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# The bovine papillomavirus E2 protein modulates the assembly of but is not stably maintained in a replication-competent multimeric E1–replication origin complex

(DNA replication/negative replication control/nucleoprotein complex/origin binding/unwinding)

MONIKA LUSKY*†, JERARD HURWITZ‡, AND YEON-SOO SEO‡§

*Hearst Microbiology Research Center, Department of Microbiology and Program in Molecular Biology, Cornell University Graduate School of Medical Sciences, 1300 York Avenue, New York, NY 10021; and ‡Graduate Program in Molecular Biology, Memorial Sloan–Kettering Cancer Center, 1275 York Avenue, New York, NY 10021

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ABSTRACT **Initiation of bovine papillomavirus (BPV) DNA synthesis *in vivo* and *in vitro* depends on the interaction of the viral initiator protein E1 with the replication origin (ori$^+$ DNA). The viral E2 protein assists this interaction, resulting in a cooperative assembly of both proteins on the replication origin. Using gel mobility-shift experiments, we demonstrate that in the presence of both E1 and E2 proteins two classes of ori$^+$ DNA complexes were formed: complex 1 (c1) and complex 2 (c2). Formation of c1 depended on both the E1 and E2 proteins and both proteins were contained within c1. The generation of c2 was dependent on the E1 protein and could be enhanced by E2, but the E2 protein was not detected within c2. At high E2/E1 ratios, c1 was the dominant complex formed. Under these conditions, E1-dependent BPV DNA synthesis *in vitro* was inhibited. At low E2/E1 ratios, the stimulation of c2 was correlated with the stimulation of BPV DNA replication by E2 *in vitro*. These data suggest that E2 assists E1 in the formation of an intermediate c1 complex, which is replication inactive. The c1 complex is converted in turn to the replication-active c2 complex, which contains E1 but lacks E2. We propose that the ratios of c1 and c2 formed in response to the levels of E1 and E2 proteins determine the potential for BPV DNA synthesis *in vitro* and *in vivo* and may contribute to copy number regulation of BPV plasmids within the cell.**

Studies of prokaryotic replication systems and small DNA viruses in eukaryotic cells have shown that initiation of DNA synthesis requires the interaction of a specific initiator protein with its cognate replication origin (see ref. 1 for review). In addition, initiator proteins such as the simian virus 40 large tumor antigen facilitate the recruitment of cellular proteins and DNA polymerases to generate an active replication complex at the origin (2–4).

Papillomaviruses, such as bovine papillomaviruses (BPV), are maintained in latently infected cells as nuclear plasmids (see ref. 5 for review) and thus provide an additional eukaryotic system to investigate the assembly of a replication complex and to study the control of copy number.

BPV DNA replication *in vivo* requires two viral proteins—the 68-kDa E1 and the 48-kDa E2 polypeptides (6). The minimal *in vivo* origin (nt 7911–27; refs. 6–8; see also Fig. 5) contains an A+T-rich region (ATR), an E1 binding site (BS) that includes a region of dyad symmetry (DSR), and the E2 BS12 (nt 16–27). E1-dependent BPV DNA synthesis *in vitro* does not require the E2 protein at high levels of E1 (9–11). At low levels of E1, E2 enhances *in vitro* DNA synthesis (9) even in the absence of a well-defined E2 BS (12).

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The E1 polypeptide specifically binds to the BPV replication origin (ori$^+$) (7, 9, 13) and this process can be stimulated and stabilized in the presence of ATP (14, 15). In addition, the E1-associated DNA-dependent NTPase and NTP-dependent DNA helicase activities lead to the specific unwinding of BPV origin-containing plasmid DNAs (10, 14).

In support of a direct role for the 48-kDa E2 transactivator (16) in viral DNA replication, E2 cooperatively assists the binding of E1 to the viral origin (9). Cooperativity is thought to result from a combination of E1–E2 protein interactions (17–20) and protein–DNA interactions, which involve the binding of E2 to the E2 BS12 (8, 14, 15, 21). It was also proposed that E2 acts as a molecular tether to sequester cellular proteins such as the replication protein A (also termed human single-stranded DNA binding protein) and, subsequently, the DNA polymerase $\alpha$–primase complex to an E1–E2-bound ori$^+$ (12).

Gel mobility-shift assays were used to examine the nature of two classes of protein complexes that are assembled on BPV ori$^+$ DNA in the presence of the E1 and E2 proteins. We also explored the functional significance of these complexes in the stimulation by E2 of E1-dependent BPV DNA synthesis *in vitro*. Our results indicate that E2 can stimulate the stepwise assembly of but is not maintained stably in a multimeric replication-competent E1 complex at the BPV origin.

