Cell-free Replication of the Human Papillomavirus DNA with Homologous Viral E1 and E2 Proteins and Human Cell Extracts*

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We have established the first homologous cell-free DNA replication system for a papillomavirus. The replication of the human papillomavirus type 11 (HPV-11) origin was achieved by using human 293 cell extracts supplemented with the HPV-11 E1 and E2 proteins purified from insect cells infected with recombinant baculoviruses. Efficient replication depends on the HPV-11 origin, the HPV-11 E1 and E2 proteins, as well as human DNA polymerase α, replication protein A, topoisomerase I, and topoisomerase II. High concentrations of E1 protein also promoted a low level of origin-independent DNA replication which was suppressed by the addition of the E2 protein, whereas E2 protein stimulated origin-dependent replication. We also show that an intact E2 protein binding site was absolutely necessary for origin activity, as a strong HPV-11 origin was rendered inactive when one half-site of each of the three E2 binding sites was mutated. In contrast, there was only a relatively small reduction in this mutant origin activity when the cell extracts were supplemented with the bovine papillomavirus type 1 (BPV-1) proteins. These results suggest that the HPV-11 E2 protein plays a primary role in HPV origin recognition. Furthermore, unlike transient replication in which HPV-11 and BPV-1 viral proteins promote efficient replication of homologous and heterologous origins, efficient cell-free replication took place only with the homologous combinations.

Small DNA viruses have long been used as models for higher eukaryotic DNA replication. SV40 is one of the most thoroughly studied DNA viruses, and the functions of both viral and host transacting factors and enzymes have been identified (Refs. 1 and 2; for reviews, see Refs. 3 and 4). The unchecked replication of these viruses, however, does not reflect the regulated host DNA replication that occurs once per cell cycle. Human papillomaviruses (HPVs), 1 which infect mucosal or cutaneous epithelium and cause hyperproliferation, have two distinct modes of DNA replication. In the basal stem cells and the parabasal layer of the epidermis and endocervix there is a proto-oncogene that produces the viral genome as a plasmid. In the upper layer keratinocytes undergoing terminal differentiation does vegetative amplification take place (5–9). Thus papillomaviruses offer a unique opportunity to study regulated DNA replication. However, HPV-1 cannot be propagated in conventional tissue cultures. When transfected into a variety of cell types, virtually all the HPV DNA is either lost or becomes integrated into host chromosomes. Only in rare transfected primary human keratinocytes does the cloned HPV DNA plasmid remain extrachromosomal for a limited period of time (10). Thus it has been difficult to study either mode of replication.

By transient transfection, we and others (11–13) have recently shown that two HPV proteins, E1 and E2, are essential for origin (ori)-specific DNA replication. This requirement is analogous to that for bovine papillomavirus type 1 (BPV-1) in transiently transfected cells (14) and in cell-free replication using extracts from mouse FM3A cells supplemented by BPV-1 proteins purified from insect Sf9 cells (15). Unlike the stringent species specificity demonstrated in cultured cells by other members of the papovavirus family, SV40 and polyomavirus, and the strict host species specificity of papillomaviruses in natural infections, mixed and matched HPV and BPV E1 and E2 proteins can replicate the ori's of many types of HPVs as well as BPV-1 and cottontail rabbit papillomavirus in transient replication assays performed in human, monkey, and rodent cells (11). These results indicate a high degree of similarity among the E1 and E2 proteins and the ori sequences of all papillomaviruses, as well as promiscuity in the ability of the viral proteins to engage the mammalian DNA replication machinery, regardless of species.

The E2 open reading frame of HPVs or BPV-1 encodes several proteins, each of which binds to a consensus origin-binding sequence of ACGN, GT (E2BS or EBBS) and regulates viral transcription (for reviews, see Refs. 16 and 17). Only the full-length E2 protein can support viral DNA replication, whereas alternative E2 proteins lacking the amino-terminal transactivating domain do not, or even act as repressors (15, 18, 19). The BPV-1 E1 protein binds to an imperfect palindrome of 18 base pairs long (ElBS) in the BPV-1 ori (14, 15, 20–23) and is a DNA-dependent ATPase and ATP-dependent helicase (24, 25). The minimal BPV-1 ori contains the E1BS, half a low affinity E2BS, and an AT-rich region in the upstream regulatory region (15, 26). However, an intact E2BS is necessary for BPV-1 ori activity if the distance between E1BS and E2BS in the minimal ori was lengthened or if the affinity between E2BS and E2 protein was reduced (27).

