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Bovine papilloma virus (BPV)-encoded E2 protein enhances binding of E1 protein to the BPV replication origin

(origin binding/origin unwinding/DNA replication)

YEON-SOO SEO*, FRIEDEMANN MÜLLER*, MONIKA LUSKY†, EMMA GIBBS*, HAE-YEONG KIM‡, BARBARA PHILLIPS*, AND JERARD HURWITZ*

*Graduate Program in Molecular Biology, Sloan–Kettering Cancer Center, 1275 York Avenue, New York, NY 10021; †Department of Microbiology, Cornell University Graduate School of Medical Sciences, 1300 York Avenue, New York, NY 10021; and ‡Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, 1300 Morris Park, Bronx, NY 10461

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ABSTRACT **The replication of bovine papilloma virus (BPV) DNA *in vivo* requires two viral-encoded proteins, E1 and E2, while all other proteins are derived from the host. We described previously the isolation of the E1 protein and showed that it contains multiple functions required for BPV DNA replication. The BPV transcription factor E2 was shown by others to stimulate BPV DNA replication *in vitro*. Here, we present results that account for the role of the E2 protein. The E1 protein bound selectively to the BPV minimal origin of replication. This process required $MgCl_2$ and ATP for maximal efficiency. The E1 protein also catalyzed a BPV origin-dependent DNA unwinding reaction. In this report, we show that at low levels of E1 protein, origin binding could be stimulated up to 40-fold by the E2 protein, provided that the DNA contained an E2 binding site. Consistent with this result, the E2 protein stimulated the origin-specific unwinding reaction catalyzed by E1, but it had no effect on the nonspecific E1-catalyzed helicase activity. In the absence of an E2 binding site, both origin-dependent binding and unwinding reactions with the E1 protein were unaffected by the E2 protein. These results suggest that E2 participates in the initiation of BPV DNA replication by enhancing E1 binding to the BPV origin via DNA–protein and protein–protein interactions.**

Bovine papilloma virus (BPV) provides an attractive model system to study the regulation of eukaryotic DNA replication. The viral DNA is maintained in BPV transformed cells as a nuclear plasmid with a constant copy number (1). Recent studies indicate that *in vivo* BPV DNA replication requires two BPV viral-encoded proteins—the 68-kDa E1 protein and the 48-kDa E2 protein (2). In addition, the minimal origin sequence that supports BPV DNA replication has been identified (nt 7911–22 of the BPV type 1 genome) (3, 4). This sequence contains a binding site for the E1 protein (E1 BS) and part of a sequence that acts as a binding site for the E2 protein (E2 BS).

The replication of BPV ori$^+$ DNA *in vitro*, using extracts of a mouse mammary tumor cell line (FM3A), was recently established in Botchan's laboratory (4). They demonstrated that DNA synthesis required the BPV minimal origin and the E1 protein, whereas all other required proteins were supplied by uninfected mouse cell extracts. At low concentrations of E1 protein, the replication reaction was stimulated markedly by the E2 protein (4).

We have described the isolation of the E1 protein and showed that it supported BPV ori$^+$ DNA replication *in vitro* (5). We also demonstrated that this protein possessed a number of different activities required for BPV DNA replication. These include (*i*) a DNA helicase activity, for which the protein translocates in the 3′ to 5′ direction, (*ii*) a BPV ori$^+$ DNA binding activity that is stimulated by ATP and $MgCl_2$, and (*iii*) the capacity to unwind covalently closed circular ori$^+$ DNA leading to highly unwound DNA products (5). Thus, the role of E1 in the BPV system is analogous to that of large tumor antigen (T antigen) in the replication of simian virus 40 (SV40) DNA (6–8). However, a notable difference between the two systems is the requirement for the E2 protein in the BPV replication system.

In this report, we have examined the interaction of the E1 protein with BPV ori$^+$ DNA fragments by nitrocellulose filter binding and unwinding assays. We observed that incubation at 37°C in the presence of ATP stimulated formation of the E1 protein–BPV ori$^+$ DNA complex and unwinding of ori$^+$ DNA fragments. Both reactions were markedly stimulated by the E2 protein, provided the DNA contained an E2 BS. These results demonstrate the involvement of the E2 protein in initiation of BPV DNA replication and account for its requirement in this system.

MATERIALS AND METHODS

Cell Cultures and Recombinant Baculoviruses. Growth of the mouse FM3A cell line and preparation of extracts and the recombinant baculoviruses containing wild-type BPV E1 and E2 genes were as described (4, 9). The E1 protein used here contained the hemagglutinin epitope of influenza virus (10) followed by a thrombin cleavage site.

