulin M (IgM), and adenovirus E1a enhancer sequences are shown beneath bracketed area delineating Py enhancer elements. Below the line are nucleotide numbers extending from the early-gene side on the left to the late-gene side on the right and arrows showing the position and orientation of the T Ag-binding consensus pentanucleotides 5′-G$^{A}_{G}$GGC-3′.

obtained from Hyclone. Radioisotopes were obtained from The Radiochemical Centre (Amersham, England), and restriction enzymes were from New England BioLabs, Inc. (Beverly, Mass.).

Preparation of Py T Ag and FM3A cell extracts. Py T Ag was partially purified by immunoaffinity procedures that were previously described (40). In general, extracts prepared from 5×10^8 CV-1 cells infected with Ad-SVR587 (5 to 10 PFU per cell) yielded 200 μg of Py T Ag as estimated from silver-stained polyacrylamide gels. Py T Ag was estimated to be about 50% pure and was the major polypeptide detected in the silver-stained gel. Immunopurified Py T Ag was stored at −20°C after dialysis against buffer containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 50% glycerol (T Ag dialysis buffer) and retained activity for at least 2 months. FM3A cell extracts were prepared from suspension cultures of mouse FM3A cells grown to a density of 5×10^5 cells per ml in Dulbecco modified Eagle medium plus 10% calf serum, according to procedures developed for the preparation of HeLa cell extracts for in vitro replication of SV40 DNA (56).

In vitro replication of Py DNA. Reaction mixtures (50 μl) contained 80 mM Tris hydrochloride (pH 7.8), 40 mM creatine phosphate, 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 mM ATP, 200 μM each CTP, UTP, and GTP, 100 μM each dATP, dCTP, and dGTP and 25 μM [*methyl*-^{3}H]dTTP (150 to 300 cpm/pmol), 1 μg of creatine phosphokinase, FM3A extract (0.2 to 0.4 mg of protein), and 10 μl of the T Ag dialysis buffer containing or lacking 0.2 to 0.5 μg of Py T Ag. The addition of larger volumes of the T Ag dialysis buffer inhibited the replication reaction.

Superhelical circular duplex (RF1) plasmid DNAs were prepared by published procedures (13, 31, 52) and were generally subjected to two rounds of CsCl equilibrium density centrifugation. DNAs were quantified both by measuring A_{260} and by comparison with standards after agarose gel electrophoresis and ethidium bromide staining. In Py replication reactions, DNAs were tested at several concentrations and were found to be maximally active as templates at 0.3 to 0.4 μg per reaction. Reaction mixtures were incubated for 2 h at 37°C, and the radioactivity incorporated into acid-insoluble material was measured. When products of the replication reactions were analyzed, [α-^{32}P]dCTP (13,000 cpm/pmol) was used instead of [^{3}H]dTTP and reaction mixtures then contained 100 μM dTTP and 20 μM dCTP.

Plasmids pBE102, pBE11, pBE22, pBE41, pPyNeoBE82, pBE27Neo, pBEBYNeo, and pBE48.19Neo have been described previously (31, 32) and contain the A2 strain of Py wild-type (pBE102) or mutant sequences extending from the *Bcl*I site at nucleotide (nt) 5022 to the *Eco*RI site at nt 1562 inserted into a pML vector containing *neo* coding sequences (pAE2E1). pPyOR, pCBYd4, and pCBYd5 were constructed as previously described (13). Mutant p48.19 was constructed by inserting an *Xho*I linker at nt 36 (A. Cowie and R. Kamen,

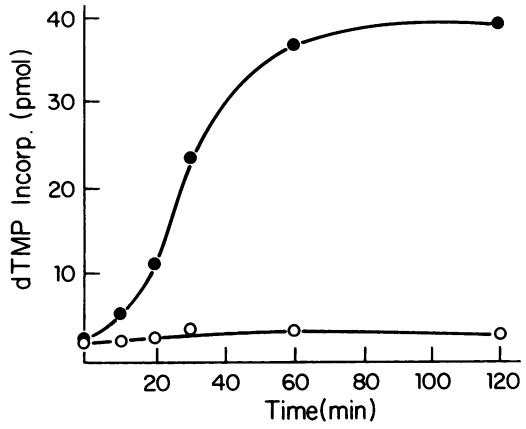

FIG. 2. Time course of Py T Ag-dependent Py DNA replication in vitro. Reaction mixtures (50 μl) containing pBE102 (wild-type Py plasmid DNA) (0.3 μg), extracts of FM3A cells (0.2 to 0.4 mg of protein), and components as described in Materials and Methods were incubated in the presence (●) or absence (○) of Py T Ag (0.3 μg) at 37°C for the times indicated. Reactions were then assayed for acid-insoluble nucleotide incorporation.

personal communication) and was inserted into a similar recombinant DNA as the pBE series (32). pBcSt was constructed by double digestion of pBE102 with *Bcl*I and *Stu*I, filling in with Klenow fragment, and recircularization by blunt-end ligation. Plasmids 3-14d, 8-142, and 9-40 contain wild-type (3-14d) or mutant (8-142, 9-40) Py sequences of the A3 strain cloned into the *Bam*HI site of pBR322 as previously described (52). p76-4 was constructed from pΔ76 (Py-pMK16) (30) containing a *Sal*I linker at nt 64 by inserting an *Xho*I linker at nt 5269. pΔ76-44 and pΔ76-30 were derived from pΔ76-4 by inverting (pΔ76-44) or deleting (pΔ76-30) the core *ori* region bounded by the *Sal*I and *Xho*I linkers, respectively. For purposes of consistency, all Py sequences are numbered according to the Py A2 strain in this paper, and for simplicity, the Neo suffix has been dropped from the plasmid names, and pPyNeoBE82 and pBE48.19Neo are referred to as pBE82 and p48.19, respectively.

