The Functions of Human Papillomavirus Type 11 E1, E2, and E2C Proteins in Cell-free DNA Replication

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We examined the functions of human papillomavirus type 11 (HPV-11) E1 and E2 proteins purified from SF9 cells infected with recombinant baculoviruses in cell-free HPV-11 origin (ori) replication. The E1 protein binds specifically to a wild type but not to a mutated sequence in the ori spanning nucleotide position 1. It also has a relatively strong affinity for nonspecific DNA. A neutralizing antiserum directed against the amino-terminal one-third of the E1 protein totally abolishes initiation and elongation, suggesting that it functions as an initiator and a helicase at the replication fork. An antiserum against the carboxyl-terminal portion of E1 protein abolished replication only when added prior to initiation. Thus this portion of E1 is hidden in the replication complexes. The HPV-11 E2 protein appears not to be essential for elongation, but it must be present in the preinitiation complex for the E1 protein to recruit host DNA replication machinery to the ori. E2 antiserum added after preincubation in the absence of the cell extracts totally abolished replication. An identical conclusion is also reached for the bovine papillomavirus type 1 E2 protein. Finally, the HPV-11 E2C protein lacking the transacting domain of the full-length E2 protein partially inhibits E2-dependent ori replication.

The large family of human papillomaviruses (HPVs) cause persistent or recurrent epitheliomatous lesions, some of which can progress to high grade dysplasias or carcinomas (1). Productive infections normally cause exophytic or flat warts in which the viruses have two distinct modes of DNA replication. A low copy number of viral DNA is maintained in basal and parabasal cells that are capable of cell division. Only in a subset of cells undergoing terminal squamous differentiation does vegetative viral DNA amplification take place (2). There is considerable interest in investigating the mechanisms of papillomaviral DNA replication; they may serve as models for host DNA replication in mammalian cells cotransfected with expression vectors for viral genes. The other is cell-free replication in the presence of viral proteins purified from insect SF9 cells infected with recombinant baculoviruses. These assays have demonstrated that replication requires a viral origin of replication (ori), virus-encoded E1 and E2 proteins and the host DNA replication enzymes including DNA polymerase α/primase, proliferating cell nuclear antigen/DNA polymerase δ or ε, single-stranded DNA binding protein RPA, and topoisomerases I and II (Refs. 3–9; for a review, see Ref. 10).

The full-length E2 proteins of HPVs and BPV-1 are also transcription regulatory proteins, each consisting of three domains, the amino-terminal transactivating domain, a hinge region, and the carboxyl-terminal DNA binding and protein dimerization domain (for a review, see Ref. 11). They bind as a dimer to a consensus sequence ACCN6GGT designated the E2-BS (12–16). The full-length HPV-11 E2 protein is the primary HPV-11 ori binding protein (8, 17) (see below). Two alternative HPV-11 E2 proteins, E2C and E1M*E2C, encoded by separate mRNAs contain the hinge and the DNA binding and dimerization domains but have different amino termini. Both are strong transcription repressors but are weak repressors in transient replication (17–19). The simplest explanation would be the inefficiency of cotransfection of four plasmids into the same cells by using electroporation. This hypothesis remains to be tested.

Aside from the ability of the purified HPV-11 E1 protein to support cell-free replication (8), the E1 proteins of various HPV types are only partially characterized. A bacterially expressed maltose/HPV-6b E1 fusion protein exhibits helicase activity (20). A glutathione S-transferase fusion protein with the HPV-31b E1 protein purified from bacteria binds to a sequence that has some homology to and is located at a similar genomic position as the BPV-1 E1 binding site (E1-BS) in the BPV-1 ori (21). Using crude cell extracts of SF9 cells infected with recombinant baculovirus, the HPV-11 E1 to bind to an ori fragment has been demonstrated by an immunoprecipitation assay (22). However, in another report, sequence-specific binding of HPV-11 E1 protein partially purified by immunoprecipitation from SF9 cells was not observed, although the protein exhibited ATPase and GTPase activities (23). A caveat of these binding results is that the functionality of the E1 protein was not corroborated by cell-free replication assays, nor was mutational analysis of the putative E1-BS performed.