We and others (18, 28, 29) have localized the minimal ori's in HPVs to a region of the viral genome comparable with the BPV-1 ori. It consists of one or more copies of E2BS, an AT-rich region and a putative E1BS. The E1 protein of various HPV types shares considerable homology with the BPV-1 E1 protein. An immunoprecipitated HPV-11 protein expressed from recombinant baculovirus exhibits ATPase activity (30) and a bacteri-

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The abbreviations used are: HPV, human papillomaviruses; BPV, bovine papillomaviruses; mAb, monoclonal antibody; RI, replication intermediates.
ally expressed HPV-6b E1-maltose fusion protein possesses helicase and ATPase activities (31). As in the BPV-1 ori, the putative E1BS of many HPV types is flanked by E2BS and has several base pair mismatches. The binding of an HPV E1 protein to the HPV ori has not been demonstrated directly with purified protein. Interestingly, the putative E1BS can be mutated or deleted and the ori still functions in transient replication (28, 29) in sharp contrast to that of BPV-1, for which insertion mutations in the E1BS completely ablate ori activity (15, 22, 26).

Attesting to the importance of the E2BS in HPV-11 ori function, the efficiency of replication increases with their copy number (13, 18). We recently found that one or more synthetic E2BS can serve as an ori in transient replication, albeit with much reduced activity, indicating that cis-elements other than the E2BS in the upstream regulatory region can increase replication efficiency. To identify these elements, we examined the mechanisms of ori recognition and to verify the results in transfected cells, is necessary to establish cell-free replication with purified HPV proteins. We report in this work the first homologous cell-free replication system for a papillomavirus by using the HPV-11 ori, extract from human 293 cells, and HPV-11 E2 and E1 proteins purified from a baculovirus expression system. We demonstrate that ori-specific replication requires E1 and E2 proteins and host proteins known to be required for cell-free replication of SV40 and BPV-1. Our results show both similarities and to differences from BPV-1 cell-free replication and transient replication in transfected cells. We also note some differences in the ability of HPV-11 or BPV-1 E1 and E2 proteins to replicate different templates that have important implications concerning the role of the HPV E2 protein.

MATERIALS AND METHODS

Plasmids—Plasmids p7730-99 and p7730-99-234 were obtained by isolating the HindIII fragments from 14-0 and 14-0-234 (32), filling in the staggered ends with the Klenow fragment of E. coli DNA polymerase I, and cloning into the HindIII site of pUC-19. Clone 14-0-1 contains the wild type HPV-11 upstream regulatory region sequence spanning nucleotides 7730-7933/1-99, whereas 14-0-234 contains the same genomic segment except that one half-site of each of the three E2BS (numbers 2, 3, and 4) was mutated and no longer binds HPV-11 E2 (28, 29) (E2 protein 28). To construct plasmids p7730-99 and p7730-99-234, HindIII fragment 7981-92 was cloned into pUC19 at the HindIII site described (26). Synthetic oligonucleotides (CCTACCATGGAGGAA-

GAGAGTATGAGCATCCGGGGCTCTGCACCC and complementary strand) that introduce an efficient eukaryotic translation initiation sequence (35), a 27-base sequence encoding the polyoma middle T antigen EE-epitope (34) and a BamHI site at the 3’ end were cloned into the HindIII site of pUC19 to generate pUC-EE. The Xbal-BamHI fragment of pUC-EE was transferred into pBluescript-SK(+) to produce pBS-EE. A fragment of DNA containing the HPV-11 E1 open reading frame flanked by a BamHI site at the 5’ end and a HindIII at the 3’ end was generated by polymerase chain reaction and cloned into pBS-EE to generate pBS-EE-E1. The XbaI-HindIII fragment from pBS-EE-E1 was recloned into the baculovirus transfer vector pVL1393 (Invitrogen, San Diego, CA) to produce pVL-EE-E1. A baculovirus transfer vector containing the E2 open reading frame (pVL-E2) was prepared by inserting the HPV-11 E2 cDNA (812-847-2622-4400) (35) into pVL1393. To prepare an affinity column for the purification of the E2 protein, plasmid p7-E2BS(7) containing a tandem copy of the E2BS was generated by cloning ligated complementary synthetic oligonucleotides: 5’ AATTCCAGC-

GGCTGCAT 3’ and 5’ GGCCGACAGGACCTGTAAATTCCAGC 3’ with one E2BS underlined) into the EcoRI site of pGem-7 (36).

Expression and Purification of Full-length E2 and E1 Proteins from the Recombinant Baculovirus Expression System—Insect cells Sf9 (ATCC) were routinely maintained in TNM-FH medium (38) with 10% fetal bovine serum and the High Five™ cells (Invitrogen) in EX-Cell 406 medium (37) at 27°C. To generate recombinant baculoviruses, 2 μg of plasmid pVL-E2 or pVL-EE-E1 together with 1 μg of wild type baculovirus DNA were cotransfected into the insect cells Sf9. The identification and purification of recombinant baculovirus by plaque assays were as described (36).

