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Cellular Factors Required for Papillomavirus DNA Replication

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In vitro replication of papillomavirus DNA has been carried out with a combination of purified proteins and partially purified extracts made from human cells. DNA synthesis requires the viral E1 protein and the papillomavirus origin of replication. The E2 protein stimulates DNA synthesis in a binding site-independent manner. Papillomavirus DNA replication is also dependent on the cellular factors replication protein A, replication factor C, and proliferating-cell nuclear antigen as well as a phosphocellulose column fraction (IIA). Fraction IIA contains DNA polymerase α-primase and DNA polymerase δ. Both of these polymerases are essential for papillomavirus DNA replication in vitro. However, unlike the case with T-antigen-dependent replication from the simian virus 40 origin, purified DNA polymerase α-primase and δ cannot efficiently replace fraction IIA in the replication reaction. Hence, additional cellular factors seem to be required for papillomavirus DNA replication. Interestingly, replication factor C and proliferating-cell nuclear antigen are more stringently required for DNA synthesis in the papillomavirus system than in the simian virus 40 in vitro system. These distinctions indicate that there must be mechanistic differences between the DNA replication systems of papillomavirus and simian virus 40.

Genomic replication is absolutely essential for successful viral or cellular proliferation. During the last decade the simian virus 40 (SV40) in vitro DNA replication system has been investigated as a general model for eukaryotic DNA replication. This viral system has resulted in the identification of a number of previously unknown human DNA replication factors, as well as confirming a role in replication for previously identified proteins (for reviews see references 29, 31, and 55).

While many factors have been identified by using the SV40 system, it is clear that other human cellular factors must also play roles in DNA replication. Previous experiences with *Escherichia coli* chromosomal DNA replication and bacteriophage models have shown that different viruses often utilize different subsets of cellular factors (for a review see reference 33). Hence, it is clear that multiple viral systems should be used when host cell systems are modeled. The development of an in vitro system for papillomavirus (PV) DNA replication has made this possible for the study of mammalian DNA replication factors (102). We have used the PV in vitro DNA replication system to further confirm the requirement for the factors identified by the SV40 system and to begin to search for additional essential human DNA replication factors.

The SV40 in vitro DNA replication system requires a plasmid containing the SV40 origin, SV40 large T antigen (Tag), and 10 cellular factors for complete replication (89, 94). Tag provides the functions of origin recognition, melting of the DNA template in the origin region, and acting as the DNA helicase at the replication fork (for reviews see references 7, 13, 17, 22, 68). Replication protein A (RPA; also known as RFA and HSSB) is an essential and specific single-stranded-DNA-binding protein that can be replaced by no other type of single-stranded-DNA-binding protein in the replication assay (21, 99, 100). The DNA polymerase α-primase complex, Tag, and RPA all interact with one another (15, 18, 19, 25, 56, 76). These interactions are essential for the RNA priming activity required for initiation and lagging-strand synthesis (15, 56). DNA polymerase α synthesizes the first nascent DNA strands. Replication factor C (RFC) is a primer binding ATPase that, with proliferating-cell nuclear antigen (PCNA), binds to recessed 3′ DNA ends (38, 86, 87). This 3′ complex prevents DNA polymerase α from binding to 3′ DNA termini and stimulates binding and extension by DNA polymerase δ (38, 87, 88). Following polymerase switching, DNA polymerase δ acts as the leading-strand DNA polymerase (84). Polymerase δ also appears to be required for extension during lagging-strand synthesis (96). Topoisomerase I acts as a swivel at the replication fork to release torsional stress on the DNA during fork progression (3, 104). Topoisomerase II allows segregation of the intertwined daughter chromosomes (81). These factors are sufficient for the initiation and elongation stages of DNA replication, but the products of these reactions remain nicked or gapped (20, 84, 98). Three additional factors, i.e., a 3′-5′ exonuclease, RNase H, and DNA ligase I, are required to generate covalently closed daughter molecules (89, 94). While these factors compose the basal SV40 DNA replication machinery, it is known that additional factors can either stimulate or regulate the replication process (68, 93, 95).

PVs are very similar to SV40 in that they are small, circular double-stranded DNA viruses that use predominantly host cell enzymes to replicate their genomes from a single origin of replication. The best studied of these viruses is bovine PV type 1 (BPV1) (for reviews see references 35, 52, 69, 82). Transfection studies using DNA templates containing the BPV1 origin region have shown that two viral proteins, E1 and E2, both of which bind DNA site specifically, are required and sufficient to support BPV1 DNA replication in vivo (91). Interestingly, this approach also demonstrated that PV DNA replication, unlike SV40 and polyomavirus DNA replication, is not cell type specific. BPV1 can replicate in a number of mammalian cell types, including human cells (14). BPV1 DNA replication has been reconstituted in vitro with cell extracts from murine, simian, and human cells (6, 60, 102, 103). One major difference between the in vivo and in vitro experiments is that the in vivo

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experiments showed a complete requirement for E1 and E2 and their cognate DNA binding sites (90–92). The in vitro systems required only the E1 protein and the E1 binding site. The E2 protein does stimulate DNA replication in vitro; however, this stimulation is independent of the presence of an E2 binding site on the DNA template and occurs primarily at low levels of E1 (60, 102, 103).

The BPV1 E1 protein is a 68-kDa phosphoprotein that provides many of the same DNA replication functions for BPV1 that Tag provides for SV40. E1 binds to a region of dyad symmetry within the PV origin in a site-specific manner. Like Tag, E1 is a DNA-dependent ATPase and a helicase that translocates in the 3′-5′ direction. E1 can unwind double-stranded DNA substrates in the presence of a topoisomerase and a single-stranded-DNA-binding protein (73, 102, 103). Like Tag, E1 also binds to DNA polymerase α-primase (64).

The BPV1 E2 protein, although having no apparent enzymatic function for viral DNA replication, seems to be important in binding E1 and the PV origin in a cooperative fashion, especially when the DNA is present in the form of nucleosomes (43, 48, 57, 73). Its role in PV DNA replication seems to be primarily to recruit E1 and stimulate the formation of the E1 initiation complex (49, 78). E2, in addition to its role in origin recognition, is a transcriptional activator and has been shown to bind to RPA (2, 42, 44, 77).

Our goal was to determine whether the known human cellular DNA replication factors are also required for PV DNA replication. We have purified the two BPV1 replication proteins, E1 and E2, from *E. coli* harboring expression plasmids (72). These proteins were used for in vitro DNA replication experiments using combinations of human cell extract fractions and purified proteins. The use of human rather than murine cell extracts has enabled us to utilize the purified human factors previously identified by using the SV40 in vitro replication system. In this study, BPV1 replication reactions were compared with reactions in parallel experiments using the SV40 in vitro replication system. In the presence of Tag and the cellular topoisomerases, RPA, RFC, PCNA, DNA polymerase α-primase, and DNA polymerase δ are sufficient to support initiation and elongation of SV40 DNA replication (20, 84, 98). We show here that these same cellular factors are also required for BPV1 DNA replication. However, in conflict with another published report (60), we find that these factors alone are not sufficient to support initiation and elongation of the PV in vitro reaction (in the presence of topoisomerases and the PV E1 and E2 proteins). Apparently there exist additional cellular factors that are essential for the initiation and elongation stages of BPV1 DNA replication, which are not essential for SV40 DNA replication.