Enzymes and DNA. Human single-stranded DNA binding protein (HSSB) (11) and SV40 T antigen (12) were prepared as described. The E1 protein and plasmids pUCOM and pKSO were prepared as described by Seo *et al.* (5). pUCOM contains E1 BS and half of E2 BS12 (nt 7911–22 of BPV type 1 genome). pKSO, a kind gift of M. Botchan (University of California, Berkeley), contains the E1 BS plus the E2 BS11 and -12 (nt 7805–100). pUCO-Xho, generated by insertion of a commercially available *Xho* I linker into the unique *Hpa* I site of the BPV origin in pUC/Msp (nt 7903–81) (3), was a kind gift of A. Stenlund (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). pUCOMΔBS12 (nt 7911–16) was generated by deleting the half E2 BS12 present in pUCOM. Plasmid DNAs were isolated by standard procedures (13) and purified by two successive CsCl gradients. Linear duplex DNA fragments used to assay the E1 origin binding and unwinding activities were prepared by isolating the 232-bp *Eco*RI/*Pvu* II DNA fragment, 296-bp *Eco*RI/*Pvu* II DNA fragment, 290-bp *Eco*RI/*Pvu* II fragment, 366-bp *Eco*RI/*Pvu* II DNA fragment, and the 242-bp *Eco*RI/*Bam*HI DNA

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Abbreviations: BPV, bovine papilloma virus; SV40, simian virus 40; T antigen, large tumor antigen; HSSB, human single-stranded DNA binding protein; BS, binding site(s); RFI, covalently closed circular duplex; DTT, dithiothreitol.

fragment by restriction cleavage of pUC19, pUCOM, pUCOMΔBS12, pUCO-Xho, and pKSO, respectively. Each plasmid (4 pmol) was cleaved with *Eco*RI and labeled with [α-^{32}P]dATP or [α-^{32}P]dCTP with T4 DNA polymerase (Pharmacia) as described by Murakami and Hurwitz (14). Each possessed a specific activity of ≈5000 cpm/fmol.

Purification and Assay of the E2 Protein. E2 protein was prepared and measured as described (15) with some modifications. Briefly, extracts from Sf9 cells, infected with a recombinant baculovirus that expressed the wild-type E2 protein, were prepared as described for the isolation of SV40 T antigen (12). The extract of infected cells (390 ml; 2.8 mg/ml) was dialyzed against buffer A [25 mM potassium phosphate, pH 6.5/10% (vol/vol) glycerol/1 mM dithiothreitol (DTT)/0.2 mg of antipain per liter/0.1 mg of leupeptin per liter/0.1 mM phenylmethylsulfonyl fluoride] containing 0.03 M NaCl and directly loaded onto a phosphocellulose (P11; Whatman) column (5 × 10 cm; 200 ml) equilibrated with buffer A containing 0.03 M NaCl. The column was washed successively with 0.03, 0.3, and 0.7 M NaCl in buffer A. The E2 protein, which eluted with 0.3 M NaCl, was diluted with buffer A to 0.25 M NaCl and loaded onto a sequence-specific DNA affinity column. This column consisted of the multimerized 21-bp oligonucleotide (5′-GGTCAAACCGTCT-TCGGTGCT-3′) containing the E2 BS10 sequence (16) coupled to CNBr-Sepharose (17). The final preparation of E2 protein complexed 1.2 nmol of a duplex DNA containing the E2 BS10 per mg of protein.

Nitrocellulose Filter Binding Assay. Reaction mixtures (20 μl) containing 25 mM potassium phosphate, pH 7.5/10% glycerol/0.1 M potassium glutamate/1 mM EDTA/0.5 mM DTT/7 mM $MgCl_2$/4 mM ATP/bovine serum albumin (250 μg/ml)/0.5 μg of a 1-kb DNA ladder (Bio-Rad) as competitor DNA/30 fmol of the substrate DNA and E1 protein, as indicated, were assembled on ice and incubated for 15 min at 37°C. These conditions are similar to those used by Yang *et al.* (4) and are referred to below as "footprinting conditions." An alternative reaction mixture (20 μl), similar to the replication mixture described below, contained 30 mM creatine phosphate (diTris salt, pH 7.5), 7 mM $MgCl_2$, 4 mM ATP, 0.5 mM DTT, 4 μg of creatine phosphokinase, bovine serum albumin (250 μg/ml), 0.5 μg of a 1-kb DNA ladder (Bio-Rad) as competitor DNA, 30 fmol of the substrate DNA, and E1 protein, as indicated and is referred to as "replication conditions." After incubation, reaction mixtures were then passed through alkaline-treated nitrocellulose filters (0.45 μm; Millipore) (18) presoaked in wash buffer (0.1 M Tris·HCl, pH 7.5). The filters were washed with three 1-ml aliquots of wash buffer, dried, and assayed for radioactivity.