Proteins were prepared from the nuclear fraction by adapting published methods (37, 38) as described below. Western blotting with E2 or E1 polyclonal antibodies were used to monitor protein fractions through the purification process. Alternatively, infected cells were harvested 48-72 h post-infection by centrifugation, washed with phosphate-buffered saline, resuspended in buffer A (10 mM HEPES-K, pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithioreth NULL (DTT), and then lysed using a Dounce homogenizer. The nuclear pellet was resuspended in buffer C (20 mM HEPES-K, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.1 mM EDTA, 0.5 mM DTT, 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin). Soluble E2 and E1 proteins were recovered after sonication and further purified as follows.

To purify the HPV-11 E2 protein, a DNA affinity Sepharose column was prepared by conjugating multimerized E2BS heptamer purified from p7-E2BS(7) to CNBr-activated Sepharose CL-4B (Pharmacia Biotech Inc.) according to Kadonaga and Tjian (39). The crude soluble E2 protein preparation was first purified by passing it through a heparin-Sepharose column (Bio-Rad Econo-Pac) before loading onto the E2BS DNA affinity column. The bound E2 protein was eluted with buffer E (100 mM HEPES-K, pH 7.5, 0.15 mM MgCl2, 1.5 mM EDTA, 500 mM NaCl, 0.5 mM DTT, 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin). The purity of the E2 protein was determined by Coomassie Blue staining. The protein concentration was determined to be 100 ng/μl by Bradford assay, and aliquots were stored at −70 °C.

Mammalian Cell Lines—Monolayer 293 cells (human embryonal kidney cells transformed by adenovirus type 5 E1A and E1B) were grown in Dulbecco's modified Eagle's medium with 10% calf serum. Monolayer HeLa cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and suspension HeLa cells in Joklik's medium (Life Technologies, Inc.) with 5% calf serum.

Preparation of Mammalian Cell Extracts and Cell-free Replication Systems—Extracts from 293 cells or HeLa cells were prepared according to Li and Kelly (41) and Stillman and Gluzman (42). The cell-free replication assay was adapted from methods described by these investigators and by Yang et al. (15). Briefly, a 25-μl reaction mixture containing the purified HPV-11 E1 protein, the E2 protein, DNA template and amounts specified in each experiment, and a fixed amount (100 μg of 293 cells extract (approximately 10 mg/ml) and 20 mM HEPES buffer, pH 7.5, 200 μM each UTP, GTP, CTP, and 4 mM ATP, 100 μM each dATP, dGTP, dTTP, and 25 μM dCTP, 40 mM phosphocreatine, and 100 μg/ml creatine phosphokinase (Sigma). In some experiments, extracts from HeLa cells (100 μg) or BPV-1 EE-E1 and E2 proteins, kindly provided by Dr. Michael Botchan (15), were used. 25 μM each of l-[3H]GTP (3000 Ci/mmol, Amersham Corp.) were added either at the beginning of the incubation or 1 h into the incubation. The reaction mixture was incubated at 37 °C for various times as indicated in each figure legend. In some experiments, antibodies to or inhibitors of various proteins were added as specified in the legend to Table 1. Replication was terminated by adding 200 μl of 20 mM Tris-Cl, pH 7.5, 10 mM EDTA, 0.1% SDS, and 20 μl RNase A, followed by incubation at 37 °C for 15 min. Protease K was then added to 200 μg/ml and incubation continued for another 30 min at 37 °C. The reaction mixture was extracted with phenol and chloroform-isooamyl alcohol (24:1), precipitated with 2.5 μl ammonium acetate, 70% ethanol, and analyzed by 0.8% agarose gel electrophoresis. Gels were stained with ethidium bromide, photographed, dried, and exposed to x-ray film for 8-16 h and quantitated using a PhosphorImager (Molecular Dynamics).

Expression and Inhibitors—Rabbit polyclonal antibodies against HPV-11 E1 and E2 have been described (11, 43). HPV-11 E1A-specific polyclonal antibodies were generated in rabbit with a TrpE-E5a fusion
RESULTS

Functional Test of HPV-11 Epitope-tagged E1 Protein in Mammalian Cells—To facilitate protein purification, we chose to tag the HPV-11 E1 protein at the amino terminus with the EE-epitope derived from polyomavirus middle-T antigen because similarly tagged BPV-1 E1 protein functions in cell-free replication (15). To confirm the expression of the EE-epitope-tagged HPV-11 E1 protein, we cloned it into the mammalian cell expression vector pMT-2 (45), as was previously done for the wild type E2 protein (11) and transfected into COS-7 cells (monkey CV-1 cells transformed by SV40 T antigen). We also examined its ability to support transient replication of HPV-11 ori reporter plasmid p7730–99 in human 293 cells cotransfected with HPV-11 E2 protein expression vector, as described (11). Western blots showed that EE-E1 was expressed at 20–30% the amount of the wild type E1 protein. It also supported replication at a level 20–30% of that promoted by the wild type E1 protein (data not shown). These results indicate that the epitope tag did not significantly alter the ability of the E1 protein to replicate the HPV-11 ori.