MATERIALS AND METHODS

Materials. [α-^{32}P]dATP (800 Ci/mmol) was obtained from Amersham. Q-Sepharose, SP-Sepharose, glutathione-Sepharose, ribonucleotides, and deoxyribonucleotides were obtained from Pharmacia. Heparin-agarose and thrombin were obtained from Sigma.

Replication factors and fractions. BPV1 E1 and E2 proteins were overproduced in *E. coli* by using the bacteriophage T7 expression system (80). The E2- and E1-overproducing vectors and purifications will be described in detail elsewhere (72). In brief, the E2 protein was purified to near homogeneity by conventional chromatography on SP-Sepharose, heparin-agarose, and finally Q-Sepharose. E1 was overproduced as a glutathione *S*-transferase (GST) fusion protein with a thrombin cleavage site positioned to allow subsequent removal of the GST domain. The GST-E1 fusion protein was purified by $(NH_4)_2SO_4$ precipitation followed by affinity purification using glutathione-Sepharose. The GST domain was cleaved from the E1 protein with thrombin. The E1 was then purified to near homogeneity by conventional chromatography on SP- and Q-Sepharose successively. SV40 Tag was purified from SF9 cells infected with a recombinant baculovirus expression vector by immunoaffinity chromatography (36, 62, 74, 79). Topoisomerases I and II were prepared from calf thymus according to published procedures with slight modifications (46, 71). RPA from human 293 cells was purified to near homogeneity by methods previously reported for yeast RPA (9) with fraction I as the starting material (65). DNA polymerase α-primase was purified from a 293 cell cytosolic extract (S100) by immunoaffinity chromatography with an anti-polymerase α monoclonal antibody (SJK 273-71)-Sepharose as described previously (61, 85). In some instances the polymerase α complex was further purified by gradient elution from a 0.1-ml SP-Sepharose column (30 mM potassium phosphate [pH 7.0], 1 mM Na_2EDTA, 10% [vol/vol] glycerol, 50 to 500 mM NaCl). PCNA, RFC, and DNA polymerase δ were prepared as described previously (54, 56, 85).

Fraction II* was prepared as described previously for fraction II but was eluted with buffer containing 667 mM NaCl rather than 1.0 M NaCl as described for fraction II (65). Fraction IIA was prepared as described previously (54, 86). Fraction IIA was depleted of DNA polymerase α-primase by being passed over an SJK 273-71-Sepharose column as described for the immunoaffinity purification of DNA polymerase α-primase. The material passing through the column was assayed for protein, and those fractions containing the highest protein levels were pooled and dialyzed against buffer A (25 mM Tris-HCl [pH 8.0], 10% [vol/vol] glycerol, 1 mM Na_2EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) with 50 mM NaCl and 20% sucrose. To prepare fractions A and B, fraction IIA was diluted to a conductivity equal to that of the equilibration buffer and applied to a Q-Sepharose column (10 mg of protein per ml of matrix) in buffer A with 150 mM NaCl. After being washed with 5 column volumes of the same buffer (flowthrough), the column was eluted with 5 column volumes of buffer A with 250 mM NaCl (fraction A) and then with 5 column volumes of buffer A with 600 mM NaCl (fraction B). Protein peaks were identified, pooled, and dialyzed against buffer A with 50 mM NaCl and 20% (wt/vol) sucrose.

DNA replication assays. Reaction conditions for PV and SV40 DNA replication were as previously described (41, 79), with the addition of polyglutamic acid at 20 μg/ml. Unless otherwise indicated, reaction mixtures were incubated for 60 min at 37°C.

The plasmid DNA substrates used for the PV replication reactions were as follows: p7914/27, which contains nucleotides (nt) 7914 to 7927 of the BPV1 genome cloned into the *Xba*I and *Hin*dIII sites of pUC19 (90); p7914/27Xho, which is the same plasmid with an *Xho*I linker inserted at the *Hpa*I site (nt 3) in the BPV1 origin, making the origin non-functional; and p7914/15, which contains nt 7914 to 7915 of the BPV1 genome also cloned into the *Xba*I and *Hin*dIII sites of pUC19. p7914/15 contains the E1 binding sites but eliminates E2 binding site 12 from the PV origin sequence insert. The SV40 plasmid DNA substrates used were pSV011, which contains the SV40 origin region from *Hin*dIII (nt 5171) to *Sph*I (nt 128) in pUC18 (2.9 kb), and pSV011(⁻), the same plasmid with a 4-nt deletion within one of the Tag binding sites in the core origin, making this plasmid deficient for SV40 DNA replication (26, 66, 79).

Each assay contained topoisomerases I and II at levels previously described (54). Where indicated, and unless otherwise specified, each standard assay also contained the following: RPA (30 ng/μl), PCNA (8 ng/μl), immunoaffinity-purified polymerase α-primase complex (4.8 ng/μl), DNA polymerase δ (2.4 ng/μl), and RFC at optimal levels. (RFC had to be titrated into the SV40 in vitro system to obtain optimal levels because of its propensity for losing activity over time.) Tag was used at 25 ng/μl, E1 was used at 10 ng/μl, and E2 was used at 5 ng/μl (unless otherwise indicated).

Incorporation of radiolabeled nucleotide was determined as the picomoles of dAMP that remained bound to DE-81 paper (Whatman) after five washes in 0.5 M Na_2HPO_4. The total volume of the reaction mixtures varied from 10 to 50 μl; incorporation levels from all experiments were normalized to reflect incorporation of a 50-μl assay in 1 h (unless otherwise indicated).

When comparisons between the SV40 and PV in vitro DNA replication systems were performed, levels of incorporation were normalized for each set of viral (SV40 or PV) reactions to 100%. This level (100%) was determined to be the level of incorporation (picomoles of dAMP incorporated per 60 min) for the appropriate positive viral (SV40 or PV) replication reaction control for that set of viral replication reactions. The levels of incorporation represented by 100% for each experiment are indicated in the figure legends.

Replication products were isolated by digestion with proteinase K and extraction with phenol-chloroform-isoamyl alcohol (25:24:1) and analyzed by agarose gel electrophoresis as described previously (54). When SV40 and PV replication products were compared by agarose gel analysis, smaller proportions of the SV40 reaction products than of the PV replication products were applied to the gel. Similar levels of incorporated nucleotide were applied to the gel for the two different viral positive control reactions. The proportion of each PV reaction mixture applied to the gel was based on the proportion of the PV positive control reaction mixture applied to the gel for that experiment. Likewise, the proportion of each SV40 reaction mixture applied to the gel was based on the proportion of the SV40 positive control reaction mixture applied to that gel. The level of incorporation for the viral positive control reactions for each experiment is given in the appropriate legend.