In contrast, the BPV-1 E1 protein, which is highly homologous to the HPV E1 proteins, is well characterized. It is a DNA-dependent ATPase and an ATP-dependent helicase (24–26). Unlike the HPV systems, it is the major BPV-1 ori recogn...
tion protein and binds to an 18-bp imperfect palindromic spanning nucleotide 1 (E1-BS) (3, 27–30). As does the SV40 T antigen, the E1 protein distorts and melts the ori upon binding (31) and interacts with the DNA polymerase α (32, 33). In addition, it interacts with the BPV-1 E2 protein in the presence or in the absence of the respective cognate binding sites (3, 31, 34–39). BPV-1 E1 protein formed two different complexes around the BPV-1 ori (40). At a high E2/E1 ratio where replication was inhibited, a c1 complex was formed that contained both BPV-1 E1 and E2 proteins. In contrast, at a low E2/E1 ratio when replication took place, a c2 complex was formed that contained no E2 protein. It was proposed that the E2 protein helps recruit and stabilize the multiple copies of E1 protein to the ori and then has to be released before initiation of DNA replication can occur. This hypothesis has not been tested experimentally.

All papillomaviruses have multiple copies of E2-BS in the upstream regulatory region (or long control region) that contains the ori (3, 5–7, 28). Site-directed mutagenesis demonstrated that one or more copies of the natural E2-BS are absolutely necessary for HPV-11 replication proteins to initiate ori-specific replication in both transient and cell-free replication assays (8, 17). Conversely, the presence of one or more copies of synthetic E2-BSs are sufficient to initiate replication by HPV-11 or HPV-18 replication proteins in either assay (22, 41). A putative E1-BS in the HPV-11 ori greatly enhances the efficiency of cell-free replication, whereas transcriptional enhancer elements have little effect in transient or cell-free replication assays (43). The natural ori of HPV-11 and BPV-1 also each contains an AT-rich region proximal to the E1-BS (3, 17, 28). However, in the absence of the E1-BS, this element had little contribution during cell-free ori replication by HPV-11 proteins.

In this report, we examined the roles of the HPV-11 E1, E2 and E2C proteins in the HPV ori-specific replication. Each protein was purified from Sf9 cells infected with a recombinant baculovirus. We show that the replication-competent HPV-11 E1 protein is able to bind specifically to the ori at the previously inferred E1-BS. It also exhibits a relatively strong nonspecific affinity for DNA. We show that E1 protein is required for both initiation and elongation but that only a small proportion of the daughter molecules entered into a second round of replication. Our results also show that E2 protein appears not to be essential for elongation. We detected no replication-competent HPV-11 or BPV-1 ori DNA complexes that were not sensitive to neutralizing anti-E2 antisera. We also show that HPV-11 E2C protein can inhibit HPV-11 ori replication, but inhibition was not very effective, even in the presence of a large excess of E2C protein.

**MATERIALS AND METHODS**

**Origin Plasmids—**HPV-11 ori plasmids pUC7730–99 (spanning nt 7730–7933/1–99), pUC7874–99 (spanning nt 7874–7933/1–99), pUC7874–20 (spanning nt 7874–7933/1–20) and pUC7874–20-OrI/H with an Hpal site generated at nt 3 were described.2 BPV-1 ori plasmid pUC-Alu and SV40 ori plasmid pSVori were described in Kuo et al. (8) and Chiang et al. (17), respectively. The HPV-11 E2C cDNA (1101–1272:3325–3900) (44) recovered from a nude mouse xenograft induced by HPV-11 by the coupled reverse transcription-polymerase chain amplification reaction was a gift from Dr. Cheng-Ming Chiang. The 3′ end of the noncoding region of this cDNA was restored to nt 4402 to include the polyadenylation signal and then cloned into the Smal site of the baculovirus transfer vector pVL1393.

**Viral Proteins—**The purification of native HPV-11 E2 protein and HPV-11 E1 protein tagged at the amino terminus with an epitope rich in glutamic acid (GluGlu) (45) from insect Sf9 cells infected with recombinant baculoviruses has been described (8). Recombinant baculovirus expressing HPV-11 E2C was selected after cotransfection with the wild type viral DNA and the E2C transfer vector (8). HPV-11 E2C protein was purified from infected Sf9 cells as described for the purification of the full-length E2 protein. The E2C protein concentration was estimated to be about 40 ng/μl and the purity at 30%. Recombinant baculovirus expressing BPV-1 E1 and native E2 proteins (3) were gifts from Dr. Michael Botchan, and the proteins were purified as for HPV-11 proteins. Purified SV40 T antigen (T-ag) was kindly supplied by Dr. Teresa Wang.