Cell-free Replication of the HPV-11 Origin—HPV-11 E1 and E2 proteins were purified from S9 or High Five™ cells infected with recombinant baculoviruses. SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining showed that the El protein (data not shown). These results indicate that the E2 proteins were purified from pCi of [cP~'P]~CTP. The reaction products were purified and pure (data not shown). The HPV-11 ori plasmid p7730–99, which spans nucleotides 7730–99 and contains three copies of the E2BS flanking a putative E1BS. The negative control was the cloning vector pUC-19, which did not replicate in transfected cells in the presence of either BPV-1 or HPV-11 proteins (14).

A time course of reaction was carried out by using 50 ng of template DNA, 80 ng of E1 protein, 20 ng of E2 protein, and 100 µg of total protein from 293 cell extracts in the presence of 2.5 µCi of [α-32P]dCTP. The reaction products were purified and separated by neutral agarose gel electrophoresis. The relative intensities of the reaction products, as determined using a PhosphorImager, are shown in Fig. 1. In the first hour of incubation, a low level of radioactivity was incorporated into Form I and Form II DNA when either template was used. We attribute the majority of this incorporation to repair synthesis due to the presence of a small amount of damaged template DNA. This repair synthesis took place without appreciable lag time, in the absence of HPV-11 proteins, and without producing detectable slow migrating replication intermediates (RI) (data not shown, but see Fig. 3, lanes 1 and 11, and Fig. 5A, lanes 11–15). RI consists of a mixture of θ form, gapped Form I, and catenated topoisomers that have mobilities between the slow migrating catenated open circles and the late θ form and the fast migrating catenated supercoils (46, 47). After the first hour of incubation, [α-32P]dCTP was incorporated into the ori+ plasmid, mostly in the RI and Form I DNA, and the amount of incorporation continued to increase and reached a plateau in another hour. In contrast, relatively little additional [α-32P]dCTP was incorporated into the ori- template in the second hour. Because of the different kinetics of the ori-independent repair synthesis and ori-dependent replication (Fig. 1), in all subsequent experiments [α-32P]dCTP was added after the first hour of incubation, and reactions were allowed to continue for another hour before termination. This protocol minimized the [α-32P]dCTP incorporation due to the nonspecific repair synthesis and facilitated quantitation of ori-specific replication products. We then carried out a series of experiments to examine the effects of varying the concentrations of the templates or viral proteins in the presence of the same fixed amount (100 µg) of 293 cell extract. In all experiments, ori+ and ori- plasmids were compared side by side to ensure specificity. Several of the experiments are presented below.

Effects of DNA Template Concentration—We tested replication efficiency with increasing amounts of DNA template in the presence of 60 ng of E1 protein and 8 ng of E2 protein. The results are presented in Fig. 2. When ori+ DNA was increased, [α-32P]dCTP incorporation into RI as well as Form I DNA increased and reached a maximum at 40 ng of template (Fig. 2A, lanes 1–5, and Fig. 2B). In contrast, replication of ori- plasmid was not apparent until the template was increased to 50 ng (Fig. 2A, lanes 6–10, and Fig. 2B).

Effects of the E1 and E2 Protein Concentrations—Using 40 ng of templates, no replication intermediates were detected in the absence of E1 and E2 proteins (Fig. 3A, lanes 1 and 11) or in the presence of E2 protein alone (lanes 2 and 12). In the absence of the E2 protein, HPV-11 E1 protein at 90 ng or higher supported a low level of ori+ plasmid replication (lanes 5 and 6). A similar and low level of ori- plasmid replication was not observed until the E1 protein was present at 120 ng or higher (lane 16 and data not shown). Addition of 8 ng of the E2 protein stimulated replication of the ori+ plasmid to a wide range of E1 protein tested (Fig. 3A, lanes 2–10). For example at 60 ng of E1, a 12-fold stimulation was observed (Fig. 3B). In contrast, the replication of the ori- plasmid was strongly suppressed by the addition of the E2 protein (compare lanes 16 and 20 of Fig. 3A and Fig. 3B).

Having demonstrated that ori-dependent replication required the presence of the E2 protein, we then tested the effects of varying the amount of E1 and E2 proteins in the presence of 60 ng of E1 and 40 ng of template (Fig. 4A, lanes 1–4). RI and daughter Form I molecules increased with increasing amounts

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3 T. F. Ho, L. T. Chow, and T. R. Broker, unpublished results.

