Human Papillomavirus DNA Replication

INTERACTIONS BETWEEN THE VIRAL E1 PROTEIN AND TWO SUBUNITS OF HUMAN DNA POLYMERASE α/PRIMASE

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Papovaviruses are valuable models for the study of DNA replication in higher eukaryotic organisms, as they depend on host factors for replication of their DNA. In this study we investigate the interactions between the human papillomavirus type 11 (HPV-11) origin recognition and initiator protein E1 and human polymerase α/primase (pol α/primase) subunits. By using a variety of physical assays, we show that both 180- (p180) and 70-kDa (p70) subunits of pol α/primase interact with HPV-11 E1. The interactions of E1 with p180 and p70 are functionally different in cell-free replication of an HPV-11 origin-containing plasmid. Exogenously added p180 inhibits both E2-dependent and E2-independent cell-free replication of HPV-11, whereas p70 inhibits E2-dependent but stimulates E2-independent replication. Our experiments indicate that p70 does not physically interact with E2 and suggest that it may compete with E2 for binding to E1. A model of how E2 and p70 sequentially interact with E1 during initiation of viral DNA replication is proposed.

Papillomaviruses are members of the small DNA tumor virus family. They cause papillomas in a wide variety of hosts and certain high risk human papillomavirus types are strongly linked to the development of cervical or penile cancer in humans (1). The mode of viral replication is closely coupled to the differentiation status of the infected squamous epithelium (for review see Ref. 2). In the basal and parabasal cells of the squamous epithelium, the virus is maintained as a low copy number extra-chromosomal episome and undergoes regulated DNA replication modulated by both viral and host proteins. As cells undergo progressive differentiation, vegetative viral replication is triggered, “late” viral genes are expressed, and progeny virions are produced in a fraction of the terminally differentiated cells in papillomas or condylomas. The latent stage of papillomaviral replication provides an ideal system for the study of regulated eukaryotic DNA replication.

We and others (3, 4) have previously reported a cell-free replication system for bovine papillomavirus type 1 (BPV-1) (3) and human papillomavirus type 11 (HPV-11) (4). Papillomaviral replication requires the viral proteins E1 and E2 (5, 6), as well as the full complement of host replication proteins that have previously been identified in SV40 in vitro replication, including DNA polymerases α and δ (7, 8). It is therefore probable that physical interactions between the host initiation enzymes and the papillomaviral initiation proteins E1 or E2 occur during initiation of viral DNA replication.

The papillomaviral E1 protein is a functional homolog of SV40 large T antigen, with origin binding activity as well as ATPase and helicase activity (9–15). It associates as a trimer or a hexamer on its cognate E1-binding site in the viral origin (ori) with relatively low affinity and low sequence specificity (3, 4, 16–23). As a result, high concentrations of E1 can bind DNA nonspecifically and initiate ori-independent replication at low efficiency in vitro (3, 4). It has been proposed that the replication competent form of BPV-1 E1 is a multimeric complex of 10–12 E1 molecules (20). Recently, the HPV-11 E1 protein has been shown to bind to the human chaperone protein Hsp40, and in its presence, a dimeric E1 complex forms on the ori (23), mirroring the structure of SV40 T antigen on the SV40 ori (24) as well as other known Escherichia coli helicases (25). In addition to its role in initiation, HPV-11 E1 is also required during elongation in vitro, suggesting its helicase activity may be critical at the replicating forks (26).

E2 is a viral transcriptional transactivator that is essential for viral DNA replication in vivo (27, 28). It binds as a dimer with high affinity to its conserved binding sites (E2-binding site) in the viral genome, including several sites in the viral origin of replication (29–34). It forms a complex with E1 (35), and this E1/E2 complex has increased sequence specificity for binding to the E1 and E2 cognate sites in the viral origin (36, 37). Thus one of the critical functions of E2 in viral DNA replication is to interact with and recruit E1 to the viral origin of replication by virtue of the stronger DNA binding affinity and specificity for E2 (20, 35–41). Therefore, the addition of E2 protein to a cell-free replication assay enhances ori-dependent and suppresses ori-independent replication. Based on these data the following model of E2/E1 interaction during initiation of bovine papillomaviral DNA replication has been proposed (20). Once the first molecule of E1 is loaded onto the origin by E2, E2 is then released from the origin, allowing E1 to multimerize into a replication-competent form. However, more recent studies in HPV replication have suggested that the role played by E2 extends beyond the recruitment of E1; E2 is necessary for the formation of the entire pre-initiation complex, although it is dispensable during elongation (26). This interaction between E2 and E1 is extremely important for HPV ori

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1 The abbreviations used are: BPV, bovine papillomavirus; HPV, human papillomavirus; pol, polymerase; ELISA, enzyme-linked immunosorbent assay; ori, origin.
replication, as only the E2-binding site is absolutely essential for origin-specific viral replication, whereas the E1-binding site is dispensable for in vivo or in vitro viral DNA replication (4, 31, 42, 43). In addition, certain heterologous combinations of E1 and E2 either do not support replication, or do so poorly, whereas the homologous pairs of viral proteins always support replication effectively (28, 40, 41), suggesting type-specific interactions between E1 and E2.