**Cell-free Replication Assay—**The conditions for cell-free replication have been described (8), except that 293 cell extracts were prepared from suspension cultures of 46. Briefly, 40 ng of pUC7874–20 and E2C plasmid together with 10 μl of 293 cell extract (approximately 10 mg/ml), NTPs, and dNTPs in replication buffer were incubated in the presence or in the absence of the following viral replication proteins: for HPV-11, 30 ng of EE-E1 and 8 ng of E2; for BPV-1, 90 ng of EE-E1 and 30 ng of E2; for SV40, 40 ng of T-ag. The viral proteins, ori plasmids, and cell extracts were preincubated at 37 °C for different lengths of time, as described in each figure legend, before 2.5 μCi of [α-32P]CTP was added. Although the length of preincubation time varied slightly among different experiments, some due to the necessity of the experimental design, we found no qualitative difference in the outcome when the experiments were repeated with slightly shorter or longer preincubation times. The deadenylated form of [α-32P]dCTP reduced the incorporation and repair synthesis and simplified the quantitative analyses of replication (8). In some experiments, the addition of cell extracts was delayed for up to 30 min during preincubation. In others, different amounts of purified HPV-11 E2C protein in place of, or in addition to, the full-length E2 protein were added at the beginning of the preincubation. In certain experiments, various antibodies were added at times specified in each experiment, 1 μl of rabbit polyclonal E1N antiserum raised against the N-terminal 190-amino acid portion of HPV-11 E1 in a Trp E fusion protein (5); 1 μl of rabbit polyclonal E1C antiserum raised against the carboxyl-terminal 402 amino acids of the HPV-11 E1 protein in a Trp E fusion protein;3 2 μl of polyclonal antiserum against HPV-11 E2 protein (4); 2 μl of monoclonal antibody against the EE-epitope. Reactions were then continued for different lengths of time as specified and were terminated by the addition of stop solution to achieve final concentrations of 1% SDS, 1 mM EDTA, and 20 μg/ml RNase A. After an incubation of 15 min at 37 °C, the proteinase K was added to 200 μg/ml, and incubation at 37 °C continued for another 30 min. Following phenol-chloroform extraction and ethanol precipitation, the DNA was electrophoresed in a 0.8% agarose gel in 1 × TAE buffer. Gels were dried and exposed to Hyper-Film (Amer sham Corp.) and quantified by PhosphorImager (Molecular Dynamics).

**Analysis of Replication Products by Digestion with DpnI Restriction Endonuclease—**To purified 32P-labeled DNA from six standard cell-free replication reactions, each with 40 ng of HPV-11 ori plasmid pUC7874–20, we added 6 μg of unlabeled template plasmid. After précipitation through a G-50 spin column to ensure the removal of all residual SDS, the DNA was precipitated with ethanol, redissolved in water, and divided into six equal aliquots. One aliquot was digested with 4 units of EcoRI alone. Four aliquots were each digested with 4 units of EcoRI and various units of DpnI at 37 °C for 1 h. All reactions were then run in 1 × TAE buffer, using 1 × TAE buffer to cleaved the DpnI-generated small DNA fragments. Under these conditions, most of the digested replication intermediates were compacted into a slow migrating band rather than the usual smeared observed in the 0.8% agarose gel. The gel was stained with ethidium bromide, photographed, and then dried and exposed to Hyperfilm and PhosphorImager.

**In Vitro Replicative Mobility Shift Assay (EMS) —**A wild type HPV-11 DNA fragment spanning nt 7874–7933/1–20 and the comparable fragment with 1-bp addition and 1-bp substitution mutation creating an Hpal site at nt 3 were generated by restriction digestion at the flanking HindIII and EcoRI sites or by polymerase chain reaction using pUC7874–20 or pUC7874–20-OrI/H3 as a template and M13 forward and reverse primers. The 150-bp products were purified and labeled at the 5′ end with [γ-32P]ATP and the T4 polynucleotide kinase (Life Technologies, Inc.). Unlabeled fragments were used as competitors. In a 10-μl reaction mixture, 240 ng of HPV-11 EE-E1 protein was mixed with 200 ng of poly(dI-dC)-poly(dI-dC) (Pharmacia Biotech Inc.) and 20 fmol (about 6000 cpm) of labeled DNA fragments in Buffer I (25 mM