Replication Assay. Reaction mixtures (40 μl) contained 30 mM creatine phosphate (diTris salt, pH 7.5), 7 mM $MgCl_2$, 4 mM ATP, 0.5 mM DTT, 4 μg of creatine phosphokinase, 100 μM rNTPs, 25 μM dNTPs, 40 μg of FM3A extract, 0.4 μg of HSSB, 80 ng of pKSO or pUCO-Xho RFI DNA, and 240 ng of pSVLD covalently closed circular duplex (RFI) DNA (19) as nonspecific competitor. Incorporation of [α-^{32}P]dCTP was monitored by formation of acid-insoluble material.

DNA Unwinding Assay. Unwinding of BPV ori$^+$ DNA fragments with the E1 protein was performed using the replication conditions described above. Reaction mixtures (20 μl) contained 30 mM creatine phosphate (diTris salt, pH 7.5), 7 mM $MgCl_2$, 0.5 mM DTT, creatine phosphokinase (100 μg/ml), bovine serum albumin (250 μg/ml), 0.4 μg of HSSB, 15 fmol of ^{32}P-labeled ori$^+$ DNA fragments, 0.2 μg of a 1-kb DNA ladder (Bio-Rad) as a nonspecific competitor, and 4 mM ATP as the only nucleotide. The reaction mixtures were incubated at 37°C for 1.5 hr and products were analyzed as described (11). The unwinding assay with supercoiled covalently closed circular DNA used to measure form U DNA (highly unwound DNA) production was carried out as described above except that either phage λ DNA or pSVLD DNA was used as the nonspecific competitor DNA instead of the 1-kb DNA ladder, and topoisomerase I (400 units) was added to the reaction mixture. Products were analyzed as described by Bullock *et al.* (20).

RESULTS

Binding of the E1 Protein to DNA. Nitrocellulose filter binding was used to examine the interaction between the E1 protein and BPV ori$^+$ DNA. Two assay conditions were used to study this reaction: one was as described by Yang *et al.* (4) used for DNase I protection (footprinting) experiments, while the other was the condition that supported BPV DNA replication.

Increasing amounts of E1 were incubated at 37°C with the ^{32}P-labeled ori$^+$ fragment isolated from pKSO. The reaction mixtures were filtered through a nitrocellulose membrane to bind DNA complexed with E1 (Fig. 1 *A* and *B*). Under the footprinting conditions, virtually no ori$^+$ DNA binding was observed in the absence of both $MgCl_2$ and ATP (Fig. 1*A*); under these conditions, binding was detected only when high levels of E1 (>0.2 μg) were used (data not shown). The addition of $MgCl_2$ stimulated complex formation, which was further increased (2-fold) by the addition of ATP and $MgCl_2$ (Fig. 1*A*). The addition of ATP alone did not affect complex formation (data not shown). At an E1/substrate DNA molar ratio of 48 (0.1 μg of E1), ≈35% of the ori$^+$ DNA fragment was bound in the presence of ATP and $MgCl_2$. Comparable amounts of complex were obtained with ori$^+$ DNA fragments from pUCOM or pUCOMΔBS12, which lack an intact E2 BS (Fig. 2). pUC19 DNA fragments lacking the ori$^+$ sequence barely supported complex formation (Fig. 2*A*). The amount of complex formed showed a sigmoidal response to the concentration of E1 added. This suggests that E1 forms multimers at the BPV origin DNA, analogous to the SV40 T antigen (12).

We also tested complex formation by using conditions that support BPV DNA replication. Under these conditions, ATP stimulated complex formation 5- to 10-fold and halved the concentration of E1 required to obtain an equivalent amount of E1–DNA complex formed by using footprinting conditions (Fig. 1*B*). ATP supported the complex formation more efficiently than did nonhydrolyzable ATP analogues, using replication conditions (Fig. 1*C*). All eight NTPs (at 4 mM) efficiently supported E1–ori$^+$ DNA complex formation (data not shown). This suggests that NTP hydrolysis may facilitate complex formation. The influence of temperature was also examined. The stimulatory effect of ATP and $MgCl_2$ was observed when reaction mixtures were incubated at 37°C but not at 0°C or 23°C (data not shown).