DNA pol α/primase is the principal enzyme for the initiation of DNA synthesis and is required for both leading and lagging strand DNA replication (44–46). Pol α/primase is essential for the initiation of cell-free replication of SV40 (50), BPV-1 (3, 51), and HPV-11 (4). DNA pol α/primase has also been shown to be the major host factor responsible for species-specific replication of SV40 and polyomaviral DNA in vitro (54–56). These findings not only indicate the critical function of DNA pol α/primase in the initiation of viral DNA replication but also reveal the important role played by this enzyme complex in the timing and control of DNA replication in eukaryotic cells. Pol α/primase consists of four subunits as follows: a polymerase catalytic subunit of 180 kDa (p180), two smaller subunits of 58 and 49 kDa (p58 and p49) containing the primase activity, and a 70-kDa subunit (p70 or B-subunit) which is a cell cycle-dependent phosphoprotein (47, 48) with no known catalytic function (44, 45). The catalytic subunit p180 has been shown to interact physically with both the SV40 T antigen (49, 50) and with the BPV-1 E1 protein (51, 52), and the interaction between p180 and T antigen is required for SV40 viral DNA replication in vitro (50). The p70 subunit has also been shown to interact physically with SV40 T antigen in vitro, although the functional significance of this interaction is not understood (53). DNA polymerase α activity is essential for the initiation of cell-free replication of SV40 (50), BPV-1 (3, 51), and HPV-11 (4). It is probable that analogous interactions between DNA pol α/primase and the host counterparts of the viral initiators may also play a critical role in the control of chromosomal DNA replication.

In this report, we describe the physical and functional interactions between HPV-11 E1 and the catalytic subunit, p180, as well as the p70 subunit of human DNA pol α/primase. HPV-11 is the causative agent of genital condylomata and laryngeal papillomas and shares extensive sequence homology with the high risk HPV types. Thus, investigation of the homo-species interaction is not understood (53). DNA polymerase α activity is essential for the initiation of cell-free replication of SV40 (50), BPV-1 (3, 51), and HPV-11 (4). It is probable that analogous interactions between DNA pol α/primase and the host counterparts of the viral initiators may also play a critical role in the control of chromosomal DNA replication.

In vitro transcription and translation of pET-EE-E1 and pET-BB-E2 were used to determine the elution profile for each protein and compared with both internal standards (aldolase, 158 kDa) as well as to external standards run in a parallel gradient. Gel Filtration—Five μg of purified proteins were layered onto a 4-ml 10–30% (v/v) sucrose gradient in 200 mM NaCl, 50 mM Tris- HCl (pH 7.5), 0.1% Tween and centrifuged for 6 h at 45,000 rpm at 15 °C in a Beckman SW60 rotor. Fractions (15 drops) were collected from the bottom of the tube using a 21-gauge needle. Ten μl of each fraction were combined with an equal amount of 2× SDS loading buffer and run on a 9% SDS-polyacrylamide gel. The sucrose density of each fraction was measured. When indicated, equimolar amounts of E1 protein were incubated together on ice with p70 for 20 min prior to loading on the gel.

In vitro transcription and translation of pET-EE-E1 and pET-BB-E2 were performed using anti-E1 monoclonal antibody against E1, or anti-p70 polyclonal antibody to determine the elution profile for each protein and compared with both internal standards (aldolase, 158 kDa) as well as to external standards run in a parallel gradient.

Concentration of Myc-tagged p70 and in Vitro Transcription and Translation of HPV11 E1 and E2, and p70—Oligonucleotides were constructed to allow polymerase chain reaction amplification of the Myc epitope from a previously existing construct. The primer for amplification of the 5′ side of the tag was constructed to contain an Nhel site (5′ GCC CGC TAG CCA TGC TGA GGA GCA A 3′). The primer for amplification of the 3′ side contains a BamHI site (5′ GCC GGC TAG CGG ATC CCA TAT GTA AGT CCT C 3′). The resulting from this amplification contains two sequential Myc epitopes (EQKLISEED). This fragment was then cloned into the Nhel-BamHI sites of pET11a (Novagen). Subsequent digestion with BamHI allowed the insertion of the coding sequence of the human p70 gene after liberation from pQE9-hup70 with BamHI digestion. Orientation and sequence of the resulting coding frame were confirmed through analysis. Construction of pET-EE-E1 and pET-BB-E2 will be described elsewhere. 2 BB epitope was derived from CMVpp65 (a phosphoprotein encoded by UL83 kindly suggested to us by Dr. William Britt).

Interaction of DNA Polymerase α/primase and HPV11 E1

**EXPERIMENTAL PROCEDURES**

**Plasmids and Proteins**—The HPV-11 ori plasmid pUC7874-99 has been described (4, 31). Native HPV-11 E2 protein and HPV-11 E1 protein tagged at the amino terminus with the Glu-rich (EE) epitope from the polyoma virus middle T antigen (57) were purified from recombinant baculovirus-infected Sf9 cells as described (4). p180 was purified from baculovirus-infected Sf9 cells using the monoclonal antibody SJK237-71 as described previously (58), except for the following modifications to accommodate the cell-free replication assay. After elution from the monoclonal antibody column with 3.2 M MgCl₂, the protein was dialyzed for two changes against 50 mM Tris- HCl (pH 8.0), 10 mM EDTA, 20% glycerol, and 1 mM β-mercaptoethanol. A final concentration step was performed by coating the dialysis tubing with dry G-50 Sephadex. The cloning of the 70-kDa subunit of DNA pol α/primase and the production in rabbit of anti-p70 polyclonal antibody will be described elsewhere. Five hundred-ml cultures of M15[pREP4] (Qiagen Inc.) transformed with an ampicillin-resistant plasmid p70 cDNA cloned into the pQE9 plasmid (Qiagen Inc.) were grown in 2× YT broth to an A₅₅₀ = 0.7–0.9, induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside and grown for 2.5 h at 20 °C. Purification of overexpressed protein was carried out as described previously (59). One-ml fractions were collected, and protein-containing fractions were immediately dialyzed against 50 mM Tris-HCl (pH 8.0), 20 mM KCl, 1 mM EDTA, and 1 mM β-mercaptoethanol for several changes. Protein was then concentrated via dialysis against 50% glycerol, 50 mM Tris-HCl (pH 8.0), 20 mM KCl, 1 mM EDTA, and 1 mM β-mercaptoethanol.