RESULTS

Time course of Py replication in vitro. Previous experiments (40) demonstrated that replication of wild-type Py DNA in vitro required extracts of mouse FM3A cells, Py large T Ag, ATP, and an ATP-generating system in addition to deoxynucleoside triphosphates, dithiothreitol, and Mg^{2+}. Evidence that the reaction observed represented DNA replication rather than DNA repair synthesis was shown by the fact that sequences near the replication *ori* region were preferentially labeled at early times in the reaction and that the majority of the labeled reaction products were resistant to the restriction enzyme *Dpn*I. The amount of Py T Ag isolated by immunoaffinity procedures from extracts of CV-1 cells infected with the helper-dependent recombinant adenovirus (37) was significantly less (5- to 10-fold) than the quantities of SV40 large T Ag isolated from this or related systems. At standard DNA concentrations (0.3 μg per reaction), DNA synthesis in vitro did not plateau at the highest concentration of Py T Ag added, in contrast to what was observed with SV40 T Ag-dependent replication in vitro. For this reason, the amount of Py T Ag added was limiting in most of the reactions described below.

The time course of the replication reaction was measured (Fig. 2). The amount of dTMP incorporated reached plateau levels within 1 to 2 h. As reported for the replication of SV40 *ori*$^+$ DNA in vitro, there was a reproducible lag in the initial rate of incorporation in the first 10 min after the start of the incubation. This most likely reflects the formation of a preelongation complex, as shown for SV40 (50, 57). The quantities of dTMP incorporated under the conditions described in Fig. 2 varied between 30 and 40 pmol. At optimum input DNA concentration this was approximately 10-fold greater than observed when Py T Ag was omitted from the reaction.

Replication of DNAs with deletions within and on the early side of *ori*. A series of mutants containing deletions extending toward the viral early region were tested for their ability to support Py DNA replication in vitro (Fig. 3). In these and subsequent experiments several concentrations of plasmid DNAs were used to measure their effectiveness in supporting replication compared with that of the wild-type DNA used, in this case pBE102. Deletion of sequences within the core *ori* (pBE27 and pCBYd4) virtually abolished Py T Ag-dependent DNA replication. However, DNAs that included the core *ori* extending to nt 41 (pCBYd5) supported very low levels of replication (two- to threefold above the incorporation observed in the absence of T Ag). Mutants pBEB8 and pBEBY in which sequences from nts 57 to 103 and nts 65 to 146 were deleted, respectively, consistently supported replication significantly more than wild-type DNA. The reason for this increased replicative activity is not clear. Both deletions remove some copies of the consensus pentanucleotide 5′-G$\overset{A}{G}$GGC-3′ that is bound by Py T Ag (12, 44, 47). It is possible that DNA lacking these regions may allow greater quantities of T Ag protein to be available for interaction at the core origin, where it has been shown (12, 47) that the binding affinity for Py T Ag is considerably reduced. Alternatively, the novel juxtapositions of sequences created by these two deletions may each permit more efficient replication from the core *ori* in vitro. The replication supported by pBEB8 indicates that the early boundary for core *ori* function in vitro lies between nt 41 and nt 57. Although plasmids pCBYd5 and pBEB8 bearing the mutant Py *ori* region sequences contain some differences elsewhere in the constructs, several different pBR322-related plasmid constructs containing wild-type Py *ori* sequences supported similar levels of replication in vitro (e.g., see below). Therefore, it is most likely that the differences between the replication of pCBYd5 and pBEB8 are due to sequences within the Py *ori* region. That deletions pBEB8 and pBEBY supported DNA replication in vitro is in keeping with studies on Py DNA replication in vivo in which it has been shown that sequences mapping between nts 41 and 57 define the early Py *ori* boundary (30). Moreover, it indicated that the full complement of high-affinity T Ag-binding sites, A, B and C, is not required for viral DNA replication in vitro.

Replication of DNAs with deletions on the late side of *ori*. The late boundary of the Py core *ori* in vivo has been mapped by measuring the replication of DNAs with small deletions in this region (35, 39, 53). These experiments indicated that discrete, noncontiguous elements on the late side of the *ori* are also required for Py DNA replication in vivo. These sequences map within the Py enhancer region, and the requirement for enhancer function in Py DNA replication was subsequently demonstrated (15, 23, 51, 54). It was therefore of interest to determine whether sequences within the Py enhancer region are required for Py DNA replication in vitro (Fig. 4). DNAs lacking sequences on the