## MATERIALS AND METHODS

**Purification of E1.** The purification of E1 (10) was revised as follows: Nuclear extracts of Sf9 cells containing baculovirus-expressed E1 protein (10) were selected based on their origin binding activity (>5 fmol per 50 ng of extract) in the presence of E2 (60 ng; ref. 14). Saturated ammonium sulfate solution (4°C; pH 7.5) was added to pooled extracts (2.4 mg/ml; 35 ml) to 30%; the supernatant was recovered by centrifugation (12,000 × *g* for 30 min), adjusted to 65% saturation, and centrifuged as described above. The pellets were dissolved in and dialyzed (12 h) against buffer A (10) containing 0.3 M NaCl. BPV ori$^+$ binding activity was enriched (>90%) in the ammonium sulfate 30–65% fraction. The E1 protein was further purified by fast protein liquid chromatography, yielding a preparation of E1 (1.24 mg/ml; 1.2 ml) with replication activity comparable to that previously reported (10).

Abbreviations: BPV, bovine papillomavirus; ori$^+$, origin of replication; BS, binding site; c1, complex 1; c2, complex 2; ATR, A+T-rich region; DSR, region of dyad symmetry.

†To whom reprint requests should be addressed.
§Present address: Samsung Biomedical Research Institute, 50 Ilwon-Dong, Kangnam-Ku, Seoul, Korea, 135-030.

**Purification of E2.** For purification of E2 (14) the sequence-specific binding step was modified: A self-complementary oligonucleotide (108-mer; 5′-biotin-GCT CAA ACC GTC TTC GGT GCA AAC CGT CTT CGG TGC AAA CCG TCT TCG GTG CAA TTG CAC CGA AGA CGG TTT GCA CCG AAG ACG GTT TGC ACC GAA GAC GGT TTG AGC-3′) containing three copies of the E2 BS10 (22) was labeled at the 5′ end with biotin using dimethoxytrityl-biotin-C11-phosphoamidate (Oligos Etc., Guilford, CT). The crude oligonucleotide (total, 2.2 mg; 22 nmol of biotinylated oligonucleotide) was coupled for 30 min to 1.5 ml of streptavidin agarose beads (2.48 mg of streptavidin per ml of packed gel; 50-nmol biotin binding capacity per ml of bead; GIBCO/Bio-Rad) in 5 ml of TEN buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA/0.1 M NaCl). About 35% of the oligonucleotide was coupled to streptavidin, determined by absorbance at 260 nm. With this DNA column, large amounts of E2 protein (1–2 mg) were adsorbed to small amounts of resin (0.8 ml); elution with 1 M NaCl yielded concentrated E2 protein (1.4 mg/ml).

**Plasmids.** Plasmids pKSO (nt 7805–100; ref. 9) and pUCOMΔBS12 (nt 7911–16; ref. 15) were described. pUC/Msp-BS12B (nt 7903–81; ref. 7), containing two point mutations in the E2 BS12, was obtained from A. Stenlund (Cold Spring Harbor, NY). The BPV ori$^+$ sequence in pUCO-BS12 (nt 7911–27; lacking E2 BS11) was inserted at the *Bam*HI/*Xba* I sites in pUC19.

**Isolation and Detection of DNA–Protein Complexes.** Nucleoprotein complexes, formed with biotinylated DNA, were added to streptavidin coupled to metal beads (5 μl; Dynabeads M-280 streptavidin; Dynal, Great Neck, NY) and incubated on ice for 15 min. Complexes were purified with a magnet particle concentrator (Dynal MPC-M) by washing the beads three times in the reaction buffer (0.2 ml each time) containing 0.05 M NaCl and 0.04% Nonidet P-40. Polypeptides were resolved on an SDS/8% polyacrylamide gel. E1 antibodies (19) combined with the enhanced chemiluminescence detection system (ECL; Amersham) were used to detect E1 within DNA–protein complexes on Western blots.

**Gel-Retardation Assays.** DNA–protein complex formation was assayed as described (refs. 14 and 15; see Figs. 1–3) except that potassium glutamate was omitted from the reaction mixtures.