**Sucrose Gradients**—Five μg of purified proteins were layered onto a 4-ml 10–30% (v/v) sucrose gradient in 200 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.1% Tween and centrifuged for 6 h at 45,000 rpm at 15 °C in a Beckman SW60 rotor. Fractions (15 drops) were collected from the bottom of the tube using a 21-gauge needle. Ten μl of each fraction were combined with an equal amount of 2× SDS loading buffer and run on a 9% SDS-polyacrylamide gel. The sucrose density of each fraction was measured. When indicated, equimolar amounts of E1 protein were incubated together on ice with p70 for 20 min prior to loading on the gel.

**Enzyme-linked Immunosorbent Assay (ELISA)**—ELISA assays were performed as described (49), with the following modifications. One-half μg of the primary protein was used to coat the 96-well plate. After blocking with 3% bovine serum albumin, increasing amounts of the second protein were added, and incubation proceeded for 1 h. The plate was washed thoroughly 5 times with phosphate-buffered saline and incubated with a monoclonal antibody against detection of E1 or anti-p70 polyclonal antibody. Gel filtration of p70 and E2 was performed using the Amersham Pharmacia Biotech SMART affinity system. Two μg of each protein were preincubated for 20 min on ice prior to injection on a Superose 6 PC 3.2/30 column at a flow rate of 0.05 ml/min. Column was eluted at room temperature in the buffer system described above. Fifty-μl fractions were collected, and protein profiles were analyzed via Western blotting using anti-p70 polyclonal antibody and anti-E2 polyclonal antibody.

**Construction of Myc-tagged p70 and in Vitro Transcription and Translation of HPV11 E1 and E2, and p70—Oligonucleotides were constructed to allow polymerase chain reaction amplification of the Myc epitope from a previously existing construct. The primer for amplification of the 5′ side of the tag was constructed to contain an Nhel site (5′ CGG CGC TAG CCA TGC TGA GGA GCA A 3′) and the primer for amplification of the 3′ side contains a BamHI site (5′ GCC GGC TAG CGG ATC CCA TAT GTA AGT CCT C 3′). The 113-base pair fragment resulting from this amplification contains two sequential Myc epitopes (EQKLISEED). This fragment was then cloned into the Nhel-BamHI sites of pET11a (Novagen). Subsequent digestion with BamHI allowed the insertion of the coding sequence of the human p70 gene after liberation from pQE9-hup70 with BamHI digestion. Orientation and sequence of the resulting coding frame were confirmed through analysis. Construction of pET-EE-E1 and pET-BB-E2 will be described elsewhere. 2 BB epitope was derived from CMVpp65 (a phosphoprotein encoded by UL83 kindly suggested to us by Dr. William Britt).
and pET-Myc-p70 were carried out using the Transcription and Translation-coupled Reticulocyte Lysate System from Promega. Due to extreme inefficiency of labeling human p70 with either [35S]methionine or [35S]cysteine, this protein was labeled using 1-(4,5-dihydroxy-1-naphthalene) (156 Ci/mmol, 1 mM/cml, Amersham Pharmacia Biotech). All other proteins were labeled using [35S]methionine (1000 Ci/mmol, 15 mM/cml, Amersham Pharmacia Biotech).

Co-immunoprecipitation—Sf9 insect cells were grown to 80% confluence in a T-150 flask. They were then infected at 10 multiplicity of infection with baculovirus expressing either p180 (AcHDPα) (58) or HPV-11 EE-E1 (4) or co-infected with both viruses. After 48 h, the cells were harvested, washed once in phosphate-buffered saline, resuspended in 20% ethylene glycol, 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM of EDTA, β-mercaptoethanol, phenylmethylsulfonyl fluoride, and sodium bisulfite, and lysed by sonication with a Branson Sonifier 450 with a 10-s pulse at 50% power three times. Lysates were cleared by centrifugation at 18,000 × g and incubated with increasing amounts of CNBr-Sepharose coupled to the anti-EE monoclonal antibody for 1 h at 4°C with gentle rocking. Beads were washed three times with 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 5% glycerol, 0.04% Triton X-100 and then assayed for polymerase activity (60) with the following modifications. To the washed beads, 80 μl of assay mix was added, and the beads were gently rocked at 37°C for 30 min. The reactions were then terminated, precipitated by trichloroacetic acid, and the acid-insoluble products were quantified as described previously (60).

Co-immunoprecipitation of in vitro translated proteins was performed as follows. Defined amounts of reticulocyte lysate containing the labeled protein of interest were mixed together in 0.5 ml of 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10% glycerol, 2% bovine serum albumin and allowed to rock gently at 4°C for 30 min, after which 5 μg of antibody (either monoclonal anti-EE or polyclonal anti-p70) that had been pre-cleared by centrifugation in a TLS-55 at 100,000 × g for 30 min was added to the mixture. Incubation continued with rocking for 1 h, after which 20 μl of protein A-agarose (Sigma) was added and rocking continued overnight at 4°C. Beads were washed 4 times with 20 mM Tris-HCl (pH 7.5), 200 mM KCl, 5% glycerol, 0.2% Triton X-100, resuspended in 2× SDS loading buffer, and run on a 10% denaturing polyacrylamide gel. The gel was stained in “Amplify” (Amersham Pharmacia Biotech), and dried before being exposed to film.