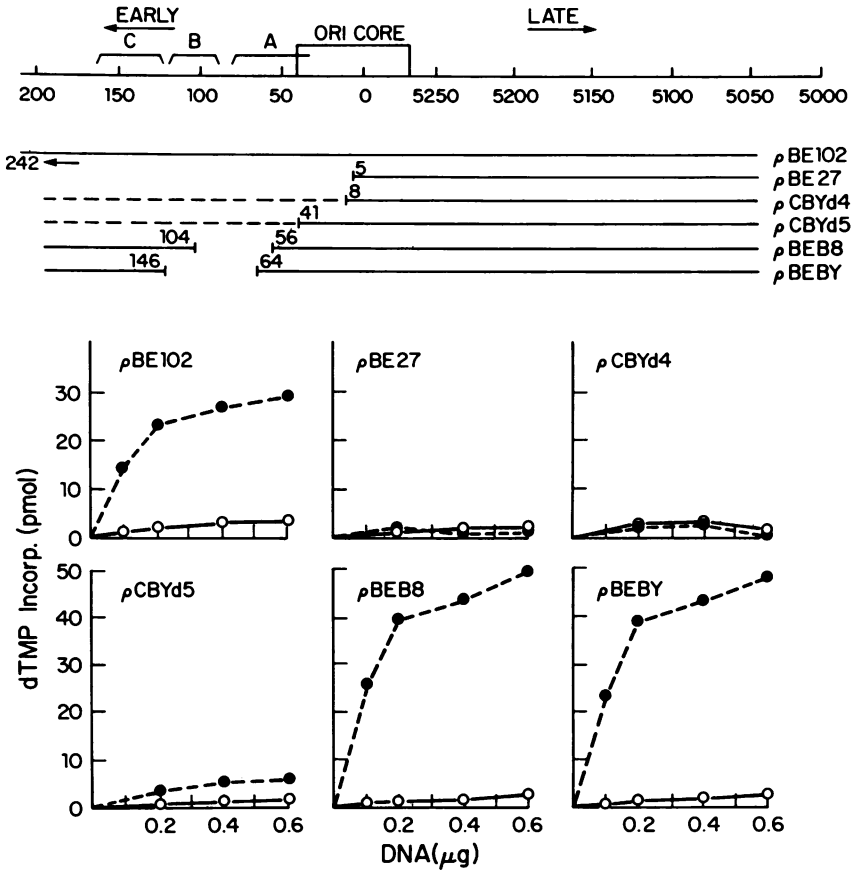

FIG. 3. Effect of deletions extending toward Py early-gene region on DNA replication in vitro. The template DNAs tested are shown in the upper portion of the figure. Plasmids pBE27, pBEB8, and pBEBY were derived from the wild-type control plasmid, pBE102, that contains a portion of the Py genome extending from the *BclI* site to the *Eco*RI site into a vector containing neomycin resistance coding sequences (32). pCBYd4, and pCBYd5 contain sequences extending from the *Bam*HI site in Py to the indicated Py nucleotide. Dotted lines depict plasmid DNA sequences (13). Reaction mixtures (50 μl) containing indicated quantities of template DNA and components as described in Materials and Methods were incubated in the presence (●) or absence (○) of Py T Ag for 2 h, followed by the determination of nucleotide incorporation.

late side of the Py enhancer (pBE11, pBE41, and pBE22) (31) all supported DNA replication in vitro. Similarly, DNA containing a deletion (pBE82) that removed sequences extending from the late boundary of the core *ori* at nt 5266 to nt 5131 at the approximate midpoint of the Py enhancer region was also as active as wild-type DNA in promoting DNA replication. An additional mutant, pBcSt, was constructed in which the entire functional enhancer region between nts 5227 and 5025 was deleted. This DNA was almost as effective as wild-type DNA in supporting replication in vitro. The extent to which this mutant replicated varied in different experiments between 80 and 100% of the replication of the wild-type pBE102 plasmid. These experiments demonstrated that sequences within the Py enhancer region are not essential in vitro, in contrast with results in vivo (15, 23, 35, 39, 51, 53, 54; this study). While this series of mutants did not permit the identification of the late core *ori* boundary for Py DNA replication, the replication of pBE82 showed that it is possible to delete sequences to nt 5266 and retain replication function in vitro.

Replication of DNAs containing mutations within core *ori* region. The minimum replication *ori*'s of both SV40 and Py contain remarkable structural similarities, including four sets of binding sites for T Ag that exist as two pairs of head-to-tail pentanucleotides opposed to each other on opposite DNA strands (Fig. 1). These structural features suggest that the position of the majority if not of all of the nucleotides in this region may be crucial for initiating DNA replication. Indeed, many base substitution or small deletion mutants within the minimum *ori* regions of SV40 (16) or Py (36, 52) abolish viral DNA replication in vivo. Three mutants containing base substitutions that map within the Py *ori* region were tested in the in vitro system (Fig. 5). Two of these DNAs, 9-40 and 8-142, were constructed to alter the Py T Ag-binding sites within the core *ori* and have been shown to be inactive in DNA replication in vivo (52). A third mutant, p48.19, in which an *Xho*I linker was inserted between nts 35 and 37 (A. Cowie and R. Kamen, personal communication) also did not support DNA replication in vivo (32). Since mutants 9-40 and 8-142 were constructed within a different wild-type Py background, the replication of the wild-type parent, 314d, and that of the pBE102 wild-type DNA used in the previous experiments (Figs. 2, 3, and 4) were compared. Both DNAs replicated to comparable extents (Fig. 5), indicating that different recombinant plasmids containing wild-type regulatory regions support Py replication similarly in vitro. It was noted, however, that the replicative form of M13mp8 containing wild-type Py *ori* replicated poorly in vitro. In contrast, this M13 derivative replicated in vivo (52). Similar observations have been made in the SV40 replication system