**Glycerol Gradient Sedimentation of DNA–Protein Complexes.** DNA–protein complexes were separated by glycerol gradient sedimentation (5 ml; 27–35% in DNA binding buffer) for 15 h at 37,000 rpm in a Beckman Sw50.1 rotor. Fractions (8 drops) were collected from the bottoms of the tubes. Aliquots (30 μl) of each fraction were subjected to gel electrophoresis as described in Fig. 1. Antibody supershift experiments were as described (15).

**Unwinding and Replication Assays.** Unwinding (10) and replication (11) reactions were carried out as reported.

## RESULTS

**Separation of Distinct BPV ori$^+$ DNA Complexes in the Presence of E1 and E2 Proteins.** We have previously reported that E1–ori$^+$ DNA complexes formed in the presence of E2 included species that migrated significantly faster during gel electrophoresis than those obtained with E1 alone (15). The nature of E1–E2–ori$^+$ DNA complexes was further investigated with a DNA substrate containing the minimal *in vivo* origin (pUCO-BS12; nt 7911–27). In the presence of E1 and E2, two distinct complexes were detected—c1 and c2 (Fig. 1). The slower c2 complex comigrated with the DNA–protein complex formed with E1 alone (lanes 2–5) and depended on the amount of E1 added (compare lanes 6–17 to lanes 2–5). In contrast, c1 increased in response to the level of E2 but decreased when the E1 concentration was raised. Thus, at high E2/E1 ratios, c1 was the predominant complex formed (compare lanes 6 and 7 with lanes 10, 11, 14, and 15). Stable

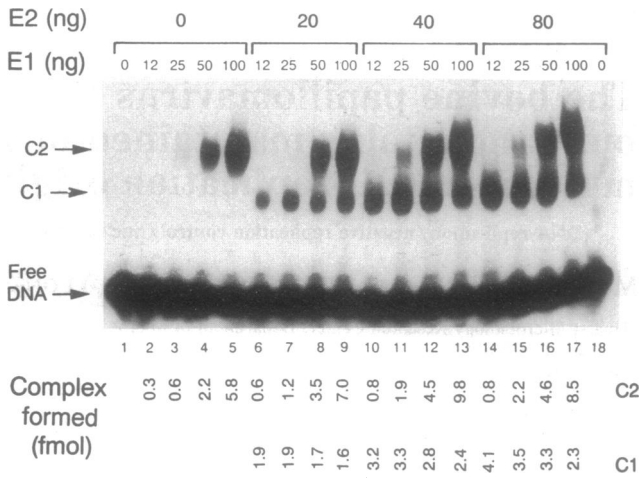


FIG. 1. Requirements for the formation of c1 and c2. E1 and E2 proteins, as indicated, were incubated with 30 fmol of the pUCO-BS12 *Eco*RI/*Hin*dIII fragment (114 bp; labeled with T4 polymerase plus [α-$^{32}$P]dCTP). DNA–protein complexes, fixed with glutaraldehyde (15), were analyzed by electrophoresis through a vertical composite agarose (0.5%)/polyacrylamide (2.4%; 29:1) gel containing 5% (vol/vol) glycerol in 0.5× TBE (45 mM Tris·HCl/45 mM boric acid/1 mM EDTA) for 1 h at 200 V. The gel was dried and subjected to autoradiography. Arrows indicate positions of free DNA, c1, and c2 in the gel. The amounts of c1 and c2 formed (fmol) are presented, and background values (1% and 2% of the input substrate) were subtracted.

complex formation with E2 alone was not detected (lane 18; ref. 15). Therefore, formation of c1 was dependent on the presence of both E1 and E2.

Reaction mixtures containing c1 and c2 were subjected to glycerol gradient sedimentation after glutaraldehyde fixation (Fig. 2 *A* and *B*). c2, formed in the presence of E1 and E2 (Fig. 2*B*), cosedimented with the E1–ori$^+$ DNA complex formed in the presence of E1 alone (Fig. 2*A*), while c1 sedimented more slowly than c2 (Fig. 2*B*). The broad peak of c2 sedimentation suggests that c2 consisted of a population of complexes containing heterogeneous amounts of E1 bound to the BPV origin fragment. Individual fractions, containing c1 and c2 from the gradient shown in Fig. 2*B*, were incubated with E1 and E2 antibodies and analyzed by gel electrophoresis. E1 antibodies retarded the migration of both c1 and c2 complexes (Fig. 2*C*, lanes 2, 5, and 8). In contrast, E2 antibodies altered only the migration of c1 (lane 9) but not that of c2 (lanes 3 and 6) or the DNA complex formed with E1 alone (ref. 15; data not shown).