Cell Extracts and Cell-free Replication Assays—Suspension 293 cells were maintained in Joklik's medium with 5% calf serum. The prepara-
tions of whole cell extracts was modified from previously published procedures. A fixed amount (0.5 ml) of protein A-agarose (Sigma) was added and rocking continued overnight at 4°C. Beads were washed 4 times with 20 mM Tris-HCl (pH 7.5), 200 mM KCl, 5% glycerol, 0.04% Triton X-100 and then assayed for polymerase activity (60). The gel was then assayed for polymerase activity (60) with the following modifications.

RESULTS

HPV-11 E1 Interacts with p180 Subunit of Human DNA Polymerase α—We have previously shown that the catalytic subunit of human DNA pol α, p180, physically interacts with BPV-1 E1 and that pol α/primase activity is required for the cell-free replication of BPV-1 and HPV-11 (3, 4, 51, 52). To test whether HPV-11 E1 interacts with p180, we initially infected Sf9 cells with either the recombinant baculovirus AcHDPα expressing the human DNA pol α p180 subunit (58) or a recombinant baculovirus expressing the HPV-11 E1 protein tagged with a Glu-rich epitope at the amino terminal called EE-E1 (4, 57), and we performed a chromatography with both viruses. The labeled proteins were then subjected to immunoprecipitation with anti-EE antibody-coupled Sepharose. Polymerase assays of the immunoprecipitates showed that a small but increasing proportion of the total polymerase activity in the lysate precipitated with increasing amounts of antibody-coupled Sepharose from cell lysates co-infected with AcHDPα and HPV-11 E1 expressing virus but not from either of the singly-infected cell lysates (Fig. 1A). The anti-EE immunoprecipitates from the singly- and co-infected insect cells were also analyzed on an SDS gel and stained with Coomassie Blue (Fig. 1B). An approximately 200-kDa insect cell protein was precipitated from all lysates, including uninfected control (lane 1). E1 protein was immunoprecipitated with anti-EE antibody in E1 recombinant baculovirus singly-infected cell lysates (lane 2). A 180-kDa protein was observed in the E1-recombinant virus and AcHDPα co-infected cell lysates (lane 4) and was not detected in the AcHDPα singly-infected lysates (lane 3). To ensure that the 180-kDa protein is indeed human pol α p180, the AcHDPα singly-infected cell lysate was immunoprecipitated with the monoclonal anti-pol α antibody SJK237. A protein of 180 kDa was immunoprecipitated (lane 5). In addition, the identities of the p180 and HPV-11 E1 proteins in the immunoprecipitates were confirmed through Western blotting (data not shown).

Together these results suggest that there is an interaction between the p180 subunit of human pol α/primase and E1 under physiological conditions in insect cells.

Since it is difficult to quantitatively control the relative levels of protein expression by these recombinant baculovirus in insect cells, we further tested the interaction by enzyme-linked immunosorbent assay (ELISA) with immunopurified p180 from AcHDPα-infected insect cells (63) and immunopurified EE-tagged HPV-11 E1 (4) as described under “Experimental Procedures.” A fixed amount (0.5 μg) of the highly purified p180 subunit was immobilized in the wells of an ELISA plate and incubated with increasing amounts of the purified E1 protein. Complex formation was detected using the monoclonal anti-EE antibody and a horseradish peroxidase-coupled goat anti-mouse secondary antibody. An increasing chromogenic signal was detected with increasing amounts of E1 (Fig. 1C). It has been previously shown by ELISA that the amino-terminal region of human DNA polymerase α p180 subunit from residues 195 to 313 interacts with SV40 large T antigen (50).

Therefore, five overlapping GST fusion peptides of p180 spanning the entire open reading frame of p180 that had been previously used for the T antigen interaction study were tested for their ability to interact with HPV-11 E1 in this assay. Contrary to the findings with SV40 T antigen-interacting amino-terminal fusion, none of the GST-human pol α fusion proteins, including the T antigen interacting amino-terminal fusion fragment (1x), yielded an equivalent signal to that of the full-length intact p180 (Fig. 1C). These results indicate that none of the GST-human pol α fusion proteins alone contains an intact interacting domain and the interaction requires a correctly folded full-length p180. Nonetheless, these GST-human pol α fusion proteins provide a negative control for the ELISA experiments. The positive signals seen between p180 and E1 by ELISA thus confirm that there is a physical interaction between these two proteins and that the interaction is specific. The region(s) of p180 necessary and sufficient for the interaction, however, is different from that required for interaction with SV40 T antigen.

The ELISA results led us to investigate further the possible interaction of HPV-11 E1 and p180 by fast protein liquid chromatography over a Superdex 200 column. The DNA polymerase activity of p180 alone eluted at a molecular mass of approximately 180 kDa, with a reproducible second peak at approximately 210 kDa. Upon preincubation of p180 with HPV-11 E1, which migrates as an 82-kDa protein in SDS-polyacrylamide gel electrophoresis (28), a portion of the polymerase activity shifted to an elution position corresponding to a molecular mass of 340–480 kDa with the peak at 340 kDa (data not shown). The shift of the polymerase activity from 180 kDa to a mass of 340–480 kDa with the peak at 340 kDa (data not shown).
higher molecular mass after preincubation with E1 again suggests that there is an interaction between HPV-11 E1 and the p180 subunit of human pol α.