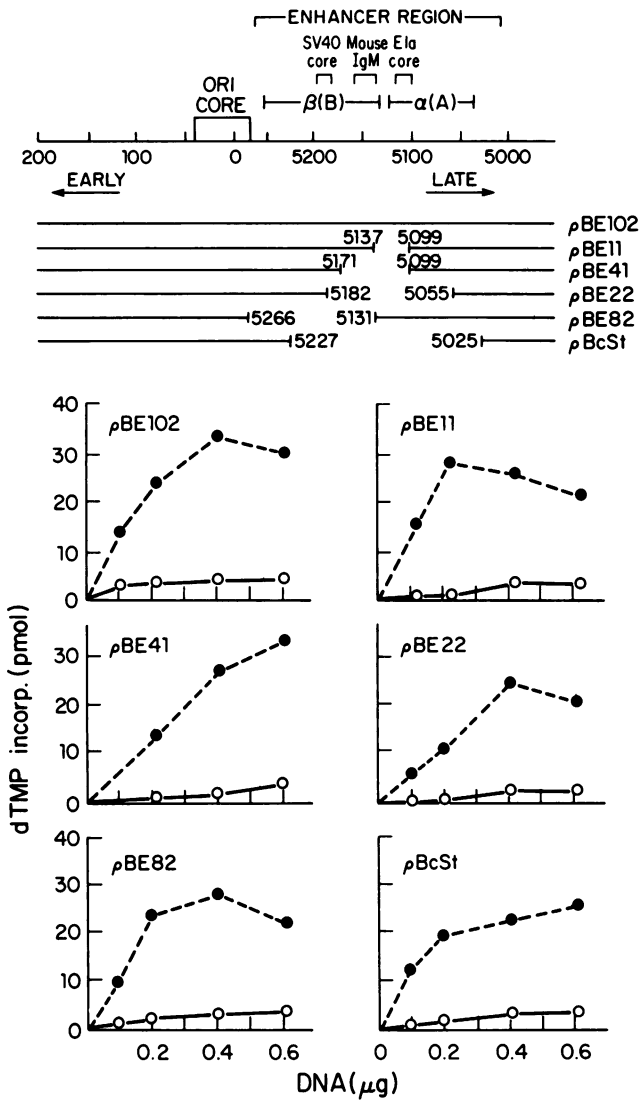

FIG. 4. Effect of deletions on late-gene side of Py core *ori* on DNA replication in vitro. Enhancer-region landmarks and template DNAs tested are shown in the upper portion of the figure. All mutants were derived from wild-type control plasmid pBE102 and contain sequences deleted as shown. Reaction mixtures (50 μl) containing indicated quantities of template DNA, FM3A extract (0.2 to 0.4 mg of protein), and additional components were incubated in the presence (●) or absence (○) of Py T Ag (0.3 μg) for 2 h, after which [^{3}H]dTMP incorporated into DNA was determined.

(R. Wobbe and J. Hurwitz, unpublished data). Thus, M13 DNA may contain sequences that are deleterious to papovavirus replication in vitro but not in vivo. All three base substitution mutants, 9-40, 8-142, and p48.19, were not replicated in vitro. Thus, three mutations within the core *ori* abolished replication both in vivo and in vitro.

Replication of DNA with inverted core *ori*. Although replication proceeds bidirectionally from both SV40 and Py *ori* regions, the actual elements in each *ori* are asymmetric. Each contains a G+C-rich palindrome on the early side and an A+T-rich region on the late side. Thus, it was of interest to determine whether the orientation of the *ori* was important for DNA replication in vitro. To test this, we constructed two mutants in which linkers were added to a fragment spanning nts 64 to 5264 that contains sequences necessary for core *ori* function in vivo (Fig. 6). This fragment was then reinserted into a vector, pΔ76-30, at the linker sites in either orientation, yielding pΔ76-4 (wild-type orientation) and pΔ76-44 (reversed orientation). These DNAs were tested for their ability to support replication. pΔ76-4 DNA replicated as well as wild-type DNA, indicating that insertion of linker sequences at the boundaries of the core *ori* did not affect replication in vitro. The reversed orientation mutant also supported DNA replication, although higher concentrations of DNA were required to achieve the same level of nucleotide incorporation found with wild-type DNA. Even at these higher DNA concentrations the reaction was T Ag dependent. This indicated that the core *ori* can function in either orientation, although there is some preference for the wild-type orientation. This is consistent with observations that the pΔ76-44 virus is viable but forms plaques of reduced size and number when compared with pΔ76-4 (M. Sullivan and W. Folk, unpublished data).

Analysis of reaction products of wild-type and mutant Py DNAs. The effects of deletion and base substitution mutations on Py DNA replication in vitro provided evidence for similarities and differences in the sequence requirements for Py DNA replication in vivo and in vitro. To further demonstrate that mutants that supported replication yielded products similar to those previously obtained from wild-type Py DNA (40), products were analyzed for resistance to the restriction enzyme *Dpn*I. This enzyme cleaves only fully methylated DNA such as that produced during plasmid propagation in the Dam$^+$ strain of *Escherichia coli*. Replication of fully methylated DNA in eucaryotic cells (or in extracts) that do not methylate DNA at the *Dpn*I site yields DNA resistant to the enzyme. To determine the extent of *Dpn*I resistance of the products formed in the in vitro Py replication system, we performed reactions with [α-^{32}P] dCTP. DNA isolated from these reactions was first linearized with *Pvu*I, and then half of the linearized DNA was digested with *Dpn*I. The majority of the input DNA, as visualized by ethidium bromide staining, was cleaved by *Dpn*I (Fig. 7, lower panels). Since parallel reactions showed that the enzyme quantitatively cleaved linearized plasmid DNA (data not shown), the small quantities of *Dpn*I-resistant material remaining in the ethidium bromide-stained gels probably represented replicated DNA. Based on estimations of quantities of nucleotide incorporated, it is estimated that newly replicated DNA in the Py in vitro system represented 20 to 30% of the input template. In contrast to the stained gels, autoradiography of the ^{32}P-labeled products showed that the labeled DNA was generally fully resistant to the enzyme (see below). The main exception to this was pΔ76-44 (the reversed *ori* orientation mutant) in which less than half the labeled products were resistant to *Dpn*I. For unknown reasons, high concentrations of this DNA were required to support maximum dTMP incorporation (Fig. 6). It is possible that replication with pΔ76-44 DNA did not lead to extensive elongation resulting in products containing long stretches of parental duplex DNA susceptible to *Dpn*I cleaveage. Since virtually all nucleotide incorporation with this DNA depended on Py T Ag, it is unlikely that this represented repair-type incorporation.