Origin of PV DNA replication in vitro. To determine where on the PV plasmid (p7914/27) DNA replication was starting, replication products from the various time points were digested with endonuclease *Dde*I and subjected to polyacrylamide gel electrophoresis (6%) as described previously (50). The gel was fixed and dried as described above for the agarose gels and analyzed with a Fuji 1000

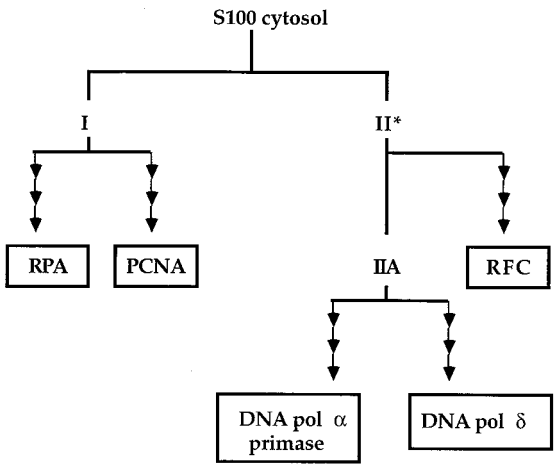


FIG. 1. Outline of fractions and factors required for SV40 DNA replication. Phosphocellulose fractionation of the crude cytosolic S100 results in two essential fractions, I and II*, required to support SV40 DNA synthesis in the presence of SV40 Tag and topoisomerases. Fraction I can be replaced by purified RPA and PCNA. Fraction II* can be replaced by fraction IIA and purified RFC. Fraction IIA can be replaced by highly purified DNA polymerases (pol) α-primase and δ to reconstitute SV40 DNA synthesis. Purified factors are shown in the boxes.

PhosphorImager. The relative incorporation was calculated by dividing the ratio of the radioactivity in a given restriction fragment to the radioactivity in all fragments at a particular time point by the ratio of the radioactivity in the same fragment at the 60-min time point to the radioactivity in all fragments at the 60-min time point. By 60 min the majority of nucleotide incorporation exists in completely replicated daughter molecules, allowing normalization against the 60-min products to determine the earliest labeled fragments (see results in reference 60).

***Dpn*I analysis.** Sixty-minute replication products from PV and SV40 reactions were each mixed with a large excess of the appropriate unlabeled template. One microgram of each mixture was digested with 7 U of endonuclease *Dpn*I (New England Biolabs) in the buffer provided with additional NaCl (to 100 mM) for 2 h at 37°C. Reaction mixtures were then separated by agarose gel electrophoresis, stained, photographed, fixed, dried, and exposed to film as described above.

RESULTS

In vitro DNA replication with human cell extracts. Unlike SV40 and polyomavirus DNA, PV DNA can be replicated in many different cell types when the viral E1 and E2 proteins are provided (14). This prompted us to investigate whether we could develop a BPV1 in vitro DNA replication system analogous to the mouse extract system reported by Yang et al. (102) by using human 293 cell extracts. Assuming that PV and SV40 DNA replication would require similar cellular factors, we attempted to replicate BPV1 origin-containing DNA in vitro by using combinations of partially purified fractions and highly purified factors capable of supporting SV40 DNA replication. The crude cytosolic extract (S100) did not support significant synthesis of BPV1 origin-containing DNA (data not shown). However, when we used highly purified topoisomerases I and II, RPA, and PCNA in conjunction with either fraction II* or fraction IIA (Fig. 1) and RFC, we detected very vigorous BPV1 DNA synthesis. As previously described for the murine cell extract system (102, 103) and more recently for monkey and human extracts (6, 60), this synthesis was clearly dependent upon the viral E1 protein and was stimulated by the BPV1 E2 protein (predominantly at low levels of E1) (Fig. 2).

To determine whether PV DNA replication exhibited a lag phase prior to the beginning of DNA synthesis similar to that seen with SV40, we compared a time course of PV DNA replication with that of a parallel SV40 reaction. As can be clearly seen, both systems experienced a lag phase of several minutes before appreciable synthesis began (Fig. 3), as noted in one other report (102) but not others (6, 60). This suggests that both systems require some presynthetic stage(s) prior to actual DNA synthesis. The replication products from these two types of reactions are very similar, predominantly replication intermediates at early times and covalently closed circular molecules at later time points (Fig. 3B). Replication with this system does not result in the preponderance of replicative intermediates seen with other cell extract systems (6, 102).

Initiation and bidirectional replication of PV DNA in vitro. To determine where DNA synthesis was initiated on the template, we digested the PV replication products isolated at various time points (from the experiment whose results are depicted in Fig. 3B) with a restriction endonuclease and separated the DNA fragments by polyacrylamide gel electrophoresis. Quantitation of the radioactivity in each of the resulting fragments showed that the majority of the labeling occurred in the PV origin-containing and adjacent fragments at the earliest time points (Fig. 3C). This clearly demonstrates that as seen with other cell extract systems (60, 102), the earliest DNA synthesis in the PV replication reaction is within the fragment containing the PV replication origin. It is also apparent that synthesis proceeds in both directions from the replication origin (Fig. 3C).

PV DNA replication in vitro is semiconservative. Template DNAs prepared from methylation wild-type *E. coli* are uniformly methylated. DNA synthesized in vitro is not methylated. DNA must be methylated to be sensitive to cleavage by the restriction enzyme *Dpn*I. Once a methylated DNA substrate is replicated in vitro the molecule is uniformly hemimethylated and therefore much more resistant to *Dpn*I cleavage than the parent molecule. If a DNA molecule is radiolabeled by a mechanism other than semiconservative replication, such as gap filling or a repair process, the labeled products will have some *Dpn*I sites that are still fully methylated. As a result, radiolabeled, nonreplicated DNA molecules will be sensitive to cleavage by *Dpn*I (41, 79).

