The cellular DNA polymerase α-primase is required for papillomavirus DNA replication and associates with the viral E1 helicase

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ABSTRACT Persistent infection by papillomaviruses involves the maintenance of viral DNA as a nuclear plasmid, the replication of which requires host DNA polymerases. The role of the cellular DNA polymerase α-primase helicase system in this process has not been completely elucidated. Monoclonal antibodies directed against the catalytic 180-kDa subunit of polymerase α inhibit DNA synthesis in this system. Addition of purified human polymerase α-primase holoenzyme to neutralized extracts restores their DNA synthetic activity. The amino-terminal 424 amino acids of E1 forms a specific protein complex with the p180 polymerase subunit. Immune complexes can be isolated with antibodies directed against E1 that contain a DNA polymerase activity. Moreover, this polymerase activity can be neutralized by anti-polymerase α antibodies. Permissivity barriers were not encountered in this in vitro system, as bovine E1 can interface with the murine and human replication apparatus. Although the large tumor antigens encoded by simian virus 40 and polyoma share limited primary sequence homology with the papillomavirus E1 proteins, the organization of functional motifs at the level of primary protein structure is remarkably similar. In addition to their origin-specific DNA-binding activity, each of these helicases may function to help recruit the cellular polymerase α-primase complex to the viral replication origin.

A central event in the initiation of DNA synthesis is the localization of one or more DNA polymerases (pol) to the origin of replication (ori). This localization appears to be accomplished through a series of protein–protein interactions in a variety of biological systems (for review, see ref. 1). Although these interactions have been well documented at the genetic and biochemical level in bacteria and their phages, little is known about how the DNA pols of higher eukaryotes recognize cellular ori. Although oris of higher eukaryotes, along with the presumed initiator proteins that recognize these elements, are ill defined, it is possible to examine how cellular DNA pols are targeted to the well-characterized oris of DNA tumor viruses (2–4).

The papovaviruses constitute a family of small, transforming DNA viruses that exhibit a complete dependence on host-cell functions to replicate (5). Each virus encodes an initiator helicase capable of assembling a specific nucleoprotein structure on the viral ori and unwinding it (for review, see refs. 5 and 6). In the case of simian virus 40 (SV40) and polyoma, the viral large tumor antigen (T) associates with the cellular pol α-primase, and this interaction may aid in localizing pol α to the ori (2–4, 7, 8). DNA pol α is responsible for initiating bidirectional DNA synthesis from the SV40 ori in vitro (9).

Papillomaviruses (PVs) behave as regulated replicons. Rodent cells transformed by bovine papillomavirus (BPV) 1 maintain the viral DNA as a multicopy nuclear chromosome that replicates in S phase with the host chromosome (for review, see refs. 10 and 11). The 68-kDa helicase/ori recognition protein encoded by E1 (12–19) and the 48-kDa E2 transactivator are required for PV DNA synthesis in vivo (20, 21) and in vitro (12, 17). E2 enhances DNA synthesis and may function as a replication factor by physically associating with E1 (22–24); moreover, this interaction may be essential for the cooperative binding of these proteins to the ori (12, 13, 16, 17, 19, 22, 25). These viral proteins must successfully interface with the host-cell replication machinery in persistently infected cells. To understand the molecular details of this process, an in vitro system was used to discern if the cellular DNA pol α was involved in PV DNA synthesis. Additionally, the ability of pure viral E1 helicase to engage the cellular pol α in a productive complex was examined.

MATERIALS AND METHODS

Proteins. Recombinant baculoviruses that express either BPV-1 E1, BPV-1 E2, human PV (HPV) 6b-E2, or the p180 subunit of DNA pol α have been described (22, 25–27). Proteins were isolated according to published procedures (22, 27, 28). The specific activity of the KB cell four-subunit pol α-primase complex was 20,000 units/mg. The recombinant p180 preparation displayed a specific activity of 40,000 units/mg. T4 DNA pol was from New England Biolabs. One unit of pol activity as referenced throughout this work represents the amount of activity to incorporate 1 nmol of labeled deoxynucleotide into acid-insoluble material in 1 hr at 37°C.