The elution profile of the p180-E1 protein complex suggests that the interaction between E1 and p180 is not stoichiometric and perhaps more than one molecule of E1 interacts with one molecule of p180, in agreement with previous observations that E1 can exist as a multimer in solution (14, 20, 21, 23). Finding that only a portion of the p180 associated with E1 during gel filtration and a small percentage of the total polymerase activity co-immunoprecipitated from lysates of co-infected insect cells (data not shown) leads us to the hypothesis that E1

![Graph](image)

**Fig. 1.** p180 subunit of the human DNA polymerase α physically interacts with HPV-11 E1 protein. A, co-immunoprecipitation with anti-EE antibody of p180 and EE-tagged HPV-11 E1 from insect cell lysates that were singly or co-infected with recombinant baculovirus AcHDPα expressing p180 or recombinant baculovirus expressing EE-tagged HPV-11 E1 protein. DNA polymerase α activity in the immunoprecipitates was measured as described under "Experimental Procedures." B, SDS gel analysis of immunoprecipitates from A. Shown are the Coomassie stains of immunoprecipitates of insect cell lysates by anti-EE antibody. Lane 1, from uninfected cells; lane 2, from cells infected by HPV-11 EE-tagged E1 HPV-11 recombinant virus; lane 3, from AcHDPα-infected cells; lane 4, from insect cells co-infected with AcHDPα and HPV-11 EE-tagged E1 virus; lane 5, immunoprecipitate by monoclonal anti-polymerase α SJK237–71 antibody from AcHDPα-infected insect cells. p180 and E1 are marked with a dot. C, confirmation of E1 and p180 complex formation by ELISA. Half of a μg of the p180 subunit, five p180 GST fusion peptides, or p70 subunit were immobilized in the wells of an ELISA plate and incubated with the indicated amounts of purified EE-tagged E1. After washing, the bound EE-E1 proteins were detected with monoclonal anti-EE antibody with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody as described under "Experimental Procedures." Shown are the increasing chromogenic signals from which background levels of each amount of E1 incubated in the absence of respective proteins have been subtracted. The chromogenic signals of each protein were normalized as percentage of the maximum signal (A_405 = 0.18).
may exist as a multimer in solution. This hypothesis will be further addressed below. Together, these results indicate that HPV-11 E1, similar to SV40 T antigen and BPV-1 E1, is able to physically interact with the human DNA pol a catalytic subunit p180.

**Purified p180 Inhibits the HPV-11 Cell-free Replication Reaction**—We have previously shown that when increasing amounts of purified p180 were added to the cell-free SV40 replication reaction, a proportional inhibition of the replication was observed (50). Therefore we hypothesized that when exogenously added p180 was included in the HPV-11 cell-free replication reaction, inhibition of replication might also be observed, through the sequestration of either E1 or other replication proteins in the cellular extracts. To test these possibilities, we conducted an HPV-11 cell-free ori-dependent replication reaction in the presence of excess p180. Inhibition was observed when 250 ng of purified p180 was added to a standard reaction containing 12 ng of E1 and 8 ng of E2 (data not shown).

To determine whether inhibition is due to a competition for cellular replication factor(s) or to interference in the interaction between E1 and E2, replication assays were performed in the presence or absence of 8 ng of E2 with 20 or 40 ng of E1, as E2-independent replication only takes place at elevated E1 protein levels (4, 10). Replication produces slow-migrating replication intermediates and fast-migrating form I and form II circular molecules. As shown in Fig. 2, there were more replication products in the presence of the E2 protein than in its absence (compare lane 1 with lane 5 and lane 3 with lane 7).

Upon addition of excess p180, a reduction of both the E2-dependent and E2-independent replication levels was observed relative to the control reactions without exogenously added p180 (lanes 2, 4, and 8). This inhibition was not completely rescued by the addition of excess cellular extracts (data not shown), suggesting the effect is mediated at least in part through the viral proteins. These data indicate that p180 may be binding to and sequestering HPV-11 E1 away from the viral origin of replication, in an interaction similar to one that occurs between p180 and SV40 T antigen.

**The 70-kDa Subunit of pol a Interacts with HPV-11 E1**—The p70 subunit of human pol a has also been previously shown to interact with SV40 T antigen (53). Given the similarity in function between T antigen and E1 as well as the observed interaction between E1 and p180, we tested whether HPV-11 E1 also interacts with p70. An affinity of the EE epitope tag of E1 to Ni$^{2+}$-nitrilotriacetic acid resin and a moderate affinity of anti-EE antibody to the His-tag on E. coli-expressed recombinant p70 precluded the possibility of testing for an interaction between E1 and His-tagged p70 by co-immunoprecipitation. Therefore the interaction between HPV-11 E1 and p70 was first analyzed by ELISA. A fixed amount of recombinant His-tagged p70 protein purified from E. coli as described under “Experimental Procedures” was immobilized in the wells of an ELISA plate. As with p180, incubation with increasing amounts of E1 resulted in increasing chromogenic signals above the background values, indicative of complex formation between E1 and p70 (see Fig. 1C).

To verify the ELISA results, we further analyzed the interaction between E1 and p70 by chromatography over the SE1000/17 Bio-Rad column. Under the conditions used, E1 alone eluted at a molecular mass >669 kDa, suggesting that E1 exists as a large homocomplex (data not shown). Upon preincubation of equimolar amounts of E1 and p70, most of the p70 eluted as a 140-kDa complex indicative of a dimer of p70, similar to that seen with p70 alone and consistent with our previous observations5 (Fig. 3, A and B). However, there was a reproducible and distinct fraction of p70 which co-eluted with the multimeric E1 (Fig. 3B). To verify the presence of E1 in the p70-containing fractions, the immunoblot in Fig. 3B was stripped and reprobed with anti-EE antibody (Fig. 3C). This profile confirms the interaction between these two proteins.