Synthesized products from both the PV and SV40 reactions

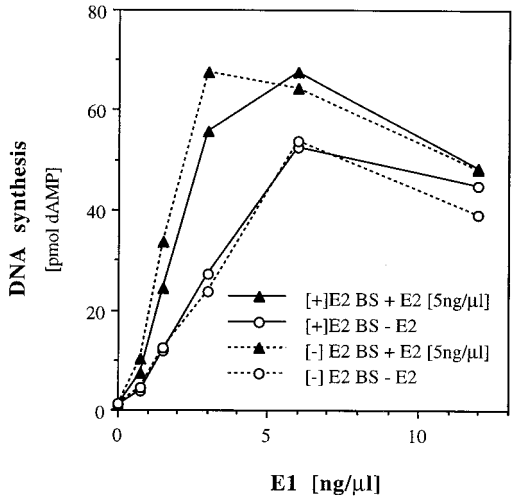
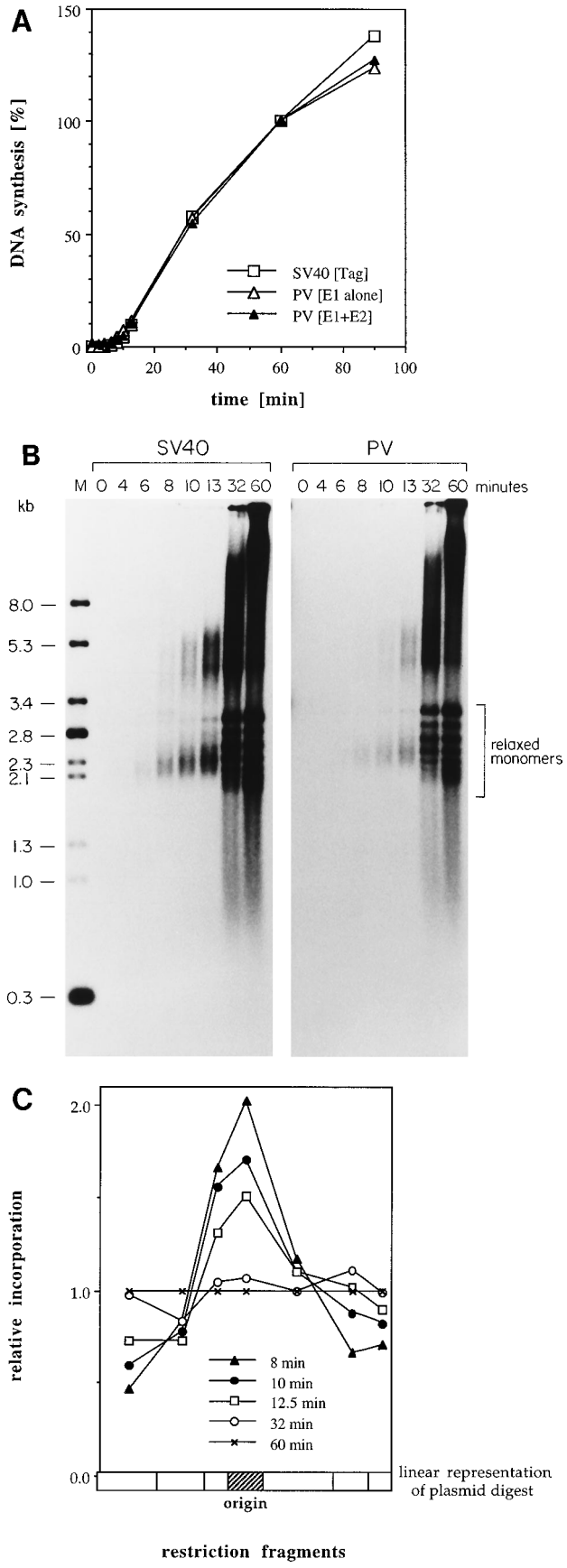


FIG. 2. PV DNA replication in vitro is E1 dependent. Increasing amounts of PV E1 protein were added to replication reaction mixtures either with or without 5 ng of PV E2 protein per μl. Each reaction mixture contained optimized levels of topoisomerases I and II, RPA, PCNA, fraction II*, and plasmid containing the BPV1 minimal origin either with or without an E2 binding site (E2 BS). DNA synthesis is expressed as picomoles of dAMP incorporated in 90 min in a 50-μl reaction mixture.



showed resistance to *Dpn*I digestion, characteristic of hemimethylated DNA (Fig. 4A, lanes 1 and 4). Since the input DNA was methylated and sensitive to *Dpn*I (Fig. 4B, lanes 1, 2, 4, and 5) and the replicated molecules were entirely hemimethylated, the radiolabeled products must have been a result of semiconservative DNA replication and not a repair-type reaction.

Requirement of cellular factors for PV DNA replication. In vitro replication of both SV40 and PV can be supported by fraction IIA and RPA, PCNA, RFC, and the topoisomerases. This IIA replication system was used to evaluate the requirement for several of these factors in PV DNA replication. Once again we compared the PV system with the SV40 system in parallel experiments. With the IIA system, SV40 and PV in vitro DNA replication reactions are both completely dependent upon fraction IIA for synthesis (Fig. 5). Furthermore, PV replication, like SV40 replication, is entirely dependent upon RPA for DNA synthesis (Fig. 5) as previously reported (42).

A notable difference between SV40 and PV in vitro DNA replication was seen in the differing dependence of the two systems on RFC and PCNA. As had been noted previously, the lack of PCNA or RFC in the SV40 in vitro replication system resulted in a decrease in synthesis, with the resulting products migrating in a smear at the normal position of the topoisomers in native gels and running as small products (average length, 100 to 500 nt) in denaturing gels (Fig. 5B and C) (20, 66, 84, 86, 98). Figure 5 shows that the lack of PCNA or RFC in PV DNA replication resulted in a decrease in synthesis more striking than that in the SV40 reactions and a nearly complete lack of the small nascent strands seen in the SV40 system.

Figure 5 also demonstrates that DNA synthesis in the PV system is dependent on the presence of a nonmutated PV origin.

Replacement of fraction IIA with purified polymerase α-primase and polymerase δ. We and others have previously reported that for the initiation and elongation stages of SV40 DNA replication, fraction IIA can be replaced by highly purified DNA polymerase δ and α-primase complexes (20, 54, 84, 98). More recently it has been reported that BPV1 in vitro DNA replication can be supported by purified proteins, including these two DNA polymerase complexes (60). Hence, we attempted to replace fraction IIA with these same two DNA polymerase complexes for our PV in vitro DNA replication system. As Fig. 6 clearly indicates, the two purified poly-

FIG. 3. Comparison of PV and SV40 DNA replication time courses. (A) All replication reaction mixtures contained optimized levels of topoisomerases I and II, RPA, PCNA, RFC, and fraction IIA. Reaction mixtures also contained either plasmid containing the SV40 origin and SV40 Tag or plasmid containing the PV origin with the E2 binding site and PV E1 protein either with or without the PV E2 protein (5 ng/μl). DNA synthesis is expressed as the percent incorporation obtained through 60 min of the reaction (for this experiment 100% represents 65 pmol of dAMP incorporated in the SV40 reactions, 12 pmol incorporated in the PV E1 reactions, and 10 pmol incorporated in the PV E1-plus-E2 reactions). (B) Reaction products from indicated time points were isolated and separated in a 0.8% agarose gel at 2.5 V/cm for 10 h along with radiolabeled DNA markers (M). Similar levels of incorporated nucleotide were applied to the gel for the two 60-min time points. Equal proportions of all PV replication reaction mixtures and all SV40 replication reaction mixtures were used as described in Materials and Methods. (C) Isolated replication reaction products from the PV E1-plus-E2 reactions (8-, 10-, 13-, 32-, and 60-min time points) containing identical levels of nucleotide incorporation were digested and electrophoresed as described in Materials and Methods. The relative level of radioactive incorporation in each fragment at each time point was calculated as described in Materials and Methods. This value was plotted against a linear representation of the plasmid (*x* axis) as digested with the restriction endonuclease *Dde*I. The seven fragments are displayed proportionally and colinearly with their positions in the plasmid, with the PV origin-containing fragment (cross-hatched) in the center.