In vitro Replication. Extracts were prepared from logarithmically growing mouse FM3A or HeLa cells as described (12). To perform antibody inhibition experiments, various amounts of SJK 132-20 or SJK 237-71 antibodies were incubated with 10 μl of FM3A extract for 30 min at 0°C. The remaining components of the replication system (12), along with E1 and E2 proteins, were then added, bringing the final volume to 25 μl. Pure DNA pol α-primase holoenzyme was also added at this time in reconstitution experiments. Reactions were incubated for 2 hr at 37°C. Product analysis and plasmid DNA templates have been described (12, 29).

BPV E1-pol α ELISA. All steps were done at room temperature. Two-hundred and fifty nanograms of purified BPV E1, d424 E1, p180 pol α subunit, or 500 ng of the

Abbreviations: ori, origin of replication; pol, polymerase; PV, papillomavirus; BPV, bovine papillomavirus; SV40, simian virus 40; SV40 T, SV40 large tumor antigen; HPV, human PV; BSA, bovine serum albumin.

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four-subunit pol α-prime complex from KB cells (this quantity of the four-subunit holoenzyme contains an equivalent molar concentration of p180 subunit as 250 ng of the purified recombinant p180 preparation) was incubated for 1 hr in a microtiter well. The wells were washed twice with PBS and blocked with PBS/3% bovine serum albumin (BSA) for 2 hr. After two PBS washes, 0–200 ng of BPV E1, d424 E1, p180, or pol α-prime complex from KB cells was added in PBS/1.5% BSA for 1.5 hr. Unbound material was removed with three washes of PBS, and a monoclonal antibody directed against either the synthetic EE epitope on E1 or p180 was added in PBS/0.5% BSA. After 1.5 hr, the wells were washed three times with PBS and a horseradish peroxidase-labeled secondary antibody was applied in PBS/3% BSA. After five PBS washes, the assay was developed in 2,2'-azinobis(3-ethylbenzthiazelinesulfonic acid)/H₂O₂, and OD₄₅₀ was measured.

**Immune Complex Pol Assay.** One and four-tenths units of either the four-subunit pol α-prime from KB cells, p180 pol α catalytic subunit, or T4 DNA pol was incubated with various amounts of pure BPV E1 in 50 μl of 20 mM Tris-HCl, pH 7.5/4 mM ATP/13 mM NaCl/0.5% acetylated BSA at 50°C for 30 min at 37°C. Twenty microliters settled-bed volume (in 100 μl of 0.25% PBS) of anti-EE monoclonal antibody cross-linked to protein G-Sepharose (22) was added, and the reactions were mixed for 1 hr at 4°C. The beads were washed with 0.5 ml of 20 mM Tris-HCl, pH 7.5/100 mM KCl/5% (vol/vol) glycerol/0.04% Triton X-100, and 80 μl of pol reaction mix (26, 27) was added to the 20 μl of washed beads. After 30 min at 37°C, acid-insoluble products were collected by filtration and quantitated by scintillation counting.

**RESULTS**

The Cellular Pol α-Prime Holoenzyme Is Required for PV DNA Synthesis in the Cell-Free System. PV DNA replication in a soluble, cell-free system is sensitive to aphidicolin, an inhibitor of cellular DNA pol (12). The role of the cellular pol α-prime in PV DNA replication was explored in this system, as this is the only known mammalian DNA pol capable of *de novo* initiation in vitro (30, 31). In the presence of viral E1 and E2 proteins, this murine cell (FM3A) extract directs the replication of plasmids that contain the BPV ori. A monoclonal antibody (SJK-132-20) directed against the 180-kDa pol α catalytic subunit (32) was preincubated with the FM3A extract. Although this antibody was raised against the human pol, it neutralizes the enzymatic activity of its murine homolog (33). The reactions were then programmed with ori DNA, E1, and E2. Reactions that received E1 alone directed basal levels of DNA synthesis, which are enhanced by E2 (Fig. 1A, lanes 2 vs. 3). Addition of SJK-132-20 to these reactions, however, inhibits viral DNA replication (Fig. 1A, lanes 4–11). One nanogram of antibody causes a 50% reduction of DNA synthesis, whereas 10 ng virtually destroys replication activity (Fig. 1B). Another anti-human pol α monoclonal that does not cross-react with the murine enzyme (SJK-237; see 33), failed to inhibit DNA synthesis at protein concentrations where the SJK-132-20 antibody abolished replication (Fig. 1A, lanes 12–14). To confirm that this inhibition was due to the binding of SJK-132-20 to pol α, a reconstitution experiment was done. Extracts neutralized with SJK-132-20 were supplemented with increased quantities of purified human pol α holoenzyme, and their ability to direct BPV DNA synthesis was examined. Seventy-five nanograms of SJK-132-20 reduced DNA synthesis to levels 2.5% that of untreated controls (Fig. 2A, lane 6). However, the addition of pure pol α restored DNA synthesis in these neutralized extracts to 56% of the level directed by untreated controls (Fig. 2A, lane 10). This level may be an underestimate, as a slight (13%), inhibitory effect of pol α storage buffer was seen (Fig. 2A, lanes 1–5). At higher concentrations of 132-20, where neutralization is even more pronounced (0.79% synthesis in lane 11 compared with 2.5% synthesis in lane 6), addition of pol α again reverses the inhibitory effect of 132-20, although at a lower level (13% synthesis at 10 units; Fig. 2A, lane 11 vs. 15).