We conclude that c1 contains both E1 and E2 proteins while only E1 is contained within c2. The E2 protein, although stimulatory for c2 formation, could not be detected within c2.

Investigation of the DNA sequence requirements revealed that generation of c1 required at least half of the E2 BS12. In addition, an intact E1 BS was not mandatory, but it stimulated c1 production. In contrast, the generation of c2 depended on the complete E1 BS (data not shown).

**Different Amounts of E1 Are Contained Within c1 and c2.** To determine the amount of E1 present in c1 and c2, the pUCO-BS12 DNA substrate, labeled with biotin and $^{32}$P, was incubated with high concentrations of E1 to yield c2 and with high E2/E1 ratios to yield c1 (Fig. 3*A*). DNA–protein complexes were separated from free protein by incubation with streptavidin beads and the E1 protein content of c2 and c1 was determined by quantitative Western blotting (Fig. 3*B*). When the same experiment was performed with ori$^-$ DNA, E1 was not detected (data not shown). For each fmol of complex formed, c2 contained 1.2 and 1.1 ng of E1 (lanes 2 and 3), while c1 contained 0.2 and 0.24 ng of E1 (lanes 4 and 5). From these values, we calculated that approximately 10–15 and 2–3 molecules of E1 protein were bound to each

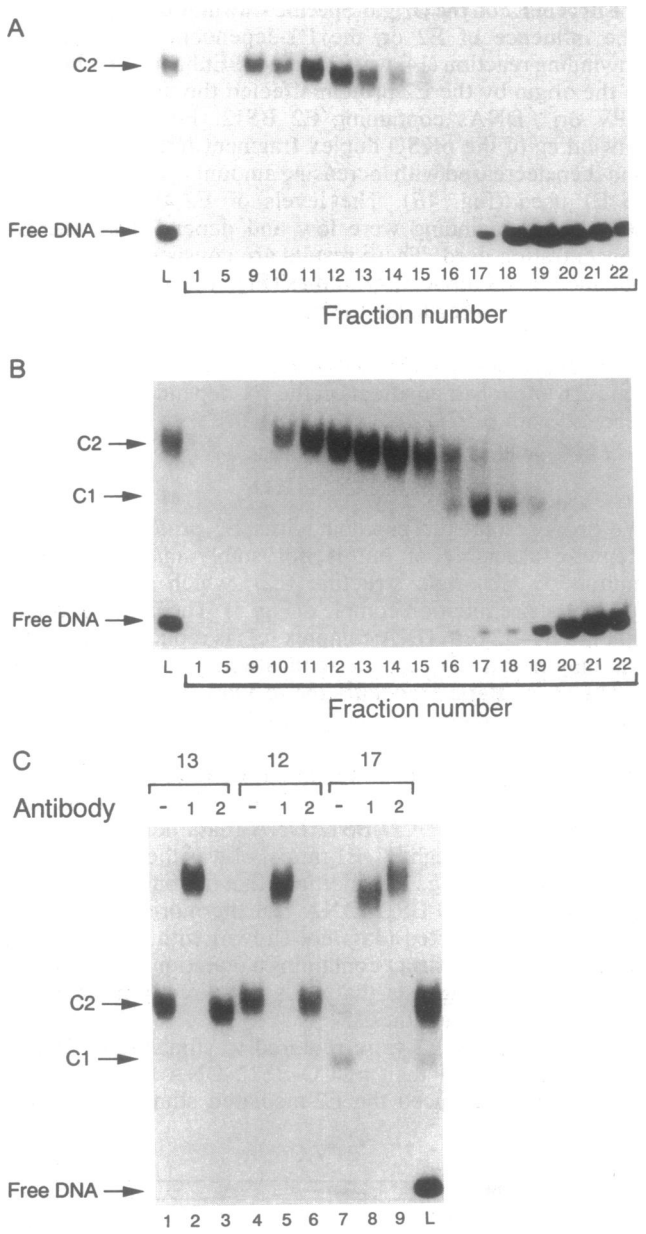


FIG. 2. Glycerol gradient centrifugation of c1 and c2. (*A*) Sedimentation of c2 formed with E1 alone. (*B*) Sedimentation of c1 and c2 formed with E1 plus E2. Binding reactions increased 10-fold; reaction mixtures contained 0.3 pmol of the $^{32}$P-labeled pUCO-BS12 DNA fragment, 1 $\mu$g of E1 protein (*A*) and 1 $\mu$g of E1 plus 0.2 $\mu$g of E2 proteins (*B*). After fixation with glutaraldehyde, DNA–protein complexes were separated by glycerol gradient centrifugation and analyzed as described in the legend to Fig. 1. In lanes L, aliquots representing 5% of the binding reaction mixtures prior to centrifugation were loaded. (*C*) Effect of E1 and E2 antibodies on migration of c1 and c2 complexes. Aliquots (30 $\mu$l) of fractions 12 (lanes 4–6), 13 (lanes 1–3), and 17 (lanes 7–9) from the gradient shown in *B* were incubated for 20 min at 37°C in the absence (lanes −) or presence of 1 $\mu$g of polyclonal E1 antisera (labeled 1) or 1 $\mu$g of polyclonal E2 antisera (labeled 2), respectively, and were analyzed by gel electrophoresis as described above. In lane L, an aliquot representing 5% of the reaction mixture used for the gradient in *B* was loaded on the gel. Arrows indicate positions of free DNA, c1, and c2 in the gels.