Comparison of replication of Py mutants in vivo and in vitro. The replication in permissive cells of many of the mutants used in these studies has been previously described (13, 31, 32, 52). In this study, several of the mutants were either tested for the first time or reexamined for their ability to replicate in WOP mouse cells (13). These cells constitu-

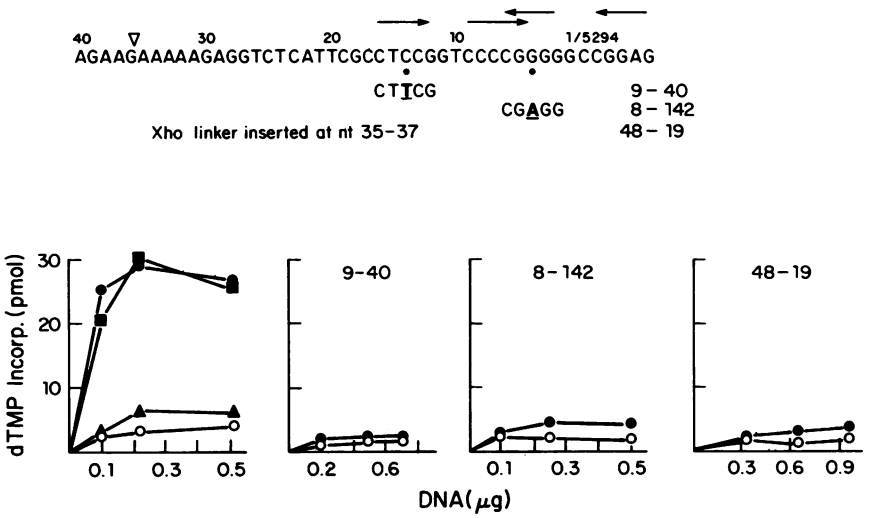

FIG. 5. Effect of base substitution mutations within the core origin on Py DNA replication in vitro. Nucleotide sequences within the core *ori* of the A2 strain of Py extending from nt 40 to nt 5289 along with the base substitutions in mutants 9-40 (C to T at nt 14), 8-142, (G to A at nt 4), and 48.19 (*Xho*I linker inserted between nts 35 and 37) are shown at top of the figure. Arrows above sequences show the position and orientation of Py T Ag-binding sites. Reaction mixtures (50 μl) were incubated for 2 h with FM3A cell extracts (0.2 to 0.4 mg of protein) and additional components including the quantities of template DNAs indicated, followed by determination of acid-insoluble radioactivity. The panel on the extreme left side shows the results obtained with plasmids containing the corresponding wild-type Py DNA origin regions: pBE102, of which mutant 48.19 is a derivative (●) (32); 3-14d, from which mutants 9-40 and 8-142 were derived (■) (52), and double-stranded M13mp8 containing Py DNA extending from the *Bcl*I site at nt 5023 to the *Eco*RI site at nt 1562 cloned into the *Bam*HI and *Eco*RI sites of the M13 vector (▲) (51). The symbol ○ in the extreme left panel refers to reactions containing pBE102 but lacking Py T Ag. The other panels show incorporation of [^{3}H]dTMP into acid-insoluble material in reactions containing (●) or lacking (○) Py T Ag (0.3 μg).

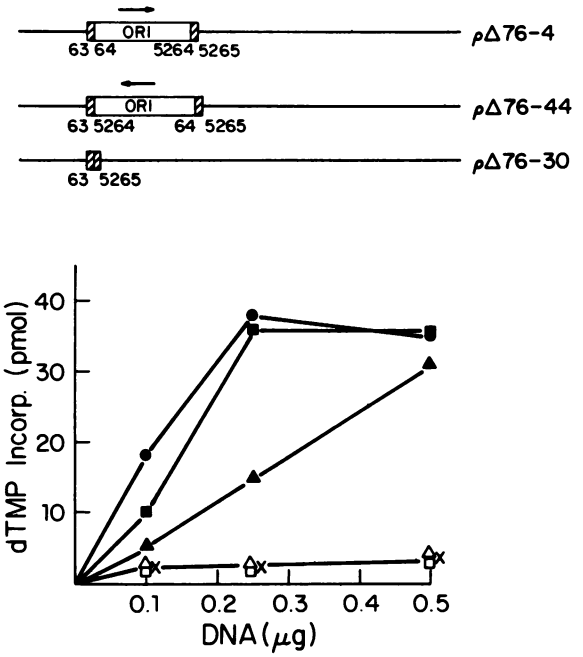

FIG. 6. Effect of Py core origin orientation on Py DNA replication in vitro. The diagram at the top depicts mutants described in the text that contain the Py core *ori* region in the wild-type orientation (pΔ76-4) or in the reversed orientation (pΔ76-44) or lack the core *ori* region (pΔ76-30). ▨, Presence of *Sal*I linker at nt 64 and *Xho*I linker at nt 5264. Reaction mixtures (50 μl) as described in Materials and Methods were incubated in the presence of Py T Ag (0.3 μg) and FM3A extract (0.2 to 0.4 mg of protein) plus the indicated amounts of pBE102 (wild-type control plasmid from previous experiments) (●), pΔ76-4 (■), pΔ76-44 (▲), and pΔ76-30 (×). The amount of acid-insoluble radioactivity was determined for each reaction. The open symbols (□ and △) show mixtures with pΔ76-4 and pΔ76-44 DNAs, respectively, and all other ingredients for replication but lacking Py T Ag.