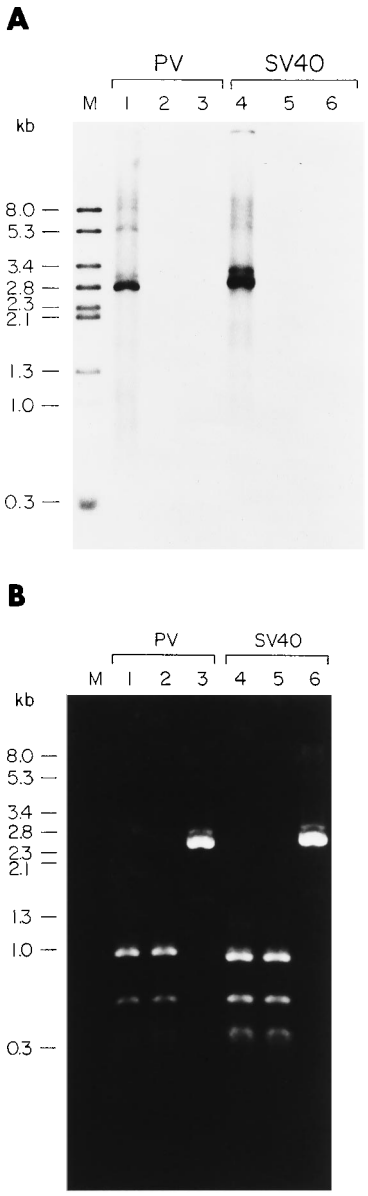


FIG. 4. Products of in vitro PV DNA replication reactions are resistant to *Dpn*I digestion. Similar levels (equal amounts of incorporated dAMP) of PV and SV40 60-min replication reaction products (described in the legend to Fig. 3) were isolated and subjected to digestion with *Bam*HI and *Dpn*I in the presence of excess (1 μg) DNA templates (lanes 1 and 4). Unlabeled DNA templates (1 μg) were digested with either *Dpn*I and *Bam*HI (lanes 2 and 5) or *Bam*HI alone (lanes 3 and 6). Products were subjected to agarose gel electrophoresis (as for Fig. 3B), and the gel was stained with ethidium bromide (250 ng/ml) for 45 min and photographed (B). The gel was then fixed, dried for autoradiography, and exposed to film (A).

merases can support synthesis in the SV40 in vitro system but not in our PV in vitro system. This is in contrast to the results of Muller et al. (60) noted above. The fact that the two polymerases were not sufficient to support the PV reaction in our experiments led us to ask whether these polymerase complexes were indeed required for PV DNA replication.

Immunodepletion of DNA polymerase α-primase from fraction IIA. To address the question of whether DNA polymerase α-primase is required for PV replication, we depleted DNA polymerase α-primase from IIA fractions by using a monoclonal antibody affinity column. The levels of DNA synthesis with these depleted IIA fractions were greatly decreased for both the PV and SV40 in vitro reactions (Fig. 7). Replenishment with purified DNA polymerase α-primase reconstituted synthesis in both systems. The same level of purified polymerase α-primase added to the reactions in place of any IIA fraction resulted in minuscule levels of synthesis (Fig. 7). These results indicate that polymerase α-primase is essential for PV DNA replication but not sufficient to replace fraction IIA. (It is important that the same amounts of purified polymerase α-primase were used for both the PV and SV40 reactions in the immunodepletion experiments for Fig. 7 and the in vitro reconstitution experiments for Fig. 6.)

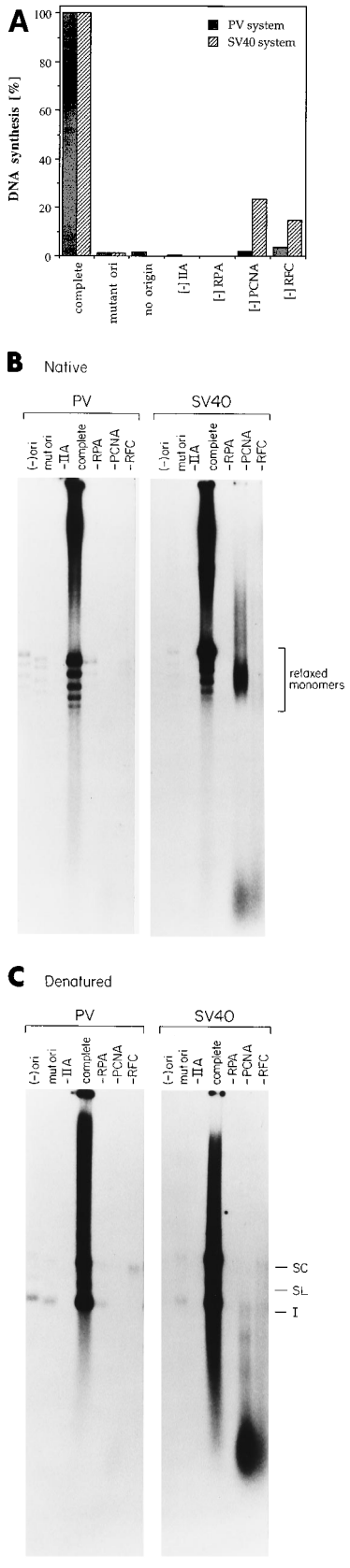
Purified polymerase δ can replace one of two essential IIA fractions. The strong requirement for RFC and PCNA in the PV reaction (Fig. 5) and their known role as cofactors for DNA polymerase δ (8, 39, 67, 83, 85) suggested that DNA polymerase δ (which is required for SV40 DNA replication) is also required for PV DNA replication. Since immunological reagents against DNA polymerase δ are not readily available, we attempted to address this question by further chromatographic fractionation of IIA.

Fraction IIA was separated into three fractions by ion-exchange chromatography (see Materials and Methods). Only two of these fractions were required to reconstitute PV synthesis at levels similar to those generated with the parent fraction (Fig. 8 and data not shown). Holding levels of either fraction constant, we attempted to replace the other fraction by the addition of highly purified DNA polymerase δ or DNA polymerase α-primase. We used levels of the two polymerases consistent with those required to support SV40 DNA synthesis or to replete immunodepleted IIA fractions for synthesis by either the PV or the SV40 system (2.4 ng of DNA polymerase δ per μl and 4.8 ng of DNA polymerase α-primase per μl). As shown in Fig. 9A, highly purified DNA polymerase δ can replace fraction A, demonstrating the requirement for DNA polymerase δ in PV DNA replication.