2 J.-S. Liu, S.-R. Kuo, T. R. Broker, and L. T. Chow, submitted for publication.

3 T. F. Ho, J.-S. Liu, L. T. Chow, and T. R. Broker, unpublished results.
reduced the efficiency of cell-free replication, we examined the
from a partial sequence homology to the BPV-1 E1-BS greatly
HEPES-K⁺, pH 7.5, 7 mM MgCl₂, and 1 mM dithiothreitol) with or without 4 mM ATP and 50 ng (25-fold excess to the probe) of competitor DNA fragments. The DNA binding reaction was conducted at room temperature for 30 min followed by the addition of glutaraldehyde to a final concentration of 0.2% and incubation for another 15 min at room temperature. EMSAs were performed in 1.5% agarose gel in 1× TAE buffer at 4°C. Gels were dried and exposed to Hyper-Film for 24–36 h.

RESULTS

HPV-11 E1 Protein Is a DNA Binding Protein—Since a mutation in a putative HPV-11 E1-BS in the HPV-11 ori inferred from a partial sequence homology to the BPV-1 E1-BS greatly reduced the efficiency of cell-free replication, we examined the possibility that the HPV-11 E1 protein binds specifically to this sequence. Replication-competent HPV-11 E1 protein purified to homogeneity from recombinant baculovirus-infected SF9 cells (8) was used in an electrophoretic mobility shift assay to examine its ability to bind the putative E1-BS in the HPV-11 ori. The addition of the E1 protein generated a slow migrating smear when the wild type DNA fragment 7874–7933/1–20 spanning the putative E1-BS was used as a probe (Fig. 1A). Formation of the complexes was dependent on the presence of ATP (lanes 2 and 3). The complexes were further retarded by the addition of anti-E2 epitope IgG (lane 4). The unlabeled homologous DNA fragment at 25-fold molar excess was able to compete for E1 protein binding. A comparable amount of a mutated fragment with an Hpal site introduced at nt position 3 within the putative E1-BS did not compete (Fig. 1A, compare lanes 5 and 6), nor did it form any complexes with the E1 protein (Fig. 1B, lane 11). These results indicate that E1 protein indeed binds to the DNA sequence spanning nt 3 previously inferred by analogy to the known BPV-1 E1-BS. We suggest that HPV-11 E1-BS spans nt 7928–7933/1–12 (10). In contrast, the HPV-11 E2C and full-length E2 proteins were able to bind to both DNA fragments, as each contains wild type E2-BS (Fig. 1B, lanes 9 and 12, and data not shown). Addition of anti-E2 antiserum led to supershifts of the bands, confirming the existence of E2 proteins in these complexes (data not shown). These results are in agreement with previous results with bacterially expressed E2C protein (48).

The E1/E1-BS interaction is, however, relatively weak, as the complexes were eliminated by poly(dI-dC) at more than 150-fold mass excess and were reduced at NaCl concentration higher than 25 mM. No complexes were detected when glutaraldehyde cross-linking was omitted (data not shown). The E1 protein also had relatively strong nonspecific DNA binding ability. In the absence of poly(dI-dC), only large complexes that did not enter into the gel were formed with DNA containing either wild type or mutated E1-BS (data not shown). In the presence of a 100-fold mass excess of poly(dI-dC) where specific binding to the E1-BS was observed, much of these large complexes remained (Fig. 1A).

This relatively small difference in the affinity of E1 protein for E1-BS and for nonspecific DNA sequence was also mani-
vested in the immunoprecipitation assays (Fig. 1C). Using the anti-EE Mab to precipitate the E1-DNA complex, there was an enrichment of the two E1-BS-containing fragments, 7874–99 (which contains the E1-BS and three copies of natural E2-BS) and 7730–99-234M (which contains the E1-BS but mutated E2-BS), over the nonspecific fragments from pUC-19 (lane 15), but only over a very narrow range of binding conditions (data not shown). Polyclonal anti-E1 antiserum disrupted the complex and led to nonspecific precipitation of all fragments (data not shown). In parallel experiments under the same binding conditions, anti-E2 antiserum precipitated only fragment 7874–99 and SN3 that contain three copies of synthetic E2-BS in association with HPV-11 E2 protein but not the other fragments (lane 14). The fragment 7730–99-234M, in which all three E2-BS were mutated, has also been shown previously not to bind E2C protein by DNase I footprinting assay (17).