![Fig. 1. Cell-free replication of HPV-11 origin-containing plasmid](image-url)
The amounts of viral proteins are comparable with those determined for the same gel with ethidium bromide, and the replication intermediates continued for another hour. Form I and Form I1 were used in lanes 6–10 except the template was the ori+ pUC-19. The quantitative results from PhosphorImager analysis are shown in B, and reactions without viral proteins (lanes 1 and 11) were set as zero intensity.

\[ \text{Form I} \]

A.

\[ \text{Form II} \]

\[ \text{R.I.} \]

**Fig. 2. Effects of DNA concentration on cell-free replication of ori+ and ori− templates.** A, 10–50 ng of p7730–99 (ori+) (lanes 1–5) or pUC-19 (ori−) (lanes 6–10) were tested in a cell-free replication assay in the presence of 60 ng of HPV-11 E1 protein, 8 ng of HPV-11 E2 protein, and 100 µg of human 293 cell extracts in the presence of 2.5 mCi of [α-32P]dCTP added at the end of the first hour, with the reaction continued for another hour. Form I and Form II DNA, identified by staining the same gel with ethidium bromide, and the replication intermediates (R.I.) were indicated. B, the relative α-32P incorporation intensity of each reaction was quantitated using a PhosphorImager.

The results are summarized in Table I. Specifically, HPV-11 ori+ replication was severely inhibited by a mAb against the proliferating cell nuclear antigen (PCNA), which is an accessory protein for DNA polymerase δ, and also by a mAb against human RP-A, which is a single-stranded DNA-binding protein. Replication was also inhibited by aphidicolin, an inhibitor of DNA polymerase α, δ, and ε. Camptothecin and etoposide, inhibitors of topoisomerases I and II, respectively, reduced replication.

**Fig. 3. Effects of E1 protein concentration in the presence or absence of E2 protein on ori+ and ori− cell-free replication.** Each reaction mixture contained 100 µg of 293 cell extract and 40 ng of template. A, p7730–99 in the absence of any viral protein (lane 1), in the presence of 8 ng of E2 alone (lane 2), 30, 60, 50, and 120 ng of E1 alone (lanes 3–6), or additionally 8 ng of E2 (lanes 7–10). Identical conditions were used in lanes 11–20 except the template was the ori− pUC-19. The quantitative results from PhosphorImager analysis are shown in B, and reactions without viral proteins (lanes 1 and 11) were set as zero intensity.

Effects of Antibodies and Inhibitors Specific for Replication Proteins—To substantiate that HPV-11 E2 protein, which was 30–50% pure, was necessary for the observed HPV ori replication, we tested the effects of anti-E2 polyclonal antibody. One pl of anti-E2 antisera was incubated with the mixture of E1, E2, and cellular extracts on ice for 15 min before DNA template and reaction buffer were added. Ori+ replication was reduced to 14%. In contrast, a polyclonal antibody raised against HPV-11 E5a protein had little effect (Table I).

To identify the host enzymes involved, we tested the effects of several mAbs against host replication proteins as well as several inhibitors specific for host RNA polymerases, DNA polymerases, or topoisomerases (for reviews, see Refs. 48 and 49). The results are summarized in Table I. Specifically, HPV-11 ori replication was severely inhibited by a mAb against the proliferating cell nuclear antigen (PCNA), which is an accessory protein for DNA polymerase δ, and also by a mAb against human RP-A, which is a single-stranded DNA-binding protein. Replication was also inhibited by aphidicolin, an inhibitor of DNA polymerase α, δ, and ε. Camptothecin and etoposide, inhibitors of topoisomerases I and II, respectively, reduced replication.
Fig. 4. Effects of E2 protein concentration on ori+ and ori− cell-free replication. A, 2-16 ng of E2 protein were added to 60 ng of E1 protein, 100 ng of 293 cell extracts, and 40 ng of ori+ p7730-99 (lanes 1-4) or ori− pUC-19 (lanes 5-8) DNA in each reaction. B, the relative α-32P incorporation intensity of each reaction was quantitated using a PhosphorImager.

Replication of HPV-11 and BPV-1 ori by Homologous and Heterologous Viral Proteins—To examine whether BPV-1 proteins can replicate HPV ori more efficiently than HPV-11 proteins as observed previously in transient replication assays (18), we conducted similar studies by using the cell-free replication system. Consistent with results presented in Fig. 5, HPV-11 proteins initiated efficient replication of the homologous HPV-11 ori+ plasmid p7730-99 (Fig. 6), but not the ori− pUC-19 (data not shown). Unexpectedly, they only marginally supported the replication of the BPV-1 ori plasmid pUC-Alu. The BPV-1 proteins replicated efficiently the homologous BPV-1 ori plasmid pUC-Alu, as reported previously (15). Surprisingly, the HPV-11 ori replicated relatively poorly in the presence of BPV-1 proteins. These results suggest that the more robust replication supported by BPV-1 proteins in transient replication assays does not reflect an intrinsic replication property of the viral proteins.

The amounts of the BPV-1 proteins used in these reactions (250 ng of E1 and 30 ng of E2) were higher than in the previous report for the BPV-1 ori (25), because little or no replication was detected when lower concentrations of the BPV-1 proteins were used (data not shown). Despite the high concentrations of the BPV-1 proteins, the ori− pUC-19 did not replicate, unlike the previous reports (21, 25). We believe that these differences are due to distinct properties of the cell extracts used in these studies.