molecule of DNA within c2 and c1, respectively. This calculation is based on a molecular mass of the E1 protein of 72 kDa, which contains the influenza hemagglutinin epitope (10). We conclude that c2 contains the E1 protein with 5–6 times the mass of that present within c1 and that the oligomeric state of E1 in c1 and c2 differs.

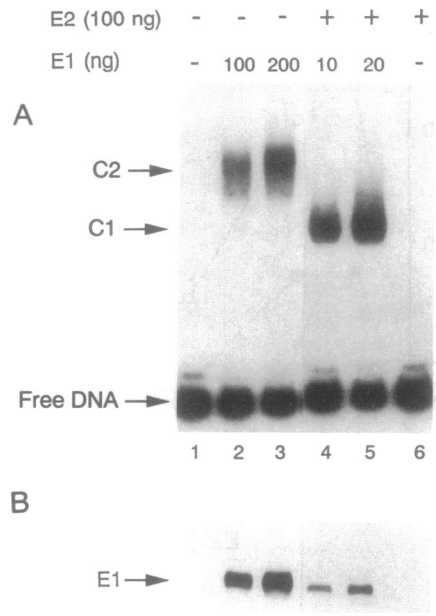


FIG. 3. Determination of the amount of E1 in c1 and c2. Binding reaction mixtures (40 $\mu$l) contained 60 fmol of the pUCO-BS12 DNA fragment {labeled with T4 polymerase plus biotin 16-dUTP (30 $\mu$M; Boehringer Mannheim) and [$\alpha$-$^{32}$P]dATP} and the indicated amounts of E1 and E2 proteins. (*A*) Gel retardation. DNA–protein complexes (10 $\mu$l of reaction mixtures), fixed with glutaraldehyde, were analyzed through a vertical 4% polyacrylamide (29:1) gel containing 5% (vol/vol) glycerol in 0.5× TBE for 1.2 h at 150 V. The amounts of c2 formed were 5.6 fmol (lane 2) and 9.6 fmol (lane 3); the amounts of c1 formed were 7 fmol (lane 4) and 12.6 fmol (lane 5). Arrows indicate positions of free DNA, c1, and c2 in the gel. (*B*) Detection of the E1 protein within c1 and c2 formed in *A*. From the remaining reaction mixtures (30 $\mu$l), DNA–protein complexes were purified and protein analysis was performed. Using enhanced chemiluminescence and densitometric analysis of the film, the E1 protein was detected and quantitated on the Western blot. In comparison to known amounts of E1 loaded on the same gel (data not shown), the amounts of E1 in c2 were 6.5 ng (lane 2) and 9 ng (lane 3), whereas 1.4 ng (lane 4) and 3 ng (lane 5) of E1 were present in c1.

**Effect of E2 on *in Vitro* DNA Synthesis of BPV ori$^+$ DNA.** To determine whether DNA synthesis *in vitro* could be related to ori$^+$ DNA–protein complex formation, replication assays were performed at various E1/E2 ratios with different BPV ori$^+$ DNAs (Fig. 4*A*). The E1-dependent *in vitro* DNA synthesis required an intact E1 BS (data not shown) and was stimulated by the E2 protein at high E1/E2 ratios.