tively express functional Py T Ag and support extrachromosomal replication of plasmids bearing the wild-type Py *ori* region. A summary of the replication of plasmids bearing wild-type and mutant Py *ori* region sequences in vitro and in vivo is shown in Fig. 8. Extrachromosomal DNA was isolated and purified from the transfected cells and tested for its sensitivity to the restriction enzyme *Mbo*I. This enzyme can cut unmethylated but not fully methylated or hemimethylated DNA and thus can be used to distinguish between input plasmid DNA and progeny DNA that has undergone at least two rounds of replication. A number of the mutants used in this study were compared in vitro and in vivo for their ability to support replication. Of these, deletions extending within the early-gene side, pBE27 (to nt 5), pCBYd4 (to nt 8), and pCBYd5 (to nt 41), were defective for DNA replication in vivo (13). This is generally consistent with the replication of these mutants in vitro. However, pCBYd5, which replicated to only 5 to 10% of wild-type levels in vitro (Fig. 3 and 7), was totally inactive in vivo (13). The ability of pBEB8, which contains sequences extending to nt 56, to replicate to wild-type levels in vivo and to greater than wild-type levels in vitro places the early-gene boundary for replication between nt 41 and nt 56. Additional mutants containing sequences extending within this region will provide more precise information and may reveal whether further discrepancies exist between the two processes. Because of the unexpected finding that pBEB8 and pBEBY, which delete portions of T Ag-binding sites A and B, resulted in levels of DNA replication approximately twofold greater

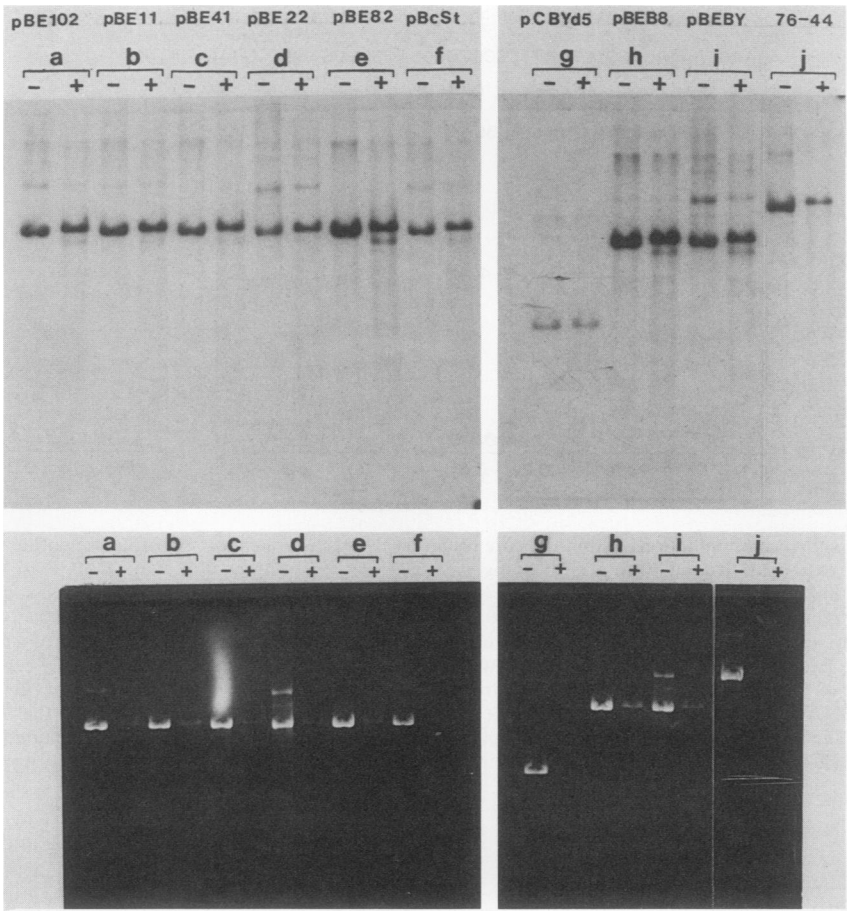

FIG. 7. Analysis of wild-type and mutant Py DNA replication products with restriction endonuclease *Dpn*I. Reaction mixtures as described in Materials and Methods but containing 20 μM [α-^{32}P]dCTP (13,800 cpm/pmol) and wild-type (pBE102) or mutant plasmid DNAs as indicated were incubated for 2 h at 37°C. Input DNA quantities were similar to those required to stimulate optimum levels of [^{3}H]dTMP incorporation, generally 400 ng/50-μl reaction mixture. A 0.1 volume of each reaction mixture was used to determine the incorporation of [^{32}P]dCMP into acid-insoluble material. The levels of dCMP incorporation closely reflected results obtained in prior experiments (Fig. 2 to 5). DNA purified from the remainder of the reaction mixtures was linearized with *Pvu*I. One half of each reaction mixture was then digested with *Dpn*I under conditions that digested the input plasmid completely. Products were then analyzed by electrophoresis in 1% agarose gels and visualized with ethidium bromide (lower panels) before autoradiography (upper panels). *Dpn*I-treated (+) and untreated (−) samples are indicated for each of the DNA templates used. The decreased size of mutant pCBYd5 relative to the other DNAs analyzed (<3,000 bp) is due to the fact that this plasmid lacks *neo* gene sequences, contains fewer Py coding sequences, and being pML, instead of pBR322, contains fewer plasmid sequences (13).

than that observed with wild-type Py DNA, these mutants were retested in vivo. At both early (30 h) and late (48 h) times posttransfection, no significant differences in replication were detected with either of these mutants when compared with wild-type DNA.

Plasmids containing base substitution mutations within the Py core *ori*, 8-142 and 9-40, as previously shown do not replicate in mouse cells (52), as did p48.19 (32). Thus, any alteration of sequences within the Py *ori* region (plasmids pBE27, pCBYd4, p48.19, 8-142, and 9-40) leads to replication-defective DNA in vivo and in vitro.