We previously reported that mutations in one half-site of each of the three E2BS in p7730-99 (p7730-99-234M) elimi-
E2 binding in vitro and also disable ori activity in transient replication assays (18). In several cell-free replication experiments using this mutated template, we observed at least a 40-fold reduction in replication to a level similar to the background incorporation obtained in reactions containing no E1 or E2 proteins (Fig. 6 and data not shown). In contrast, there was only a 3.3-fold reduction in the replication of this ori mutation when the cell extracts were supplemented with the BPV-1 proteins. Neither HPV-11 nor BPV-1 proteins initiated replication from the ori-pUC-19 template, as no RI was detected. No ori+ replication was detected when viral proteins were omitted (data not shown). These results indicate that the replication of HPV-11 ori in the homologous cell-free system also requires at least one intact E2BS. This result is consistent with the hypothesis that replication of p7730–99–234M initiated by the BPV-1 proteins is dependent on the presence of the putative E1BS. It is possible that the remaining three half-E2BS in the mutated ori was able to stabilize the binding of BPV-1 E1 to the putative E1BS. These possibilities remain to be tested.

Comparison of Different Cell Extracts—We prepared monolayer and suspension HeLa cell extracts and tested their ability to support HPV-11 ori replication in parallel experiments with extracts of 293 cells. Extracts from monolayer HeLa cells did not support the HPV-11 ori replication at any site near the efficiency of the 293 cell extracts (Fig. 7, compare lanes 1 and 3), whereas the intensity of nonspecific labeling in Form I DNA was the same (compare lanes 2 and 4). Extracts from suspension HeLa cells functioned even more poorly, although they were able to support a similar level of repair synthesis (data not shown).

DISCUSSION

We have established the first homologous cell-free DNA replication system for a papillomavirus by using an HPV-11 ori reporter plasmid, the HPV-11 E1 and E2 proteins purified from recombinant baculovirus-infected insect cells, and extracts from human 293 cells. The E1 protein has been purified to near-homogeneity, whereas the E2 protein is about 30–50% pure. The system has been characterized for conditions under which only ori-dependent replication took place efficiently (Figs. 2–6). Results with antibodies to or inhibitors of viral or host replication proteins (Table I) demonstrated that efficient ori replication depended on the viral E1 and E2 proteins and also required human DNA polymerase α and the associated primase, proliferating cell nuclear antigen and the associated DNA polymerase δ, RP-A, and topoisomerase I and II (Table I and data not shown), all of which are known to participate in the replication of SV40 and BPV-1 DNAs (1–3, 15). There was a 1-h lag before substantial RI were detected. Whether this lag time represents a relatively slow assembly of initiation complexes due to the low affinity of the HPV-11 E1 protein for the ori, a period during which post-translational modifications (such as phosphorylation or dephosphorylation) of the added viral proteins takes place or a deficiency in certain host factors in the cell extracts remains to be determined. During the second hour of incubation, the appearance of replication intermediates increased linearly and preceded that of Form I DNA, kinetics which are consistent with successful completion of replication and accurate segregation of supercoiled daughter monomers.

Many of our results are similar to those observed for cell-free BPV-1 DNA replication with mouse cell extracts supplemented with BPV-1 proteins (15, 21, 25). They include (a) a strict dependence on the presence of an ori, the viral E1 and E2 proteins, and the host chromosomal replication machinery (Figs. 2–5 and Table I); (b) the concentrations of viral and host proteins needed (Fig. 5); (c) the extent of replication and the level of conversion of replication intermediates to Form I daughter molecules; (d) the ability of high concentrations of the E1 protein alone to support a low level of either ori- and ori+ replication (Fig. 3); (e) a suppression of ori-independent replication by the addition of the E2 protein (Fig. 3); and (f) a strong stimulation of the ori-dependent replication by the E2 protein (Fig. 3) (see also Ref. 21). The stimulating effect of the E2 protein on ori replication is consistent with an interaction between the E1 and E2 proteins, as reported previously between the BPV-1 proteins and between the BPV-1 and HPV-6b proteins (21, 50–54). The BPV-1 E2 protein can stimulate and stabilize the binding of BPV-1 E1 protein to the E1BS, enhancing the E1 unwinding activity on the ori+ plasmid (15, 21, 54, 55). Our results suggest similar interactions also exist for HPV-11 proteins during ori recognition and initiation of replication. Experiments are in progress to examine these activities.