HPV-11 E1 Protein Is Necessary for both Replication Initiation and Elongation but HPV-11 E2 Protein Appears Not to Be Essential for Elongation—In this and all subsequent experiments, the amounts of E1 protein and strong ori template, pUC7874–99 (which contains three copies of E2-BS and the E1-BS), used only promote ori-dependent and E2-dependent replication (8). To investigate the specific roles of E1 and E2 proteins during initiation and elongation, we examined the effects of rabbit polyclonal antisera raised against the amino-terminal portion of the HPV-11 E1 protein (5) or the HPV-11 E2 protein (19). Antisera (left panel) or stop solution (right panel) was added at different times as indicated by the arrow, and the reactions continued until termination at 105 min by the addition of stop solution. B, total incorporation into replication products (minus repair synthesis) were quantified by PhosphorImager for the three experiments. C, incorporation into RI or Form I molecules in the presence of E1N or E2 antiserum after subtracting the counts in the control experiment generated by the addition of stop solution at each time point.
times after the addition of \([\alpha\text{-}32\text{P}]dCTP\). Incubation was allowed to continue to 105 min and was then terminated by the addition of the stop solution of SDS and proteinase K. The extent of replication that took place up to the moment of antiserum addition was determined in parallel experiments by the addition of stop solution (Fig. 2A, right panel). We reasoned that if the viral proteins are required for both initiation and elongation, the addition of neutralizing antiserum should, ideally, block further replication as effectively as the stop solution. The quantitative analyses of total incorporation (Fig. 2B) of all three sets of reactions (after subtraction of repair synthesis) were determined by PhosphorImager. The amounts of Form I and replication intermediate (RI) accumulated after the addition of E1N antisem or E2 antisem in excess over the control experiments are shown in Fig. 2C. The E1N antisem virtually abolished all subsequent \([\alpha\text{-}32\text{P}]dCTP\) incorporation into both RI and Form I molecules, indicating that E1 is required for both initiation and elongation and must remain in active form in association with the replication intermediates until the completion of replication. In contrast, the addition of E2 antisem did not prevent the incorporation of \([\alpha\text{-}32\text{P}]dCTP\) into RI or Form I DNA. We interpret these results to mean that elongation was not inhibited, at least not extensively, by E2 antisem, and consequently the E2 protein appears not to be essential for elongation. The accumulation of RI increased for 30 min and then steadily decreased, whereas Form I DNA plateaued at 60–75 min. This delayed accumulation of Form I relative to RI is consistent with a product-precursor relationship. Furthermore, the decline in RI is in dramatic contrast to their continued accumulation in the absence of antiserum (8). These results suggest that RI that matured into Form I DNA was not replenished by newly initiated RI in the presence of E2 antisem. We concluded that E2 antisem inhibits initiation but not elongation (see also Fig. 3).

Nonspecific inhibition of replication by either antiserum was ruled out on the basis of several observations. Preimmune serum or serum raised against TrpE protein that is present in the immunizing antigens (TrpE-E1N or TrpE-E2), had little or no effect on cell-free replication (data not shown), nor did a polyclonal antiserum against a Trp E-HPV-11 E5a fusion protein (8). Neither antisem significantly affected cell-free SV40 replication initiated by the SV40 T antigen (Fig. 3B, lanes 21–23).

Postinitiation Replication Complexes Are Relatively Insensitive to HPV-11 E1C Antiserum—Experiments illustrated in Fig. 3A were conducted to test the inhibitory effect of the polyclonal antiserum against the carboxyl-terminal two-thirds of HPV-11 E1 protein (E1C), which contains the ATP binding and ATPase domain by analogy to the homologous BPV-1 E1 protein (24–26). ATPase activity has indeed been detected in HPV-11 E1 protein (23). When added at the beginning of the incubation, the E1C antisem was as effective as E1N or E2 expression plasmids (5).