Consistent with the results shown in Fig. 6, we could not obtain equivalent levels of synthesis when we tried to replace the second fraction, fraction B, with highly purified DNA polymerase α-primase (Fig. 9B). At high levels of DNA polymerase α-primase (twofold greater than those required to replete immunodepleted IIA for PV DNA replication) some synthesis was seen (approximately 30% of that in the positive reaction). This suggests that either the immunopurified DNA polymerase α-primase contained low levels of the unknown essential factor(s) required for PV DNA replication or the addition of high levels of DNA polymerase α-primase can generate appreciable nonspecific synthesis in this reaction.

DISCUSSION

PVs are important human pathogens that are at least in part responsible for many human cancers (1, 27, 32, 34, 59, 70, 106). Furthermore, it is also becoming apparent that PVs, like SV40, are valuable viruses to study as models of cellular processes (for reviews see reference 69 and references therein). SV40 has proven to be an excellent model system for studying eukaryotic DNA replication systems (for reviews see references 29, 31, and 55). Indeed, virtually all of the homologs of the human cellular factors required for SV40 DNA replication have been detected in *Saccharomyces cerevisiae* and shown to be essential for and apparently involved in cellular DNA replication (4, 5, 10, 24, 28, 30, 47, 75, 105). However, as noted previously, it is imperative that multiple viral systems be used when host cell systems are modeled. This report presents our findings con-



cerning the requirements for the known cellular DNA replication factors with the in vitro system for PV DNA replication.

PVs are similar to SV40 in a number of ways. Both have small, double-stranded circular DNA genomes that are replicated in the host cell nucleus by using primarily cellular factors (for reviews of PV see references 35, 52, 69, and 82). Both make use of a viral factor that can bind to its cognate origin in a site-specific manner, open the DNA helix, and act as a strand-separating enzyme to generate single-stranded DNA regions around the origin. However, the two types of viruses have very different life cycles. Nonetheless, the mechanistic similarities led us to surmise that SV40 and PVs would utilize a number of similar cellular factors to replicate their viral genomes. Several combinations of extracts, partially purified fractions, and highly purified replication factors, all capable of supporting SV40 DNA replication, were tested for their ability to support PV DNA replication in vitro. Although the systems using the crude hypotonic cell lysate or purified proteins did not support significant synthesis, several combinations of purified factors and partially purified fractions were capable of supporting vigorous PV DNA replication.

These efficient PV in vitro DNA replication systems were analyzed by a number of criteria to determine whether they represented authentic DNA replication. Replication in these systems was dependent upon the viral E1 protein and a nonmutated viral origin. Synthesis started at or near the viral origin, apparently proceeding in a bidirectional fashion, and resulted in products that were the result of semiconservative DNA synthesis. Such characteristics indicate that this synthesis represents genuine PV DNA replication similar to that seen with crude lysate systems (6, 60, 102).

With these replication systems in hand we were able to address the question of whether the various purified factors, initially identified by using the SV40 in vitro replication system, were indeed required for PV DNA replication. It was determined that, like SV40 replication, PV DNA replication requires RPA, PCNA, RFC, DNA polymerase α-primase complex, and DNA polymerase δ. However, unlike the case with the SV40 in vitro system, these five cellular factors, topoisomerases I and II, and the viral replication factors are not sufficient to support in vitro PV DNA replication (Fig. 6).

FIG. 5. Requirement of RPA, PCNA, RFC, fraction IIA, and the PV origin for PV DNA replication in vitro. (A) The PV replication reaction mixtures contained the PV E1 and E2 proteins. The SV40 replication reaction mixtures contained SV40 Tag. All replication reaction mixtures contained optimized levels of topoisomerases I and II, RPA, PCNA, RFC, and fraction IIA unless otherwise indicated. All reaction mixtures also contained a DNA plasmid substrate. The PV reactions utilized either a plasmid containing the BPV1 origin (p7914/27), a plasmid containing a nonfunctional interrupted BPV1 origin (p7914/27XhoI) (mut ori), or a plasmid with no PV origin (pSV011) [(−)ori]. The SV40 reactions utilized either a plasmid containing the SV40 origin (pSV011), a plasmid containing a nonfunctional SV40 origin with a small deletion [pSV011(⁻)] (mut ori), or a plasmid with no SV40 origin (p7914/27) [(−)ori]. DNA synthesis is expressed as the percent incorporation of dAMP in a 60-min incubation compared with the positive reaction (complete) (for this experiment 100% represents 50 pmol of dAMP incorporated in the SV40 reaction and 15 pmol of dAMP incorporated in the PV reaction). (B) Reaction products from the indicated reactions were isolated and separated by agarose gel electrophoresis (as for Fig. 3B). Similar levels of incorporated nucleotide were applied to the gel for the two viral positive control reactions (complete). As described in Materials and Methods, equal proportions of all PV replication reaction mixtures and all SV40 replication reaction mixtures were applied to the gel. (Although there are products in the SV40 −PCNA and −RFC lanes that run in the same area of the gel as relaxed replicated monomers, these products are abortive replication initiation or lagging-strand products that do not represent relaxed replicated monomers [see panel C and Results].) (C) The same amounts of the products described for panel B were subjected to electrophoresis in an alkaline agarose gel as described in Materials and Methods.

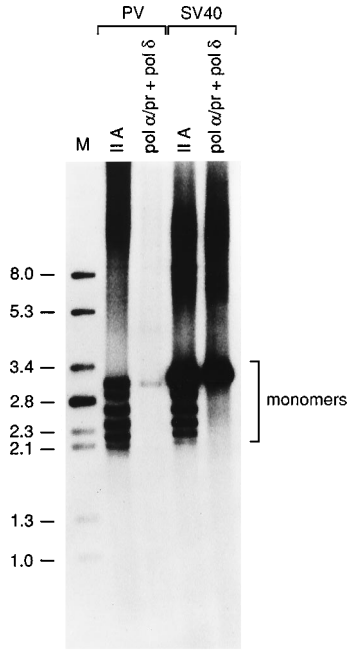


FIG. 6. DNA polymerase α-primase and DNA polymerase δ cannot support PV DNA synthesis in place of fraction IIA in in vitro reactions. Positive PV and SV40 replication reactions were performed with the appropriate DNA templates and viral proteins as described in the legend to Fig. 5 (IIA). In each case a second reaction in which fraction IIA was replaced by highly purified DNA polymerase α-primase (4.8 ng/μl) and DNA polymerase δ (2.4 ng/μl) was performed (pol α/pr + pol δ). Products were isolated and subjected to agarose gel analysis (as for Fig. 3B). As described in Materials and Methods, similar levels of incorporated nucleotide were applied to the gel for the two viral positive control reactions (IIA). Equal proportions of the PV replication reaction mixtures and the SV40 replication reaction mixtures were applied to the gel (for this experiment the amount of incorporation in the IIA positive controls represents 80 pmol of dAMP incorporated in the SV40 reaction and 26 pmol of dAMP incorporated in the PV reaction).