**Bovine and Human Viral Proteins Direct DNA Synthesis in Both Murine and Human Extracts.** The ability of human cell extracts to support BPV DNA synthesis was investigated, as human pol α interfaces with both the murine replication apparatus and the bovine viral proteins to replicate BPV DNA in vitro (Fig. 2B group a). Fig. 2B group b demonstrates that extracts prepared from HeLa cells support BPV DNA replication when supplemented with PV E1 and E2 (lane 5). Furthermore, murine and human extracts can be mixed together, and these hybrid extracts also function (lanes 6 and 7). As permissivity barriers were not identified in vitro at the host-cell level, the ability of HPV E2 to function in concert with BPV E1 was examined. Pure HPV E2 stimulates replication from the BPV ori in a manner almost indistinguishable from its bovine counterpart (Fig. 3A, lanes 5–7 and 8–10). Finally, BPV E1 can initiate DNA synthesis from the HPV-6b ori (Fig. 3A, lanes 15–20). The reduced level of basal synthesis promoted by BPV E1 on the human ori may reflect a reduced affinity of BPV E1 for the human ori relative to its bovine counterpart. Although both HPV and BPV oris bind E1 and E2, they differ considerably with respect to the number of E2-binding sites, the divergence of these E2 sites from consensus, and their potential E1 recognition motifs. Further studies are necessary to fully understand the nature of the elements responsible for generating these functional differences.

The stimulation promoted by either BPV or HPV E2 on human ori templates is similar to that seen with bovine ori templates. (Fig. 3A, lanes 5–7 plus 8–10 vs. 2–4; lanes 15–17).
Fig. 2. Addition of purified human DNA pol α-primase restores replication activity to neutralized extracts. (A) FM3A extracts were preincubated at 0°C (lanes a) in the absence of SJK-132-20, (lanes b) in the presence of 750 ng of SJK-132-20, or (lanes c) in the presence of 100 ng of SJK-132-20. In addition to a supplement that contained replication buffer, nucleotides, and BPV E1 and E2, the reactions received increased amounts of DNA pol α from human KB cells (lanes b and c) or increased amounts of pol α storage buffer. After incubation at 37°C, the replication products were analyzed on an 8.8% agarose gel and visualized by autoradiography. Quantitation referred to in text was done on a Molecular Dynamics PhosphorImager. The control reaction in lane 16 was preincubated with 100 ng of SJK-237 antibody, which does not inhibit pol α. (B) Human and murine extracts support BPV DNA synthesis in vitro. Reactions were programmed with extracts prepared from HeLa cells (lanes 1 and 5), FM3A cells (lanes 4 and 8), or a mixture of FM3A and HeLa extract (lanes 2, 3, 6, and 7). BPV E1 and E2 proteins were absent from group a (lanes 1–4) reactions but were present in group b reactions (lanes 5–8). The arrow at right denotes form I DNA product using the pKSO BPV template. R.I., replicating intermediates. Isolated products were analyzed as described for A.