Several points are worth noting: (*i*) The largest E2-mediated stimulation (7-fold) of BPV DNA synthesis was observed in the absence of a well-defined E2 BS (pUCOMΔBS12), whereas the lowest extent of stimulation (3- to 4-fold) occurred in the presence of both E2 BS11 and E2 BS12 (pKSO). Intermediate levels of stimulation were observed in the presence of the wild-type E2 BS12 (4- to 5-fold; pUCO-BS12) or a mutant E2 BS12 (5- to 6-fold; pUC/Msp-BS12B). (*ii*) Maximal stimulation with each plasmid DNA was reached at different E1/E2 ratios. For example, with E1/E2 at a molar ratio of 12:1, E2 stimulation of pKSO DNA synthesis had plateaued, whereas the stimulation of pUCOMΔBS12 DNA synthesis still increased with E1/E2 at a molar ratio of 6:1. (*iii*) For plasmid DNA molecules containing the E2 BS12, the stimulation of DNA synthesis at high E1/E2 ratios corresponded to an increase in the ratio of c2/c1 complexes. (*iv*) High levels of E2 (high E2/E1 ratios), conditions that favor c1 formation with BPV ori$^+$ DNAs that contain the E2 BS12 (see Fig. 1), inhibited replication and unwinding (see below) of all BPV ori$^+$ DNAs examined. The extent of inhibition appeared to be related to

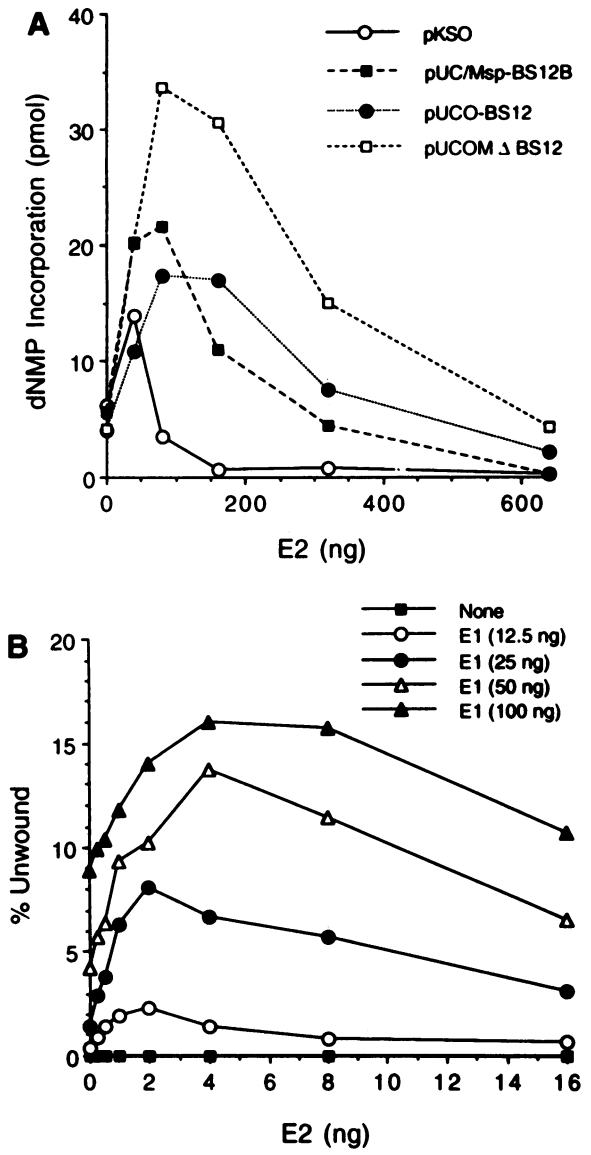


FIG. 4. Effect of E2 on *in vitro* synthesis of BPV ori$^+$ DNAs and on origin-specific unwinding activity of E1. (*A*) DNA synthesis in response to the strength of the E2 BS at the BS12 position. Replication reaction mixtures, with the indicated BPV ori$^+$ DNAs, contained 140 ng of E1 and the indicated amounts of E2. (*B*) Origin-specific unwinding in response to E2. Increasing amounts of E1, in the absence or presence of the indicated amounts of E2, were incubated with the $^{32}$P-labeled *Bam*HI/*Eco*RI fragment of pKSO DNA (232 bp). To prevent origin-independent unwinding, the reactions were carried out in the presence of a 50-fold molar excess of nonspecific competitor DNA. Unwinding was measured as the percentage of duplex DNA fragment converted to single-stranded DNA.

the strength of E2 binding. Marked inhibition was observed with pKSO (containing both BS11 and BS12), while high levels of E2 were less inhibitory for DNA synthesis with pUCOMΔBS12 DNA.