These results conflict with those of Muller et al. (60) that indicate that the above-mentioned combination of purified factors does support PV DNA replication in vitro. There are several possible explanations for this difference. One is that in the two studies several of the human cellular factors were purified from different human cell types. In the Muller study the cellular factors were purified from cervical carcinoma (HeLa) cells. In our study the cellular factors were purified from transformed human embryonic kidney (293) cells. Differentially modified factors could be present in the different cell types, and this could explain the different results for the two in vitro DNA replication systems.

Different purification procedures were used to purify several of the replication factors used in the two studies. Since many of the purified protein preparations used in biochemical studies of viral DNA replication must be purified from host cell sources, these purified protein preparations all contain small amounts of additional cellular proteins. Since several of the purified protein preparations used in the two studies were purified by different procedures, it is likely that the small amounts of additional proteins present in these preparations represent different trace activities. The different results could therefore be attributed to either trace inhibitors in our purified protein preparations or trace unknown essential replication factors in the purified protein preparations of Muller et al. (60).

Our results suggest that it is unlikely that the difference between the two studies is attributable to the presence of trace inhibitors in our highly purified protein preparations. The only difference between our IIA replication system, which supports PV DNA replication, and synthesis with purified factors, which does not support PV replication, is the replacement of fraction IIA by the two purified polymerase complexes. When both of the purified polymerases are added to the positive PV replication reaction (using the IIA replication system), no inhibition is seen (53), showing that no potent PV DNA replication inhibitors are present in our purified polymerase preparations. Furthermore, it is clear from our results that both of our purified polymerase preparations are capable of reconstituting PV DNA replication in vitro (Fig. 7 and Fig. 9A).

In further support of the presence of additional DNA replication factors in the IIA fraction, it has recently been shown

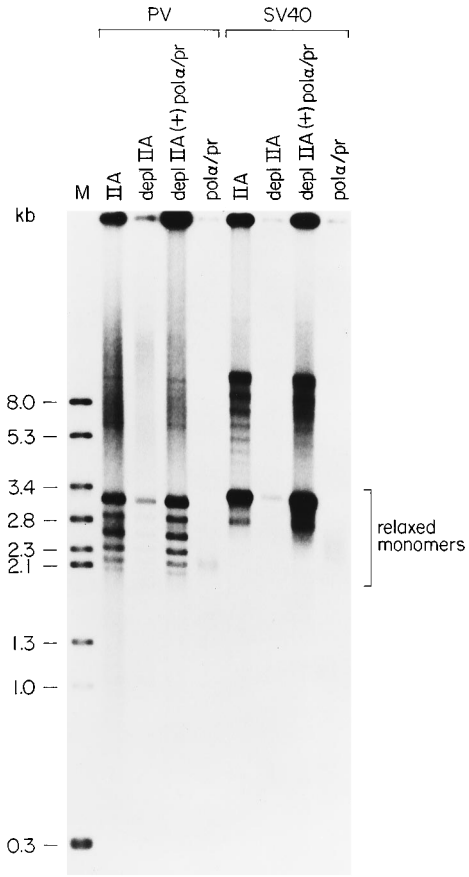


FIG. 7. DNA polymerase α-primase is required for PV DNA synthesis in vitro. Positive PV and SV40 replication reactions were performed with the appropriate DNA templates and viral proteins as described in the legend to Fig. 5 (IIA). Fraction IIA was depleted of DNA polymerase α-primase by immunoaffinity chromatography as described in Materials and Methods. Immunodepleted IIA was used in PV and SV40 replication reactions in place of fraction IIA (equal amounts of protein in all cases) (depl IIA). In each case an identical immunodepleted reaction mixture was prepared and supplemented with highly purified DNA polymerase α-primase (4.8 ng/μl) as indicated [depl IIA (+) polα/pr]. For each viral system control reactions were also performed in the presence of purified DNA polymerase α-primase (4.8 ng/μl) with no added IIA fraction (polα/pr). Products were isolated and subjected to agarose gel analysis (as for Fig. 3B). As described in Materials and Methods, similar levels of incorporated nucleotide were applied to the gel for the two viral positive control reactions (IIA). Equal proportions of all the PV replication reaction mixtures were applied to the gel, as were equal proportions of all the SV40 replication reaction mixtures (for this experiment the amount of incorporation in the IIA positive controls represents 43 pmol of dAMP incorporated in the SV40 reaction and 13 pmol of dAMP incorporated in the PV reaction).

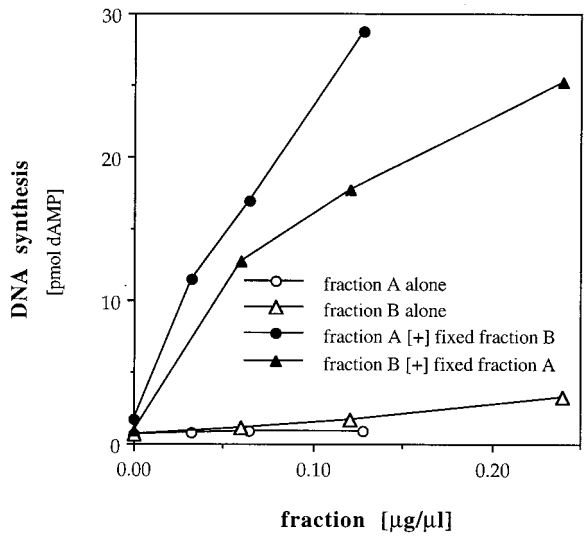


FIG. 8. Fraction IIA can be separated into two components, both essential for PV DNA replication in vitro. Fraction IIA was separated into two bound fractions, A and B, by anion-exchange chromatography as described in Materials and Methods. Increasing amounts of fraction A or fraction B were added to replication reaction mixtures containing optimized amounts of topoisomerases I and II, RPA, PCNA, RFC, E1, E2, and plasmid containing the BPV1 replication origin. Increasing amounts of fraction A were added to the above-described reaction mixtures with fraction B present (at 0.12 mg/ml). Increasing amounts of fraction B were added to the above-described reaction mixtures with fraction A present (at 0.064 mg/ml). Optimized levels of fraction IIA generated 28 pmol of incorporated dAMP in this experiment. DNA synthesis is expressed as picomoles of dAMP incorporated in 60 min in a 50-μl reaction mixture.

that adeno-associated virus DNA can be replicated in vitro by using a combination of purified cellular and viral proteins and partially purified extracts, including fraction IIA. Adeno-associated virus DNA replication in vitro, like our in vitro PV DNA replication system, is not supported by our preparations of highly purified DNA polymerase α-primase and δ in place of the IIA fraction (63). These studies strongly indicate that an additional replication factor(s) is evidently required for PV as well as adeno-associated virus DNA replication in vitro. Since potent replication inhibitors are evidently not present in fraction IIA, it seems likely that these additional replication factors are present in trace amounts in the purified protein preparations used by Muller et al. (60).