The Py enhancer region has been shown to contain two functional elements, α (A) and β (B), mapping within nts 5039 to 5126 and 5131 to 5265, respectively, that provide complementary functions for transcription and DNA replication (26, 39). Mutations that delete sequences within the β (B) element such as pBE82 or within the α (A) element such as pBE11 did not affect replication in WOP cells. However, mutations deleting sequences spanning regions within both α (A) and β (B) elements such as pBE22 and pBE41 caused marked reductions in Py DNA replication in vivo.

The mutant plasmid pBcSt in which the greatest portion of the enhancer region was deleted exhibited negligible levels of replication (<0.1%). These data confirmed earlier in vivo studies showing that deletion of the enhancer region virtually abolished Py DNA replication. Mutations that deleted sequences within this region on the late side of the Py core *ori* did not significantly affect replication in vitro; these results provided the strongest contrast between sequences required for replication in vitro and in vivo.

DISCUSSION

The core *ori* regions of the human (BKV and JCV), monkey (SV40), and murine (Py) papovaviruses are markedly similar (see reference 16 for a review). All possess an A+T-rich region of 14 to 20 bp on the late-gene side, a

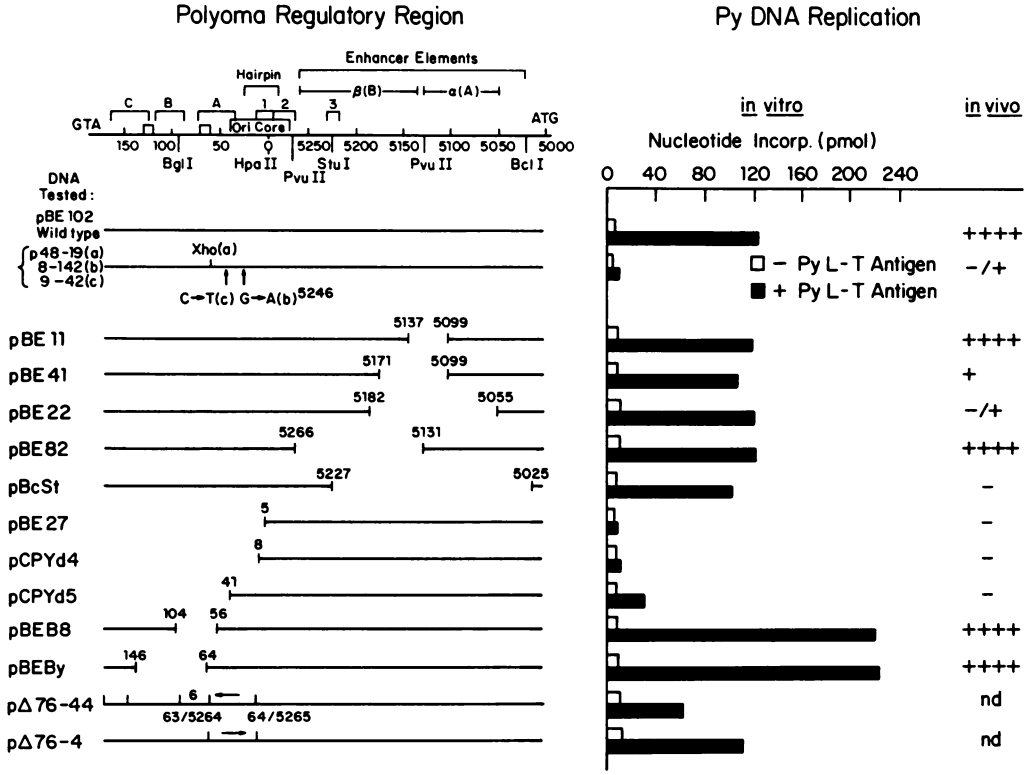

FIG. 8. Summary of data showing effects of alterations in Py regulatory sequences on Py DNA replication in vitro and in vivo. On the top left side of the figure, the Py regulatory region is summarized, while the template DNAs used are shown below. In this diagram the Py nucleotide number (A2 strain) and some restriction sites are shown below the line. On the early-gene side of core *ori* (nts 40 to 170), above the line, high-affinity T Ag-binding sites A, B and C as well as low-affinity T Ag-binding sites 1, 2, and 3 are bracketed. The positions of TATA and CAAT boxes are shown as open boxes. The core *ori* region is also bracketed above the line. On the late-gene side, the enhancer region (*Pvu*II at nt 5264 to *Bcl*I at nt 5025) is shown with α (A) and β (B) enhancer elements shown above the line. On the right side of the figure is a bar graph showing the maximum replication of template DNAs observed in the presence (■) or absence (□) of Py T Ag. Most templates were examined in at least three different experiments, and results presented are average figures. The results did not vary more than 10% between experiments. Results obtained from in vivo Py replication studies with different template DNAs depicted on the right side were compiled from published data and this study. −/+, Replication levels less than 1% of that of wild type.

G+C-rich palindrome of 23 to 69 bp in the center region, and an inverted repeat of 14 bp on the early-gene side of the core *ori*. The primate viruses have a second palindrome of various sizes that Py lacks. It is likely that the organization of sequences within these regions and their interaction with the viral T Ag is responsible for providing a strong initiation signal that allows the viral DNA to replicate to 10^5 to 10^6 copies during the time interval that host DNA has replicated only once. The organization of the regulatory regions of the two best studied of these viruses, SV40 and Py, is summarized in Fig. 1, which includes sequences that make up the core *ori* regions of both genomes (5, 30, 34–36, 39, 49, 52, 53).