We note some interesting differences from the BPV-1 cell-free replication that may have significant implications concerning the role of the HPV E2 protein in ori replication. (a) An intact E2 site is absolutely required for HPV-11 ori replication in that a highly active HPV-11 ori (p7730–99) was incapacitated when one half-site of each of the three E2BS was mutated (p7730–99–234M) (Fig. 6), identical to the result in transient replication (18). In contrast, there was only a relatively small reduction in the ability of the BPV-1 proteins to replicate this mutated HPV-11 ori plasmid (Fig. 6). The BPV-1 proteins replicate efficiently a BPV-1 ori mutation in which the half-site of a low affinity E2BS in close proximity to the E1BS is mutated (15, 26). The BPV-1 E1 protein can bind to certain substitution mutations in the BPV-1 E1BS in vitro and replicates these mutated DNA in transfected cells (22). We suggest that, despite the 4-base pair sequence divergence between the two viruses in the E1BS, the BPV-1 E1 protein may bind to the HPV-11 site with an affinity higher than the homologous HPV-11 E1 protein. The remaining half-sites of the E2BSs in p7730–99–234M may also play a role in stabilizing the BPV-1 E1 binding to this

| Table 1 |
| Effects of antibodies and inhibitors on replication |
| The total incorporation of the complete reaction (minus repair synthesis) was taken to be 100% activity. Antibodies and inhibitors were added prior to replication reactions, [α-32P]dCTP was added at the end of the first hour of incubation, and reactions continued for another hour. Relative intensities were quantitated using a PhosphorImager. |

| Relative activity (%) |
|-----------------------|
| Complete reaction     | 100 |
| E1 E2                 | 80  |
| Human RP-A            | 20  |
| T3 DNA                | 20  |
| DNA polymerase α      | 25  |
| Inhibitors            |     |
| Aphidicolin, 5 µg/ml  | 0   |
| Aphidicolin, 10 µg/ml | 0   |
| Aphidicolin, 100 µg/ml| 0   |
| α-Amanitin, 10 µg/ml  | 0   |
| α-Amanitin, 100 µg/ml | 0   |
| Camptothecin, 40 µg/ml| 0   |
| Camptothecin, 80 µg/ml| 0   |
| Camptothecin, 150 µg/ml| 0   |
| Camptothecin, 300 µg/ml| 0   |
| Camptothecin + Etoposide | 0 |

(b) The concentrations of viral and host proteins needed (Fig. 5); (c) the extent of replication and the level of conversion of replication intermediates to Form I daughter molecules; (d) the ability of high concentrations of the E1 protein alone to support a low level of either ori- and ori+ replication (Fig. 3); (e) a suppression of ori-independent replication by the addition of the E2 protein (Fig. 3); and (f) a strong stimulation of the ori-dependent replication by the E2 protein (Fig. 3) (see also Ref. 21). The stimulating effect of the E2 protein on ori replication is consistent with an interaction between the E1 and E2 proteins, as reported previously between the BPV-1 proteins and between the BPV-1 and HPV-6b proteins (21, 50–54). The BPV-1 E2 protein can stimulate and stabilize the binding of BPV-1 E1 protein to the E1BS, enhancing the E1 unwinding activity on the ori+ plasmid (15, 21, 54, 55). Our results suggest similar interactions also exist for HPV-11 proteins during ori recognition and initiation of replication. Experiments are in progress to examine these activities.

We note some interesting differences from the BPV-1 cell-free replication that may have significant implications concerning the role of the HPV E2 protein in ori replication. (a) An intact E2 site is absolutely required for HPV-11 ori replication in that a highly active HPV-11 ori (p7730–99) was incapacitated when one half-site of each of the three E2BS was mutated (p7730–99–234M) (Fig. 6), identical to the result in transient replication (18). In contrast, there was only a relatively small reduction in the ability of the BPV-1 proteins to replicate this mutated HPV-11 ori plasmid (Fig. 6). The BPV-1 proteins replicate efficiently a BPV-1 ori mutation in which the half-site of a low affinity E2BS in close proximity to the E1BS is mutated (15, 26). The BPV-1 E1 protein can bind to certain substitution mutations in the BPV-1 E1BS in vitro and replicates these mutated DNA in transfected cells (22). We suggest that, despite the 4-base pair sequence divergence between the two viruses in the E1BS, the BPV-1 E1 protein may bind to the HPV-11 site with an affinity higher than the homologous HPV-11 E1 protein. The remaining half-sites of the E2BSs in p7730–99–234M may also play a role in stabilizing the BPV-1 E1 binding to this
HPV-11 ori mutation. It is conceivable that an adventitious E2BS in pUC-19 vector (56) also contributed BPV-1 E2 binding to the mutated HPV-11 ori, even though pUC-19 itself did not replicate in the presence either BPV-1 or HPV-11 proteins. Bacterially expressed BPV-1 E2, but not HPV-16 E2 protein, binds to this E2BS in pUC-19 in vitro (56) . (b) high amounts (120 ng and more) of the HPV-11 E1 protein did not replicate the HPV-11 ori+ plasmid more effectively than the ori- control template in the absence of E2 protein (Fig. 3B and data not shown). In contrast, mouse cell extracts supplemented with BPV-1 E1 protein alone in amounts similar to or higher than the HPV-11 E1 protein used in our experiments replicate the BPV-1 ori+ template many fold more efficiently than the ori- template (21, 25).