Further studies are required to understand the E2-promoted stimulation of the *in vitro* synthesis of pUCOMΔBS12 DNA, an origin construct that lacks a cognate E2 BS and that is unable to replicate *in vivo* (8). With this DNA, c2 formation was only slightly stimulated by E2; however, at high E2/E1 ratios, c2 was inhibited (15). A complex of the c1 class was not observed (data not shown). It was previously reported that E2 enhanced DNA synthesis *in vitro* with a similar template (12).

**Effect of E2 on the Origin-Specific Unwinding Activity of E1.** The influence of E2 on the E1-dependent origin-specific unwinding reaction (14) was examined. Enhanced E1 binding to the origin by the E2 protein affected the unwinding of all BPV ori$^+$ DNAs containing E2 BS12. For example, the unwinding of the pKSO duplex fragment initially increased and then decreased with increasing amounts of E2 at all levels of E1 used (Fig. 4*B*). The levels of E2 that maximally stimulated unwinding were low and depended on the E1 concentration used. These results are consistent with those obtained in the replication studies (Fig. 4*A*) and suggest that the E1/E2 ratio regulates the replication of ori$^+$ DNAs that contain an E2 BS at the BS12 position by controlling the DNA unwinding reaction catalyzed by E1. As reported previously, the E2 protein had no effect on the E1-dependent unwinding reaction with pUCOMΔBS12 DNA (14).

## DISCUSSION

We propose that the papillomavirus E2 protein orders the stepwise assembly of but is not stably maintained in a multimeric E1 origin structure (c2), which serves as the functional preinitiation complex (Fig. 5). The model predicts that the E1–E2 ori$^+$ DNA complex (c1) is replication inactive and may negatively regulate replication.

**The c1/c2 Ratio Modulates the *in Vitro* Replication Potential.** The E2 protein was not required for but stimulated the formation of c2 at the minimal *in vivo* replication origin (pUCO-BS12) 2- to 4-fold at high E1/E2 ratios. Under these conditions, E2 stimulated the *in vitro* replication (Fig. 4*A*) and unwinding of pUCO-BS12 DNA (data not shown) to a similar degree. At high E2/E1 ratios, c1 was the predominant complex formed (Fig. 1), conditions that inhibited the *in vitro* synthesis of pUCO-BS12 DNA. Furthermore, c1 was the only complex detected (data not shown) with a replication-deficient origin construct containing a mutation within the E1 BS (7, 9). This suggests that c1 is not directly involved in initiation of DNA synthesis.

Lower levels of E2 were required to stimulate or inhibit synthesis of pKSO than pUCO-BS12 DNA (Fig. 4*A*). E2 BS11 further enhanced the E2-mediated stimulation of E1

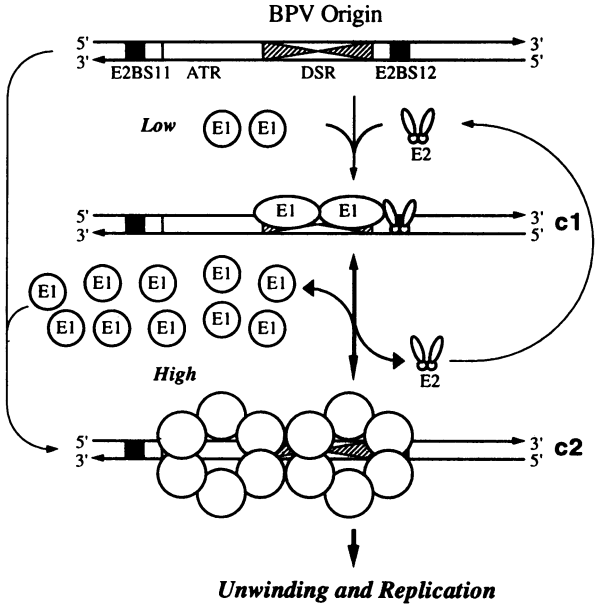


FIG. 5. Model for the E2-induced stepwise assembly of E1 multimers at the BPV origin. The E2 BS11, the ATR, and DSR, which contain the E1 BS and the E2 BS12 within the BPV origin, are depicted. See text for details.

binding (data not shown). Thus, E2 interaction with BS11 might stabilize E1–E2 interactions that lead to c1 complexes, perhaps by DNA looping, which has been reported to occur between E2 binding sites over distances (23).