plus 18–20 vs. 12–14). The reduction in the absolute amounts of synthesis observed on the HPV ori may reflect the lower basal levels of synthesis directed by bovine E1 on the HPV 6b ori. Alternatively, structural differences between the two oris may contribute to these effects. Although both human and bovine E2 proteins enhance replication to similar levels on the bovine pKSO origin, BPV E2 displays consistently higher activity on the pKSO T template, which lacks a proximal E2-binding site. The human E2 protein also displays greater activity than the bovine E2 protein on the HPV origin (Fig. 3C). Continued probing of these subtle differences may yield information regarding the nature of these hybrid interactions. The promiscuity displayed by E1 and E2 in their ability to recognize oris from other PV types and species in vitro is consistent with in vivo data (21, 34, 35). Moreover, it provides a functional role for the interspecies E1/E2 complexes that form in vitro (25). Finally, in vitro data eliminates potential concerns over variable template concentrations and protein-stability effects associated with the in vivo data. The apparent lack of host permissivity barriers between BPV and HPV replication proteins stands in contrast to the rigid constraints displayed by the murine polyoma and SV40 viruses (36, 37).

Viral E1 Protein Binds to the Cellular pol α-Primase. Having established a pivotal role for the pol α-primase holoenzyme in PV DNA synthesis, it was important to discern how this cellular complex is recruited to the viral ori. Microtiter plates were prepared that contained either the multisubunit human pol α-primase from KB cells or the purified 180-kDa catalytic subunit (purified from a recombinant baculovirus) immobilized in individual wells. The retention of pure E1 onto the solid phase was quantitated by immunochemical methods. Fig. 4A demonstrates that E1 is retained in wells that contain either the p180 subunit or the holoenzyme but is not retained in wells that did not contain immobilized pol α. This result was confirmed by using plates that contained immobilized E1 (Fig. 4B). p180 was efficiently retained only in wells that contained the viral E1 polypeptide. A deletion mutant, d424, which removes the carboxyl-terminal 182 aa from E1 (19), retains the ability to interact efficiently with pol α. These carboxyl-terminal 182 residues, thus, do not contain the critical determinants for complex formation. The enhanced signal generated in Fig. 4A by d424 is probably due to the increased number of epitopes on the
smaller d424 relative to equivalent weight amounts of wild type E1 (605 aa) (data not shown).

To further probe the specificity of this interaction, E1 was incubated in solution with either the pol α holoenzyme p180 or T4 DNA pol. This phage-encoded enzyme shares some homology to its eukaryotic counterparts in the α family (38, 39). Immune complexes were isolated by using antibodies directed against E1, and the complexes were assayed for the presence of DNA pol activity. Only complexes formed with the eukaryotic holoenzyme or the recombinant p180 subunit displayed DNA pol activity (Fig. 4C). The viral E1 helicase is thus associating with specific determinants on the eukaryotic enzyme. Similar complexes can be isolated from replication extracts programmed with E1. Moreover, the DNA pol activity observed in those complexes can be neutralized by antibodies directed against pol α (data not shown). At E1 concentrations from 250 to 500 ng, the enhanced activity of immune complexes isolated in the presence of the holoenzyme, as opposed to the p180 catalytic subunit, may reflect interactions with additional pol components. A detailed investigation of this phenomenon, however, will require proteins from homologous sources, as the p180 subunit was expressed in insect cells, whereas the holoenzyme was isolated from human sources. The 58-kDa and 48-kDa holoenzyme subunits contain the primase activity, whereas the precise role of the 70-kDa subunit remains to be elucidated. Studies with SV40 T have demonstrated that the 70-kDa subunit promotes the formation of the p180–T complex and mediates the stimulation of pol α activity observed in the presence of T (40).

**DISCUSSION**

Papovaviruses have evolved a specific strategy for targeting the host-cell machinery to the viral ori. Unlike larger DNA viruses that encode numerous DNA replication proteins, these small DNA viruses encode DNA helicases that recognize the viral ori (for review, see ref. 5). For SV40 and polyoma, this is the large T antigen, whereas in PVs it is the E1 polypeptide. Furthermore, these origin-binding viral helicases associate with the cellular pol α–primase, and this interaction may be crucial in establishing a replication complex at the viral ori. Sequestering pol α–primase at an active ori is critical, as this is the only eukaryotic pol capable of initiating new DNA chains by virtue of its associated primase activity (30, 31). The initiator protein–pol interaction may be the nucleation point for the assembly of other cellular replication proteins and is thus likely to be critical for the establishment of a persistent infection and the maintenance of the viral chromosome as an episomal element.