PV DNA replication in vitro exhibits a greater dependence on PCNA and RFC than SV40 replication does. Specifically, the anomalous lagging-strand synthesis (300- to 500-nt [average length] fragments) generated in the absence of either of these factors in SV40 reactions is not seen in PV reactions (Fig. 5C) (20, 37, 66, 84, 86, 98). It is not clear why this is so. Other studies have indicated that the DNA polymerase α-primase complex may be responsible for synthesizing only a 30-nt RNA-DNA primer during SV40 replication in crude cell extracts (12, 16). Furthermore, recent studies have indicated that DNA polymerase δ appears to be required for efficient completion and ligation of lagging strands synthesized in SV40 in vitro reactions (96). Taken together, these studies suggest that the production of 300- to 500-nt lagging strands by the DNA polymerase α-primase complex, in the absence of any of the polymerase switching or leading-strand factors (PCNA, RFC, and DNA polymerase δ), may be a specific characteristic of the SV40 in vitro system and may not be representative of other eukaryotic DNA replication systems. If this is the case, it could explain why so little relative synthesis is seen in the PV in vitro replication system in the absence of RFC or PCNA compared with the SV40 system. Similar numbers of initiations may be occurring in the PV reactions and in the SV40 reactions, but the synthesis of 30-nt fragments would result in 15- to 25-fold less total synthesis compared with the SV40 reactions (300- to 500-nt fragments). This difference between the two systems is currently under further investigation.

The greater requirement for PCNA and RFC in PV DNA replication in vitro may provide a clue to the identity of one of the possible additional cellular factors required for PV replication. RFC and PCNA, in addition to being cofactors for DNA polymerase δ, are also cofactors for DNA polymerase ε (40). Polymerase ε has been shown to be essential in *S. cerevisiae* and may be involved in cellular DNA replication (11, 45, 58, 97). It may be that polymerase ε provides a critical role in

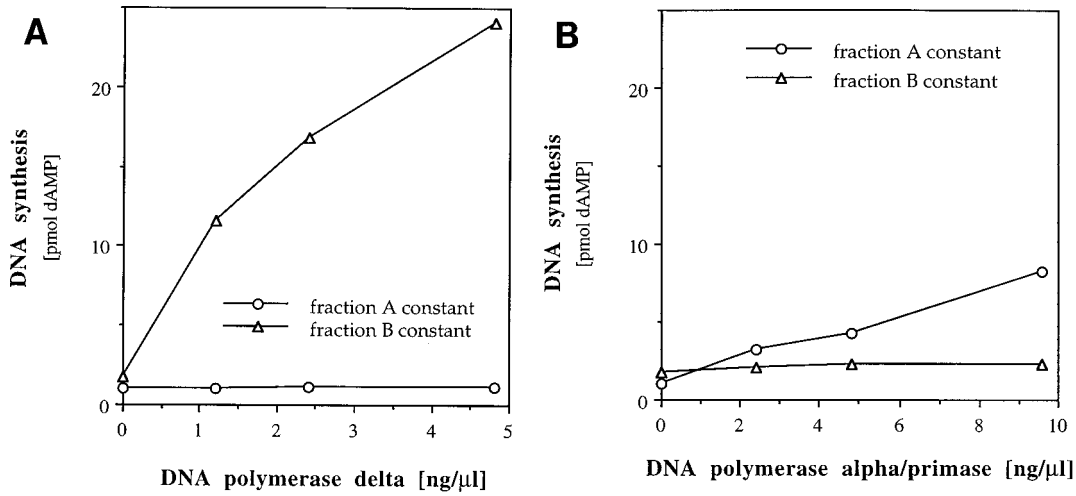


FIG. 9. DNA polymerase δ can replace fraction A and is therefore required for PV DNA replication in vitro. (A) Increasing amounts of DNA polymerase δ were added to replication reaction mixtures containing optimized amounts of topoisomerases I and II, RPA, PCNA, RFC, E1, E2, plasmid containing the BPV1 replication origin, and either fraction A or fraction B. (B) Increasing amounts of DNA polymerase α-primase were added to the reaction mixtures described for panel A. The combination of fraction A and fraction B generated 26 pmol of incorporated dAMP in these experiments. DNA synthesis is expressed as picomoles of dAMP incorporated in 60 min in a 50-μl reaction mixture.

cellular DNA replication that is carried out by one of the other DNA polymerases during SV40 DNA replication but not during PV DNA replication. Such a role could be the extension of the 30-nt primers synthesized by DNA polymerase α-primase. This function may be carried out by DNA polymerase ε (with PCNA and RFC) during cellular and PV DNA replication, but it can be carried out by DNA polymerase δ (with PCNA and RFC) during SV40 DNA replication.

Although it is possible that DNA polymerase ε is the unknown essential factor in fraction IIA required for PV DNA replication, there are other factors and/or functions that may be required for this process. While the PV E1 protein, like SV40 Tag, provides 3′-5′ helicase function, *E. coli oriC* DNA replication requires an additional 5′-3′ helicase function during replication (for reviews see references 22, 23, 33, 51, 68). Hence, a cellular 5′-3′ helicase may be essential for PV and/or cellular DNA replication. SV40 Tag provides the primosome loading functions of both the DnaB and DnaC proteins of *E. coli* and both the gene 41 and gene 59 proteins of bacteriophage T4 (56). It is possible that the PV E1 protein may not carry out both primosome loading functions; a cellular factor may be required to carry out the function analogous to that of the DnaC or gene 59 protein. Alternatively, cellular factors may be required to modify one of the other PV DNA replication factors. It is possible that the viral E1 protein (as is known for SV40 Tag) needs to be phosphorylated at particular amino acids before it can fully support PV DNA replication. It is unclear at this time which of these activities represent the hitherto unknown cellular factors required for PV DNA replication.

Clearly there exist additional cellular DNA replication factors other than those indicated by the SV40 in vitro DNA replication system. Preliminary studies indicate that multiple factors in fraction IIA (in addition to the two known factors, DNA polymerases α-primase and δ) are required for PV replication (53). The different requirements for DNA replication in the two viral systems may reflect the differences in the two viral life cycles—PV replication is a much more regulated process than SV40 replication. The unknown factors required for PV DNA replication may also be involved in cellular chromosomal DNA replication. The aforementioned findings suggest that further studies of the PV in vitro DNA replication system may help to identify these factors and provide further insights into the general mechanisms of both viral and eukaryotic DNA replication.

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