The interaction was further substantiated by sedimentation analysis in 10–30% sucrose density gradient. E1 alone sedimented at a range between 300 kDa to a very large complex of >1000 kDa, indicating again that under these conditions HPV-11 E1 exists as multimers in solution (Fig. 4A). In the absence of E1, p70 sedimented as a dimer and was not detectable in the higher sucrose density fractions even upon overex-

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5 K. L. Conger and T. S. F. Wang, unpublished observation.
FIG. 4. p70 subunit associates with HPV-11 E1 during sucrose density gradient sedimentation. Sucrose gradient sedimentation analysis was performed as described under “Experimental Procedures.” Shown are aliquots of each fraction immunoblotted by their respective antibodies. Sucrose density of fractions are indicated under the elution profiles. A, sedimentation profile of EE-tagged E1 alone. B, sedimentation profile of p70 alone. C, sedimentation profile of p70 after preincubation with EE-tagged E1. Molecular mass standards: bovine serum albumin (68 kDa) sediments around 15.4% (w/w), catalase (232 kDa) around 19.0%, and thyroglobulin (669 kDa) around 23.8%.

Purified p70 Inhibits Cell-free HPV-11 Ori-dependent Replication—To evaluate whether the physical interaction between HPV-11 E1 and p70 is functionally significant in HPV-11 ori-dependent replication, we tested the effect of adding purified p70 to a standard cell-free reaction. Increasing amounts of purified p70 inhibited replication in a dose-dependent manner (Fig. 5, lanes 2–7). In contrast, addition of identical amounts of the recombinant p58/p49 primase subunit complex had little or no effect on replication, although it was expressed and purified from E. coli in an identical fashion as p70 (Fig. 5, lanes 2–4). This suggests that the primase subunits of DNA polymerase α do not interact with E1, and the inhibitory effect of p70 on HPV-11 cell-free replication is specific.

To investigate possible mechanisms of this inhibition, we conducted replication assays with a constant amount of E2 (8 ng) and increasing amounts of E1 (Fig. 6A) or, conversely, a constant amount of E1 (12 ng) and increasing amounts of E2 (Fig. 6B). In the absence of p70, increasing amounts of replication products were observed when the E1 concentration in the reaction was increased (Fig. 6A, lanes 1, 3, 5, 7, and 9), in agreement with our previous results (4). However, with the addition of p70, a similar reduction in replication products was observed (Fig. 6A, lanes 2, 4, 6, 8, and 10). In reactions with a constant 12 ng of E1, it appears that increasing, saturating, levels of E2 were able to reduce slightly the inhibition by p70 (Fig. 6B, lanes 2, 4, and 6).

p70 Does Not Interact with HPV-11 E2—One possible explanation of the above result is that p70 competes with E2 for binding to E1. If E2 is unable to bind E1 and bring it to the origin, replication would be inhibited. Increasing amounts of E1 should increase both the amount of E1 available for p70 binding as well as for binding to E2, resulting perhaps in a minimal rescue of replication. An alternative explanation for the slight rescue by E2 is that p70 may also bind to E2. Thus, at higher concentrations of E2, more E2 is available to bind to E1 and recruit it to the origin, resulting in a decrease in inhibition.

To distinguish between these two possibilities, we analyzed the ability of p70 to bind E2 by gel filtration on a Superose 6 column. As shown in Fig. 7, both the 43-kDa E2 protein and the p70 subunit eluted as homodimers (Fig. 7, A and C). Upon preincubation of equimolar amounts of E2 and p70, neither E2 nor p70 shifted to an elution volume of a larger complex (Fig. 7, B and D). This result rules out the possibility that p70 is sequestering E2 away from the productive reaction. Together
with the data in Fig. 6B, it suggests that p70 may be competing
with E2 for binding to E1 and that the inhibition by p70 may be
due to this competition.

**p70 Stimulates the E2-independent Replication Activity of**
**E1—**If the above hypothesis is correct, no inhibition by p70
should be detected under conditions allowing E2-independent
replication. However, if the inhibitory effect of p70 is due to
interference by p70 in an interaction between E1 and the pol
α/primase holoenzyme or other host factors, p70 inhibition
should persist in E2-independent replication reactions. To dis-
tinguish between these two possibilities, we tested the effect of
excess p70 in E2-independent replication reactions.

Elevated amounts of E1 (required in the absence of E2) were
used in reactions with either an ori-containing plasmid
(pUC7874-99) (Fig. 8A, lanes 1–8) or pUC-19 (lanes 9–12). In
the control reaction with 8 ng of E2, replication was inhibited
by the addition of 250 ng of purified p70 (Fig. 8A, compare
lanes 1 and 2, and lanes 3 and 4), consistent with the above results.
Interestingly, in the absence of E2, the addition of p70 stimulated
the replication of both the ori-containing template and
pUC-19 in an identical fashion (Fig. 8A, compare lanes 5–12).
Thus, in the absence of E2 but in the presence of high concen-
trations of E1, p70 stimulates replication in both ori-dependent
and ori-independent replication.

To gain further insight into the opposing effects of p70 on
HPV-11 replication under different conditions, we compared
the relative efficiency of E2 and p70 to stimulate ori-specific
replication at levels of E1 that replication by E1 alone is very
inefficient. In a reaction containing 10 ng of E1, no replication
was observed in the absence of E2 (Fig. 8B, lane 1), but exten-
sive replication took place upon the addition of 8, 16, and 32 ng
of E2 (Fig. 8B, lanes 9–11). In contrast, the addition of up to
250 ng of p70 failed to stimulate replication at 10 ng of E1 (Fig.
8B, compare lanes 2–4 to lane 1). When 20 ng of E1 protein
were used in the reactions, a low level of E2-independent rep-
lication was observed (Fig. 8B, lane 5). At this concentration of
E1, the addition of p70 also stimulated the replication activity
(Fig. 8B, lanes 6–8) but not nearly as efficiently as the E2
protein in the presence of 10 ng of E1 (Fig. 8B, lane 9). Interest-
ingly, the level of stimulation decreases at the highest amount
of p70 added (Fig. 8B, lane 8), suggesting that the stoichiome-
try between the two proteins may be important. This point will
be addressed in greater detail below. These results demon-
strate that the stimulation by p70 is rather weak relative to
that by E2.