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the end of preincubation at 0 min. Together with antiserum against E2 protein were then added at 293 cell extracts containing the host replication proteins to result in clearly demonstrated that the addition of E2 antiserum previously proposed, the addition of E2 antiserum together with increasing longer periods of preincubation (Fig. 4). The absence of the host replication proteins.

increased BPV-1 (40), we performed the experiment outlined in Fig. 4. Appropriate amounts of HPV-11 E1 and E2 proteins were purified from Sf9 cells and preincubated at 37°C for 30, 20, 10, or 0 min (lanes 2-10). The presence (+) or absence (−) of viral proteins or E2 antiserum is indicated at the top of each lane.

BPV-11 E2 Protein Is Required during Assembly of Preinitiation Complexes—To examine whether BPV-1 E2 protein is also required during the assembly of preinitiation complexes, cell-free replication experiments with BPV-1 ori plasmid pUC-Alu similar to those just described for HPV-11 were performed with BPV-1 E1 and E2 proteins that were purified from Sf9 cells (Fig. 5A). The amounts of DNA template and E1 and E2 proteins used promoted only E2-dependent and ori-dependent replication (Fig. 5B, lane 2, and data not shown). The results show clearly that polyclonal antibodies against BPV-1 E2 added together with cell extracts after up to 30 min of preincubation of the template DNA and BPV-1 E1 protein totally inhibited replication (Fig. 5B). We ruled out a nonspecific effect because it did not inhibit SV40 ori replication initiated by the SV40 T-ag (data not shown). These results indicate that, as is the HPV-11 E2 protein, the BPV-1 E2 protein is also required for the formation of preinitiation complex.

E2C Is a Negative Factor in HPV-11 Cell-free Replication—In the presence of a low E1 protein concentration where ori-dependent replication is strictly dependent on the stimulation by the intact E2 protein (8), E2C could not replace the E2 protein, as it did not stimulate ori replication of pUC7874−99 (data not shown). Addition of E2C inhibited E2-dependent ori replication (Fig. 6A). However, a 15-fold molar excess of E2C (60 ng) over E2 (8 ng) was required to exert a 40% inhibition (Fig. 6A, lane 10). This result may be explained by the ability of any of the three copies of E2-BS in the ori plasmid to initiate cell-free replication. We therefore tested the effect of E2C on E2-dependent replication on a weak ori that contains only a single copy of the E2-BS. The results were similar, and a large molar excess of E2C was needed to achieve repression (Fig. 6A, lanes 1-5). To rule out the possibility that the inhibition by the E2C protein was due to contaminating nonspecific factor, we tested the E2C protein in SV40 ori replication initiated by purified SV40 T-ag from plasmid pSVori. Up to 60 ng of E2C protein has no effect on SV40 ori replication (Fig. 6B). Quant-
titative analysis with the PhosphorImager is presented in Fig. 6C.

Cell-free Replication Products Are Mostly Semimethylated Resulting from a Single Round of Semiconservative Replication—The results presented in Fig. 2 indicated that E1 protein remains associated with the elongation complexes throughout the replication process. We are interested in determining whether the daughter molecules from the first round of replication underwent a second round of replication. We analyzed the purified replication products by digestion with different amounts of DpnI restriction endonuclease in the presence or absence of EcoRI after the addition of unlabeled template as carrier DNA as described under “Materials and Methods.” DpnI cleaves the unreplicated template DNA at the sequence GATC with methylated A residues and will also digest semimethylated DNA when the enzyme is present in excess (New England Biolabs). The addition of carrier DNA not only allowed us to monitor the completeness of the digestion by ethidium bromide staining, it also permitted us to adjust accurately the amounts of DpnI enzymes relative to the DNA.