In both SV40 and Py DNAs, the orientation and arrangement of T Ag-binding sites within the core *ori* are remarkably similar, consisting of two pairs of closely spaced head-to-tail pentanucleotides that are opposed to each other on opposite DNA strands. Tegtmeyer and colleagues (14) have proposed a model for SV40 in which the orientation and spacing of binding pentanucleotides determines the interaction between and function of T Ag monomers once bound to DNA. The overall similarities between the binding sites within the core *ori* regions of both SV40 and Py suggest that this model also applies to the latter. Certainly, sequence variations such as base substitutions within the core *ori* of both viruses frequently abolished replication in vivo. Furthermore, in the core *ori* regions of both SV40 and Py, all mutations that inactivate replication in vivo behave similarly in vitro. The precise points of Py initiation in vivo have not yet been determined as they have been for SV40 (24), although the homology between the core *ori* regions of both viruses suggests that Py DNA synthesis also probably initiates in this region. However, it is interesting to note that while the SV40 TATA box and early mRNA start sites are located within the SV40 core *ori*, both are located outside of the Py *ori* core on the early-gene side (18, 25, 29).

The complete high-affinity binding region (in SV40, site 1; in Py, sites A, B, and C) is not required for DNA replication in vivo, although deletion of sequences within the portion of these regions that is closest to the core *ori* causes some reduction in DNA replication (3, 13, 30, 34, 50). We observed that deletion of sequences within the Py-binding sites A and B (mutants pBEB8 and pBEBY) led to increased Py DNA replication in vitro but not in vivo. It has been reported that the affinity of Py T Ag for sequences within the core *ori* is approximately 10-fold lower than that for sequences within the high-affinity binding sites A, B, and C (11, 47). It is possible that when quantities of Py T Ag are limiting in vitro, as was the case in our experiments, the presence of all three high-affinity binding sites sequesters Py T Ag and

reduces the amount available to bind to the core *ori*. Indeed, in a competition experiment, replication-defective Py DNA containing Py sites A, B, and C inhibited the replication of equivalent quantities of wild-type Py DNA in vitro, while Py DNA lacking these sites did not (C. Prives, unpublished data).

The relative juxtaposition of the conserved regions within the core *ori* regions of all papovaviruses is similar. The inverted repeat, palindrome, and A+T-rich region are always oriented from the early-gene side to the late-gene side, respectively. To determine whether this arrangement is an absolute requirement for viral DNA replication, a mutant (pΔ76-44) was constructed in which the orientation of the core *ori* was reversed. We observed that this sequence rearrangement supported viral DNA replication in vitro but that higher levels of DNA were required for this activity. This suggests that the arrangement of these elements within the *ori* region is not essential but is required for optimal replication.

The most dramatic discrepancy between sequences required for Py DNA replication in vivo and in vitro that was observed was on the late-gene side of the core *ori* where the Py enhancer region lies. The major difference between Py and SV40 replication in vivo is that Py requires enhancer sequences for DNA replication, while SV40 does not (5, 34, 50). Various reports (26, 52) have shown that the Py enhancer consists of multiple elements that fall within two regions, α (A) and β (B), that can complement each other for transcription and replication. Base substitution mutations in the β (B) element result in their ability to allow Py to replicate in the mouse embryonal carcinoma cell line F9 (see reference 1 and references therein), presumably owing to increased enhancer function. DNase I-hypersensitive sites are located in both α (A) and β (B) regions (7, 27), and nuclear proteins capable of binding to sites within the enhancer have been described (6, 20, 41, 43). It has been speculated that the binding of specific proteins to the enhancer region modifies the interaction of the core *ori* with the replication complex (16). However, since our data have established a striking difference between the requirements for enhancer sequences in vivo and in vitro, it is likely, at least in the latter case, that these proteins do not function directly in DNA replication.

Why is there no requirement for enhancer sequences in vitro? Several possible explanations can be suggested. First, it has been proposed that the role of the Py enhancer in DNA replication is to activate transcription and, in doing so, prime the core *ori* for replication (16). However, marked differences in the requirements for transcription and replication in cell extracts argue that little if any viral transcription is occurring during SV40 or Py DNA replication in vitro. While in vitro transcription systems have been utilized for both SV40 and Py, conditions were developed recently in which SV40 enhancer sequences can augment this process (55). Therefore, it may be possible eventually to establish conditions in which a requirement for enhancers for Py DNA replication can similarly be observed.

Second, one striking difference between DNA templates in vivo and in vitro is that viral DNA is replicated as chromatin in the former but not the latter (48). The existence of DNase I-hypersensitive sites in the regulatory region of viral chromosomes is highly correlated with transcription and replication. However, SV40 enhancers have been shown to be associated with the presence of DNase I-hypersensitive regions even in the absence of promoter or *ori* sequences (19). This suggests that enhancer sequences trigger signals in DNA to both replication and transcription complexes by providing nucleosome-free areas in chromatin.

Third, enhancers may function in vivo to localize DNA at cellular structures such as the nuclear matrix. It was reported that newly synthesized cellular DNA (4), subpopulations of SV40 (10) and Py (8) T Ags, and some cellular enhancer sequences (9, 21) are all associated with the nuclear matrix. Demonstration of an association of either the viral enhancer region or replicating chromosomes with the nuclear matrix would clearly suggest an explanation for the discrepancies in enhancer requirements in cells and in soluble extracts. However, a negative correlation between the quantities of nuclear matrix-bound SV40 T Ag with viral DNA replication in infected cells has been observed (10). Furthermore, no preferential association of sequences containing the SV40 enhancer region with the nuclear matrix could be demonstrated (45).

Our studies have provided evidence that while the Py core *ori* sequences may function similarly in vivo and in vitro, those within the viral enhancer region do not. To understand the basis for the differences we observed, we are performing studies to determine whether conditions can be established that demonstrate dependence on enhancer sequences for Py DNA replication in vitro.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants GM34559 (J.H.), CA26905 (C.P.), CA38538 (W.F.), and CA42568 (C.B.) from the National Institutes of Health.

M. Sullivan is thanked for construction of the pΔ76 series of plasmids.

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