**p70 Competes with E2 to Bind E1—**In order to evaluate
better the relative interactions between p70, E1, and E2, we
utilized radioactively labeled, in vitro transcribed and trans-
lated proteins. p70 was constructed with a Myc tag to avoid the
cross-reactivity between the anti-EE antibody and the histi-
dine tag on the recombinant p70. Due to internal initiation
sites within the Myc tag, p70 appears as a triplet, whereas E1 is
translated as a doublet (Fig. 9A and B). Immunoprecipita-
tion of the anti-EE antibody precipitates E1 and the associ-
ated p70 protein (Fig. 9B, lanes 3–5). Paradoxically, more E1 is
immunoprecipitated by the anti-EE antibody in the presence of

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**Fig. 6.** Inhibition of the cell-free replication reactions by the p70 subunit is independent of E1 concentration but partially
alleviated by elevated E2 levels. HPV-11 ori-dependent cell-free replication reactions were performed as described under “Experimental
Procedures.” A, increasing amounts of E1 protein as indicated were mixed with 8 ng of E2 proteins in the absence (odd-numbered lanes) or the
presence (even-numbered lanes) of 250 ng of p70 protein. B, increasing amounts of E2 protein as indicated were added in reactions with 12 ng of
E1 in the presence (even-numbered lanes) or in the absence (odd-numbered lanes) of 250 ng of the p70 subunit. R.I., replication intermediates.

**Fig. 7.** p70 was unable to associate with HPV-11 E2 protein during gel filtration. Two μg of p70 and E2 each were preincubated together
and fractionated on a Superose 6 PC 3.2/30 column as described under “Experimental Procedures.” Proteins in each fraction were analyzed by
immunoblot using anti-p70 polyclonal antibody and anti-E2 polyclonal antibody.
p70 than in its absence (Fig. 9B, compare lanes 2, 3, and 4). Furthermore, this effect is dependent on a particular stoichiometry of the two proteins, as the higher amounts of p70 caused a reproducible decrease in the amount of E1 immunoprecipitated (Fig. 9B, compare lanes 4 and 5). Perhaps the multimeric conformation of E1 is stabilized by the inclusion of p70 in the reaction, allowing each antibody molecule to precipitate multiple molecules of E1 protein. Alternatively, p70 may induce a conformational change in E1 that makes the EE tag more accessible. This effect may help in part to explain the stimulation of E2-independent replication by p70 seen in Fig. 8. The reduction in precipitable E1 at a high ratio of p70 to E1 seen in Fig. 9B (lane 5) appears to parallel the reduced stimulatory effect by p70 on E2-independent replication described in Fig. 8B (lane 8) and may emphasize the importance of the dual role played by p70 in viral replication initiation in our assays. However, the nature of the in vitro transcription and translation reactions and the fact that p70 and E1 are radiolabeled with different isotopes preclude reliable quantification of the relative amounts of E1 and p70 in this assay.

To assess the potential competition between p70 and E2 for E1 binding, we performed co-immunoprecipitation of E2 in the presence of E1 using the anti-EE antibody. Increasing amounts of unlabeled His-tagged p70 were preincubated with in vitro translated labeled E1 protein prior to the addition of E2. Interaction between E1 and E2 is decreased at 20 ng of p70 (Fig. 9C, compare lane 1 to lane 2). This reduction suggests that p70 competes with E2 for binding to E1. Again, at high amounts of p70 (100 and 400 ng), lower levels of E1 were precipitated, similar to that observed in Fig. 9B, lane 5.

Results of these experiments indicate the following: (i) p70 inhibits an HPV-11 cell-free replication reaction that contains optimal levels of E1 and E2 and the ori-containing template; (ii) in the absence of E2, p70 stimulates, rather than inhibits, the replication activity of E1, and this stimulation is not dependent on the presence of an HPV-11 origin; (iii) stimulation by p70 of the replication reaction requires higher levels of E1 than stimulation by E2, but the level of stimulation achieved is much lower than that by E2; (iv) p70 does not physically interact with E2; and (v) excess p70 can interfere with the interaction between E1 and E2. Collectively, these results strongly suggest that the apparent inhibitory effect of p70 on replication reactions containing both E1 and E2 proteins may be due to competition between p70 and E2 for binding to E1.

**DISCUSSION**

In this study, we have shown by ELISA, gel filtration, sucrose density gradient sedimentation, and co-immunoprecipitation that HPV-11 E1 can exist as a multimeric complex in solution and can physically interact with two DNA pol α/primase subunits, p180 and p70. Furthermore, the HPV-11 cell-free replication assay demonstrates that both of these interactions are functionally significant. Here we discuss these interactions and propose a model of how these proteins participate in initiation of viral DNA replication.

E1 as well as other viral helicases has been postulated to function in large oligomeric complexes (for review see Ref. 64). Electron microscopy revealed that HPV-11 E1 exists in solution as particles of different sizes. When bound to ori, only particles of hexameric sizes were observed. However, in the presence of Hsp40, the majority of the DNA-bound E1 is a dihexamer (23). In this study, we observed that HPV-11 E1 exists as a large multimer in solution (Figs. 3 and 4), in excellent agreement with the previous observations. Thus, in the absence of E2 and DNA, E1 may exist as a mixture of monomeric and oligomeric complexes. This equilibrium could be affected by many factors, including protein concentration, the presence of DNA, or the interacting viral or host proteins. Perhaps the high concentrations of E1 protein, trace amounts of DNA in our E1 preparations, or buffer conditions used in the studies favor the formation of oligomeric complexes. Collectively, our results suggest that a dynamic interplay occurs between E1, E2, DNA, and the DNA polymerase α holoenzyme during the initiation of viral DNA replication.