Although limited sequence homology was noted in the carboxy-terminal nucleotide-binding regions of SV40 T, Polyoma large tumor antigen, and PV E1 (41), the biochemical functions of these proteins in initiating DNA synthesis are strikingly similar. Furthermore, the linear arrangement of functional domains on each polypeptide is highly conserved (Fig. 5). Additional refinement will reveal if a small amino-terminal domain of E1, similar in structure to T antigen, has sufficient information to bind pol α. The extent of homology among papovaviral initiator proteins at the level of tertiary structure may prove even more striking, as the constraints on constructing a multifunctional helicase that specifically binds the ori and interfaces with pol α may be enormous.

Further studies will be necessary to determine whether identical surfaces on p180 are involved in interfacing with each of these viral polypeptides. This region of pol α, which has been demonstrated to contact SV40 T (residues 195–313; ref. 7), may be involved in contacting the cellular replicative helicase. Alternatively, there may be more than one region of p180 capable of interfacing with helicases. Some of these
putative regions may display marked interspecies differences, which could thus limit viral host range, whereas other regions could interface with helicases across species lines.

The host range permissivity barriers defined with SV40 and polyoma appear not to be an issue for PVs. While SV40 T binds to primate pol α-prime, it cannot form a productive complex with the murine enzyme. Polyoma large tumor antigen, on the other hand, recognizes the murine machinery but does not recognize the human equivalent (36, 37). BPV E1 interfaces with both the murine and human machinery and directs PV DNA synthesis in extracts from both sources. Furthermore, \textit{in vitro} replication directed by BPV E1 is stimulated by HPV E2 proteins on both bovine and human viral oris. This result is consistent with \textit{in vivo} studies that demonstrate that BPV E1 and E2 direct replication from HPV oris (21, 34, 35).

The dissection of replication systems from bacteria, their phages, and eukaryotic DNA tumor viruses has revealed a remarkably conserved set of processes. A variety of protein–protein interactions have been discovered that shape our current understanding of replication machines (1, 42, 43). While it has been useful to think about these complex processes as a series of ordered biochemical reactions on a sequential pathway, the significance of partial reactions reconstituted apart from the complete synthesis of a replicon is unclear. Further studies are needed to illuminate the mechanism by which such a diverse ensemble of proteins are coordinately instructed to initiate DNA synthesis from an ori. Specifically, is a preinitiation complex assembled before ori unwinding and which factors, such as pol α-prime and replication protein A (RPA), must be correctly positioned along with the initiator protein to permit the subsequent initiation of DNA synthesis? The assembled structure could drive a concerted process, the net result of which is the initiation of DNA replication. For example, the E1-pol α-E2-RPA complex (this work and ref. 29) may preexist before the onset of DNA synthesis and be stably poised over the ori, hovering on the brink of initiation. Replication protein A could thus be available to function in the initiation reaction once the ori unwinds.

The stability of these preinitiation structures may be important for a complete understanding of the molecular events associated with transit from the G1 phase of the cell cycle to S phase. The potential dynamics of assembling and disassembling replication structures remains relatively unexplored. Along these lines, the Epstein–Barr virus-encoded nuclear antigen 1 initiator protein remains stably associated with its cognate replication origin throughout the cell cycle (44), and the yeast origin recognition complex (45) remains bound to autonomously replicating sequence elements (46). The limiting event for initiation could thus be either the modification of this stable initiator–ori complex or the association of the initiator nucleoprotein locus with the remaining components of the cellular replication machinery. Stable initiator protein–replication ori complexes may represent the ultimate targets of many signal-transduction pathways that lead to proliferation. They may also ensure that each ori fires only once per S phase. A detailed understanding of both when and how DNA pol interface with these complexes could thus lead to new modalities of interfering with proliferation. Further investigation of viral replicons and ultimately of chromosomal origins and their trans-acting factors will eventually reveal the true nature of these machines.

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