The results are shown in Fig. 7A. All of the digestions were complete (compare lane 3 to lanes 2 and 4–7). PhosphorImager recording revealed $^{32}$P-labeled molecules before and after digestions (compare lane 9 to lanes 8 and 10–13). Undigested replication products migrated as Form II, various topoisomers of Form I, and slow migrating replication intermediates (lane 9) as shown previously (8) and throughout this study (Figs. 2–6). Digestion with EcoRI alone, which cuts once in the polylinker just outside the ori fragment at nt 396 (see Fig. 7B), generated $^{32}$P-labeled, unit-length linear DNA (Form III) (lane 10) derived from the Form I and Form II DNA. The partially replicated intermediates were converted to slow migrating molecules attributable to their longer than unit length and forked ends after digestion of ori form molecules. When the replication products were digested with both EcoRI and 0.5 or 1 unit of DpnI, much of the Form III-labeled DNA remained (lanes 11 and 8). The replication intermediates were converted to fragments shorter than unit length, most of which were, however, longer than the largest unlabeled fragment $a$ of 1,100 bp generated from a complete digestion of the carrier DNA (compare lanes 8 and 11 with lanes 2 and 5–7; see also panel B). A small amount of slow migrating material persisted, probably for reasons stated above. Thus, these results confirm that both the $^{32}$P-labeled Form I and the slow migrating molecules generated in the replication reaction are products of replicative DNA synthesis rather than repair synthesis, which would have yielded small fragments similar to those shown in lanes 5–7 upon DpnI digestion. Increasing amounts of DpnI to 2 or 4 units decreased Form I DNA and also correspondingly reduced the sizes of the majority of the other $^{32}$P-labeled materials.
E2-dependent to bind to the E1-BS explains the 6-fold stimulation of HPV-11 same genomic location (10). This ability of HPV-11 E1 protein papillomaviruses appear to have this putative E1-BS at the nucleotide sequence to the E1-BS in BPV-1. We show that E1 protein binds to a sequence in the assay (Fig. 1). The HPV-11 E1-BS is analogous in location and in nucleotide sequence to the E1-BS in BPV-1 or1. All the papillomaviruses appear to have this putative E1-BS at the same genomic location (10). This ability of HPV-11 E1 protein to bind to the E1-BS explains the 6-fold stimulation of HPV-11 E2-dependent ori activity by the presence of E1-BS observed in cell-free replication.2 It may also contribute to the E2-indepen- dent ori replication promoted by high concentrations of E1 protein in the cell-free system (8). The HPV-1 and BPV-1 E1 proteins also exhibited E2-independent replication in transient replication or cell-free replication, presumably through the same mechanism (9, 24, 33, 39, 49, 50).

Our experiments with neutralizing antiserum to the amino-terminal portion of the E1 protein demonstrate that E1 protein is required not only for initiation but also for elongation, as the addition of the antiserum completely prevented initiation and caused an immediate cessation of elongation (Figs. 2 and 3). The BPV-1 and HPV-6 E1 fusion proteins are helicase (20, 24, 25). Together with the results just described, the E1 protein of HPVs and BPV-1 most likely remains at the replication forks, unwinding the parental DNA strands in an ATP-dependent manner as elongation proceeds. A similar conclusion has been reached for the SV40 T-ag (for a review, see Ref. 51). This interpretation is consistent with the relatively strong nonspecific DNA affinity of E1 protein (Fig. 2), as may be expected for a helicase required throughout the replication reaction. In turn, this nonspecific DNA binding could account for the ori-independent cell-free replication by high concentrations of HPV-11 or BPV-1 E1 protein (8, 24, 33, 39, 49, 50). Ironic experiments with antiseras against BPV-1 E1 protein in BPV-1 ori replication, however, did not lead to a complete cessation of replication, perhaps because the particular antiserum did not recognize the vulnerable region of the E1 protein (data not shown). Interestingly, using DpnI sensitivity as a probe, we demonstrated that most of the replication products resulted from a single round of semiconservative replication, which initiated from sequences containing the HPV-11 ori, generating semi-methylated daughter molecules (Fig. 7). Although the majority of the replication products were resistant to DpnI digestion, they were sensitive to excess enzyme. Thus the E1 protein dissociates from the finished products and then reinitiates from different template molecules. Alternatively, the majority of viral or host proteins become inactivated during incubation, and thus little reinitiation occurred. These possibilities remain to be examined.

The polyclonal antiserum against the carboxyl 2⁄3 of the HPV-11 E1 protein did not completely inhibit replication when it was added postinitiation. However, complete inhibition was observed when it was added prior to initiation (Fig. 3, B and C). We infer that the amino-terminal portion of the E1 protein remains relatively exposed and accessible to antisem inactivation throughout initiation and elongation, whereas the carboxyl-terminal portion including the predicted ATP binding and ATPase domains remains partially hidden in the elongation complex due to oligomerization of the E1 protein or to interaction with host replication enzymes. SV40 T antigen functions as a double hexamer at the replication fork (52). The c2 complex of the BPV-1 E1 protein is also proposed to be a hexamer (40). Interestingly, the monoclonal antibody against the EE-epitope on the E1 protein had very little effect when added during preincubation or postinitiation (data not shown), indicating that the very N terminus of the E1 protein was not in functional contact with viral and host replication proteins, nor with the DNA template.