The interactions between E1 and the DNA pol α/primase subunits, p180 and p70, appear to be of a different nature. Gel filtration and sucrose density gradient experiments suggest that many more molecules of E1 are present in the complex than either p180 or p70, indicating the two cellular DNA polymerase subunits are able to associate with E1 in its multimeric conformation. It has been reported that the hexameric T antigen must be assembled on the origin-containing DNA for productive interactions to occur, whereas pre-formed T antigen hexamer in solution is replication-incompetent (65). Similarly, the E1 multimers alone or the E1 multimers complexed with p180 or p70 formed in solution may not be replication-competent.

**p180 and p70 Exhibit Differential Effects in HPV-11 Cell-free**
Replication—We have shown in this study that exogenously added p180 inhibits cell-free HPV-11 viral replication. This inhibition is similar to that seen in the SV40 in vitro replication reaction in the presence of excess p180 (50) and is independent of E2 function (Fig. 2). Increasing amounts of cellular extracts were not able to completely rescue replication, indicating that E1 is limiting. The simplest explanation for the inhibition is that p180 sequesters the E1 oligomers, shifting the equilibrium between monomeric E1 and oligomeric E1, so that there is less E1 available for the formation of a productive pre-replication complex on the ori.

We observed two opposing effects when exogenously added p70 was included in the HPV-11 cell-free replication reaction. In the presence of both E1 and E2, p70 inhibited replication in a manner similar to the inhibition by p180 (compare Fig. 2 with Fig. 5). Increasing amounts of E1 could not alleviate this inhibition (Fig. 6A). In contrast, increasing levels of E2 in the replication reaction may partially restore replication activity (Fig. 6B). One explanation for this effect supported by our experimental data is that p70 and E2 may compete for binding to the E1 protein (Fig. 9C), either through an overlapping recognition site on E1 or through a conformational change that precludes simultaneous binding by E2 and p70. During the preparation of this manuscript, Masterson et al. (66) reported an interaction of HPV-16 E1 with only the p70 subunit of DNA polymerase α/primase but not the p180 subunit. Their results also suggest that HPV-16 E2 competes with p70 for binding to HPV-16 E1. Increasing E1 concentration would increase both the highly replication-efficient E1-E2 complex and a less replication-efficient E1-p70 complex, resulting in no apparent increase in replication. The fact that the exogenously added p70 is in excess in the reaction and the unique stoichiometry-dependent interactions between the two molecules observed in Figs. 8B and 9B may also explain why increasing amounts of E1 are unable to overcome replication inhibition. In contrast, an increase in E2 levels would tip the balance toward the formation of replication-competent E1-E2 complexes, leading to a slight restoration of activity. An alternative explanation is that although p70 and E2 do not physically interact in vitro (Fig. 7), it is possible that E2 may recruit other host proteins to the ori-bound E1 (26). The exogenous p70 may somehow interfere with these interactions between E2 and host proteins, inhibiting the assembly of the preinitiation complex.

Stimulation of the cell-free replication by p70 in the absence of E2 at elevated levels of E1 (Fig. 8) suggests that the mechanism of p70 stimulation of E2- and ori-independent replication is different from that employed by E2. First, E2 stimulates ori-specific replication and represses ori-independent replication (4). Second, E2 stimulation is much stronger than that achieved by p70 (Fig. 8B). Third, the stimulatory effect of p70 is observed only at a concentration of E1 where E2-independent replication occurs (Fig. 8). In the presence of E2, the two proteins compete for binding to E1, and the presence of p70 at these low levels of E1 inhibits replication activity. However, the
exact cause of the stimulation of replication by p70 remains to be investigated.

A Proposed Model for Replication Initiation—The possibilities presented above for stimulation and inhibition of replication activity by p70 are not mutually exclusive. Together with previous studies of both BPV-1 and HPV E1 and E2 interactions we suggest a model of sequential associations between viral and host proteins during normal viral origin replication.

We propose that E1 exists in solution in a dynamic equilibrium of monomers and multimeric complexes. The high affinity of E2 for the E2-binding site allows it to recruit E1 to the ori at relatively low E1 concentrations. Interactions between E1 and the pol α holoenzyme subunits may then displace E2 and replication is initiated.

In the absence of E2, elevated levels of E1 can multimerize on DNA nonspecifically and initiate replication, albeit in a very inefficient manner. We propose that the exogenous p70 forms nonproductive complexes with E1 multimers in solution. But the small amounts of E1 that successfully assemble on the DNA are stabilized by the exogenous p70, allowing subsequent productive interactions with the DNA pol α/primase to occur. Thus, p70 was able to stimulate replication weakly but only at higher E1 concentrations (Fig. 8). However, the stimulatory effect is much lower than that seen with E2 (Fig. 8), perhaps due to the relatively inefficient binding of E1 to the DNA in the absence of E2 or to the important role of E2 involvement in pre-initiation complex formation (26). In the presence of E2, the exogenous p70 competes with E2 for binding to E1. The formation of a non-productive p70-multimeric E1 complex in solution then results in a decrease in the amount of highly productive E1-E2 complex, inhibiting replication.

Overall, the elucidation of the multiple interactions among viral and host proteins may aid in the understanding of the initiation of chromosomal replication as well. Given the important role played by the p70 subunit of human DNA polymerase α, it will be interesting to investigate the protein partners of p70 in an uninfected cell.

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