Both of these differences between HPV-11 and BPV-1 cell-free replication suggest that the HPV-11 E1 protein has a relatively weak affinity for the putative E1BS in the HPV ori. In fact sequence-specific DNA binding by the HPV E1 protein has not been consistently observed (28, 30). Moreover, the putative HPV E1BS is not essential for replication in transfected cells, and vectors containing synthetic E2BS can replicate (28, 29).2 Taken together, these observations suggest the two viruses may use different primary ori recognition proteins during the assembly of the initiation complex. For HPV-11, or perhaps HPV6 in general, it is the E2 protein, whereas for BPV, both E1 and E2 proteins recognize ori elements, with the E1 protein playing a more important role than the E2 protein. The role of the putative E1BS in HPV-11 ori replication and the ability of the purified HPV-11 E1 protein to bind to this sequence are being investigated.

An important distinction between cell-free replication and transient replication in transfected cells is noted. Both E1 and E2 proteins are absolutely required for BPV or HPV ori replication in transfected cells (11-14). In contrast, E2 protein is not necessary in cell-free replication of either ori+ or ori- templates when high concentrations of E1 protein are present (Ref. 21 and 25; Fig. 3 of this study). The ori-independent replication might be attributed to an inability of the E1 protein to bind and unwind DNA nonspecifically, as observed with the BPV-1 E1 protein (24, 25). This difference between the two replication assays has been interpreted to results from the lack of nucleosomes in the cell-free replication system, whereas there is a need in vivo for the E2 protein to disrupt nucleosomes which block the assembly of the initiation complex at the ori (57, 58), as also suggested for the role of enhancer binding proteins in SV40 replication (Refs. 59 and 60; for review, see Ref. 4). However, in the case of HPV-11 ori replication by the homologous viral proteins, we believe that E2 protein plays a more critical role in ori recognition than the BPV-1 counterpart, as just discussed.

Another interesting difference has been observed between the transient replication and cell-free replication. In many of the cell lines tested, BPV-1 proteins replicate the ori's of human and animal papillomaviruses more efficiently than HPV-11 proteins (11), whereas in the cell-free system, only the homologous combination replicated efficiently (Fig. 6). There are differences in the number and sequences of the viral protein binding sites and in the distance separating them, all of which affect the interactions among the BPV-1 proteins and BPV-1 ori and the efficiency of ori replication (15, 22, 27). Undoubtedly these factors also influence the efficiency with which HPV-11 proteins assemble the initiation complex on the homologous or heterologous ori. We believe that the results of cell-free replication may reflect the co-evolution of the ori and the homologous viral proteins to achieve optimal DNA-protein and protein-protein interactions for regulated replication. In contrast, the results in transient replication may be a consequence of a more stable and efficient expression of BPV-1 proteins that more than compensated for the reduced affinities for the heterologous ori. A second simplistic explanation is that the conditions for the heterologous combinations have not been optimized. A third possibility is that the BPV-1 ori's used in the previous transient assays contained the entire BPV upstream regulatory region, whereas the present study used a shorter plasmid. This is however not likely because this shorter plasmid contains all the
cis-elements to function as an efficient ori for BPV-1 proteins in transient and cell-free replication assays (15, 26). Furthermore, the HPV-11 ori template used in these two assays was one the same and therefore cannot account for the observed difference. Further examinations are needed to distinguish among these possibilities.

It is noteworthy that, relative to the extracts of 293 cells, identically prepared monolayer or suspension extracts from HeLa cells support HPV-11 ori replication very poorly (Fig. 7). A similar observation has also been reported in cell-free replication of SV40 (44). HeLa cells are derived from a cervical cancer and contain integrated HPV-18 DNA. We have previously shown that HPV ori's replicate poorly in this and other HPV-containing cervical carcinoma cell lines when cotransfected with either HPV or BPV-1 E1 and E2 protein expression vectors (11). We have speculated that perhaps these cells had some deficiency which affected interactions with viral ori recognition proteins and caused the integration of HPV DNA into the host chromosomes in the first place. The results of cell-free replication are consistent with this hypothesis.

In summary, we have established the first homologous cell-free replication system for papillomavirus DNA using highly purified viral ori recognition proteins and homologous host cell extracts. A number of similarities and differences were observed between this system and previously reported transient replication assays in transfected cells as well as with BPV-1 cell-free replication. A comparison of mixed and matched combinations between ori and viral proteins further suggests possible differences in the roles of viral ori recognition proteins in the assembly of the replication initiation complexes for HPV and BPV. Additional investigation is needed to elucidate the basis for these differences.

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