Unlike the anti-E1N antiserum, the addition of HPV-11 E2 antiserum resulted in total inhibition of replication only when the antiserum was added prior to but not after initiation (Figs. 2 and 3). There are two possible explanations. We favor the interpretation that E2 protein is needed only for initiation and that it is not present in the elongation complexes. This interpretation is consistent with the continued accumulation of Form I daughter molecules after antiserum addition (Fig. 2C) and the ability of high concentrations of E1 protein alone to initiate replication from ori+ or ori− templates (8). An alternative possibility that cannot be formally ruled out is that E2 protein is present in the elongation complexes but becomes less accessible to the antiserum than when it is in the preinitiation complexes.

We also demonstrated that the presence of E2 protein of HPV-11 or BPV-1 in the preinitiation complex is absolutely necessary, as the addition of HPV-11 or BPV-1 E2 antiserum to the mixture of DNA and viral proteins after a 30-min preincuba- tion in the absence of cell extracts totally abolished HPV-11 or BPV-1 ori replication (Figs. 4 and 5). We suspect that the E2 protein not only stabilizes E1 binding to the ori (3, 28, 37–39), it also interacts with host DNA replication machinery during the assembly of the preinitiation complexes. The BPV-1 E2 protein binds weakly to the single-stranded DNA binding protein RPA (53). Considering the functional similarities of the E2 proteins among animal and human papillomavirus, it is very likely that HPV-11 E2 also has a similar activity. Alternatively, E2 may aid indirectly the recruitment of host replication proteins to the ori by the E1 protein. Binding of E2 proteins introduces a sharp bend into the DNA (54, 55). This change of DNA conformation may increase E1/E1-BS affinity, stabilize E1/host protein interactions, or facilitate ori unwinding.

The role of E2 in transient replication cannot be fulfilled by the E2C protein devoid of the transacting domain; rather it inhibited replication in transient assays (17). We now have identical results using the cell-free replication assay (Fig. 6). Thus, the N-terminal domain of the intact E2 protein is required for initiation of replication, perhaps through interaction with the E1 protein or the host replication machinery. Interactions between BPV-1 E1 and E2 proteins in the presence or in the absence of an ori have been reported (3, 31, 34–39). Presently, we have not been able to demonstrate any interaction between HPV-11 E1 and E2 proteins under the conditions described in Fig. 1 where specific interactions between DNA and each protein were detected, probably due to a very weak affinity between the two proteins and between the E1 and E1-BS. The relatively strong E1 affinity for nonspecific DNA also presented additional difficulties. However, the transient...
replication assay with matched or mixed pairs of E1 and E2 proteins from different papillomaviruses suggest that such an interaction must exist to account for the differential ability to replicate an ori consisting of synthetic E2-BS alone or a natural ori with both E2-BS and E1-BS. In principle, the E2C might inhibit replication by competing with the full-length E2 for the E2-BS, as concluded for BPV-1 proteins (3, 37, 42). Whereas E2C may not be able to do so. This enhanced E2 affinity for E2-BS relative to E2C could account for a high ratio of E2C to E2 to effect inhibition. Alternatively, heterodimers of E2 and E2C may support replication, as observed with BPV-1 E2 proteins.\(^5\) We have no information about whether such heterodimers could form under our cell-free replication conditions.

In summary, we have used cell-free replication to investigate the functions of papillomavirus E1 and E2 proteins. We show that the E1 protein of HPV-11 is required for initiation and elongation, that HPV-11 E2 is not essential for elongation, and that the HPV-11 E2 protein and the BPV-1 E2 protein are each required for the assembly of preinitiation complex for HPV-11 ori or BPV-1 ori replication. The HPV-11 E2C protein is capable of inhibiting ori replication, albeit very inefficiently.

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\(^5\) D. A. Lim and M. R. Botchan, personal communication.