**E2 Induces the Stepwise Assembly of a Multimeric E1 Complex.** Two pathways for the assembly of a multimeric E1 structure at the origin are proposed (Fig. 5). (*i*) At low levels of E1, E2, indicated in the dimer form, assists the binding of E1 to the DSR, resulting in formation of c1. Consistent with the cis requirements for cooperative binding (14, 15), both E1 and E2 contact the DNA; the protein contacts are shown to occur asymmetrically between E1 and one N-terminal domain of the E2 dimer, suggesting that an E2 heterodimer [consisting of the full-length E2 and an E2 repressor protein (24)] might be sufficient to target E1 to the origin. We suggest that the interaction of E1 and E2 within c1 induces conformational changes within E1 (indicated by the different shapes of free E1 molecules and those bound to DNA in Fig. 5), which accelerate further loading of E1. The resulting multimeric E1 origin (c2) complex occupies both the DSR and the ATR. In support of this, DNase I protection assays indicated that E2 induces a biphasic binding of E1 to the origin, first to the DSR and then to the ATR (25). During the formation of c2, E2 is displaced and can recycle in a reversible process (double arrow), which is facilitated by a weak E2 binding site at the BS12 position. (*ii*) High levels of E1 bypass the requirement for E2.

From the data presented in Fig. 3, it is likely that c1 contains an E1/E2 molar ratio of 2 (two E1 molecules per one E2 dimer). In view of the E1 content of c2 (Fig. 3), the biphasic E1 origin footprinting protection pattern (25), and the different sedimentation properties of c1 and c2 in glycerol gradients (Fig. 2), the c2 complex is depicted as a double multimeric E1 structure analogous to that observed with simian virus 40 large tumor antigen (26, 27).

Consistent with previous results (14, 15), we observed that the formation of c2, but not c1, is enhanced by ATP. In the absence of $MgCl_2$ and ATP, c1 was the predominant complex formed even at high E1/E2 ratios (M.L., unpublished observations). ATP, but not nonhydrolyzable ATP analogues, was required to induce E1-dependent structural changes at the BPV origin, visualized by $KMnO_4$ hyperreactivity within the origin sequence (25). Thus, ATP binding to E1 might lead to conformational changes within an E1–E2 ori$^+$ DNA complex (c1), mediating the formation of a multimeric E1 structure (c2).

Our model predicts that the E2 protein is displaced from the origin during generation of c2. Since both E1 and E2 are required to form c1 (Fig. 1) and since E2 can be detected only in c1 (Fig. 2*C*), we interpret the decrease in c1 with increasing E1 (Fig. 1) to reflect the release of E2. Furthermore, the challenge of a preformed E1–ori$^+$ DNA complex with increasing amounts of E2 resulted in the formation of c1 accompanied by a decrease in c2, indicating a dynamic relationship between the two complexes (data not shown).

The c1/c2 ratio within the cell might also influence E2-mediated transcriptional activation. It was recently reported that enhancement of E2 transactivation *in vivo* required low levels of E1 in trans and the E1 BS plus the E2 BS12 in cis to a reporter gene, while repression of E2 transactivation occurred at high levels of E1, regardless of the presence of the E1 BS (28). The requirements for E2-dependent transactivation *in vivo* (28) appear to be similar to the conditions that favor formation of the replication-inactive c1 complex, but not the replication-competent c2 complex, *in vitro*. Therefore, we suggest that only c1, which contains E1 and E2, can be involved in the kind of transcriptional activation observed by Le Moal *et al.* (28).

**BPV DNA Replication *in Vitro* and *in Vivo*.** Transient replication of BPV DNA *in vivo* was directly proportional to the strength of an E2 BS at the BS12 position (8). However, these (8) and other studies (21, 29–31) do not indicate whether the E1/E2 ratio in the cell influences papillomavirus DNA replication *in vivo*. Recently, it was demonstrated that the E1/E2 expression vector ratios modulated the transient replication of human papillomavirus HPV18 DNA in human C33A cells (32). These data are consistent with our *in vitro* results and suggest that the E1/E2 ratio in the cell may also modulate the replication competence of BPV DNA and contribute to the regulation of copy number *in vivo*.

Our data indicate that the E2 protein, although serving a critical role in assembly (at low levels of E1), is not stably maintained in the developing E1–origin preinitiation complex. The role of E2 resembles the pathway involved in the recruitment of the replicative *Escherichia coli* DnaB helicase by the DnaC and the bacteriophage λ P proteins to the *E. coli* origin (oriC) or the λ origin, respectively (see ref. 1 for review). Further investigations of viral and chromosomal replicons will reveal whether the assembly of preinitiation complexes underlie a general principle.

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