E2 Protein Stimulates Formation of E1 Protein–ori$^+$ DNA Complex Only in the Presence of an E2 BS. The influence of the E2 protein on E1–ori$^+$ DNA complex formation was examined. As shown in Fig. 2*A*, binding of E1 to the pKSO ori$^+$ fragment, which contained both E2 BS11 and -12, was markedly stimulated by the E2 protein. The greatest effect (>40-fold) was observed at the lowest level of E1 used (25 ng) (Fig. 2*A*). Even though two E2 BS were present in the DNA, E2 alone (60 ng) did not form a stable complex with the pKSO DNA fragment (<1 fmol). The stimulatory effect of E2 decreased with increasing amounts of E1, suggesting that E2 is not required for the ori$^+$ DNA binding in the presence of excess E1 protein. The influence of E2 was also tested with the pUCOM ori$^+$ DNA fragment, which contains half of E2 BS12. The stimulatory effect of E2 with this fragment was considerably lower (2- to 3-fold) than observed with the pKSO fragment. In the absence of E2, however, E1 interacted with both ori$^+$ DNA fragments identically (Fig. 2*A*). In contrast to the stimulatory effect of the E2 protein with ori$^+$

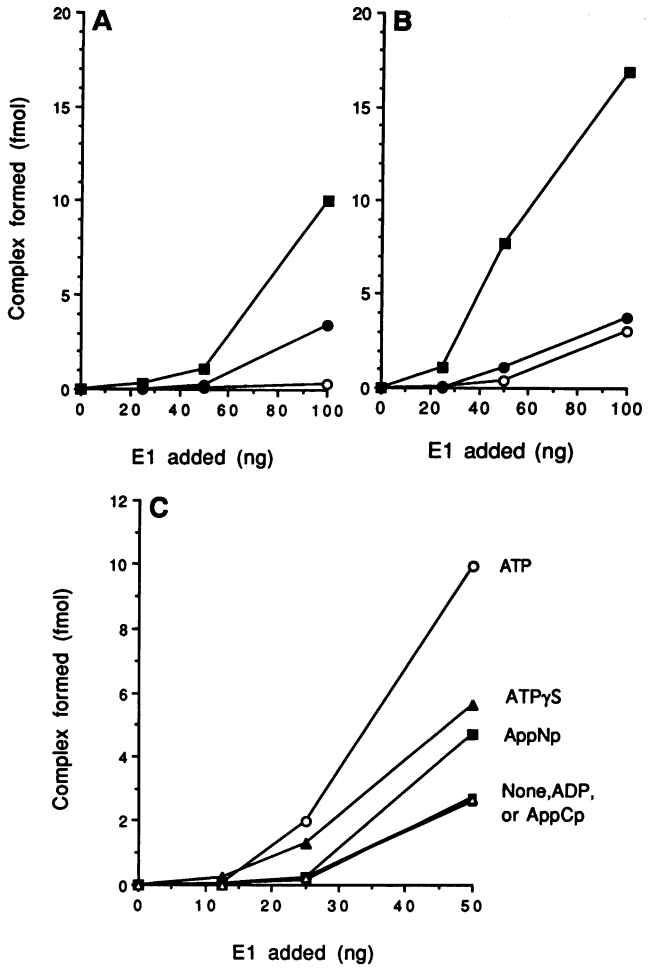

FIG. 1. Influence of $MgCl_2$, ATP, and ATP analogues on binding of the E1 protein to BPV ori$^+$ DNA. Reactions were carried out under footprinting conditions (*A*) or under replication conditions (*B*). Various concentrations of E1 were incubated at 37°C for 15 min with the ori$^+$ DNA (pKSO) fragment in the presence of $MgCl_2$ alone (solid circle), in the presence of both ATP and $MgCl_2$ (solid square), or in the absence of both (open circle). After incubation, reaction mixtures were adsorbed to nitrocellulose filters, washed, and then assayed for radioactivity. (*C*) Effects of ATP analogues on binding of E1 to the origin. Reactions were carried out under replication conditions as described above. With ADP, creatine phosphokinase was omitted from the reaction mixture. The nucleotides used (4 mM) are indicated. ATPγS, adenosine 5′-[γ-thio]triphosphate; AppNp, adenosine 5′-[β, γ-imido]triphosphate; AppCp, adenosine 5′-[β, γ-methylene]triphosphate.

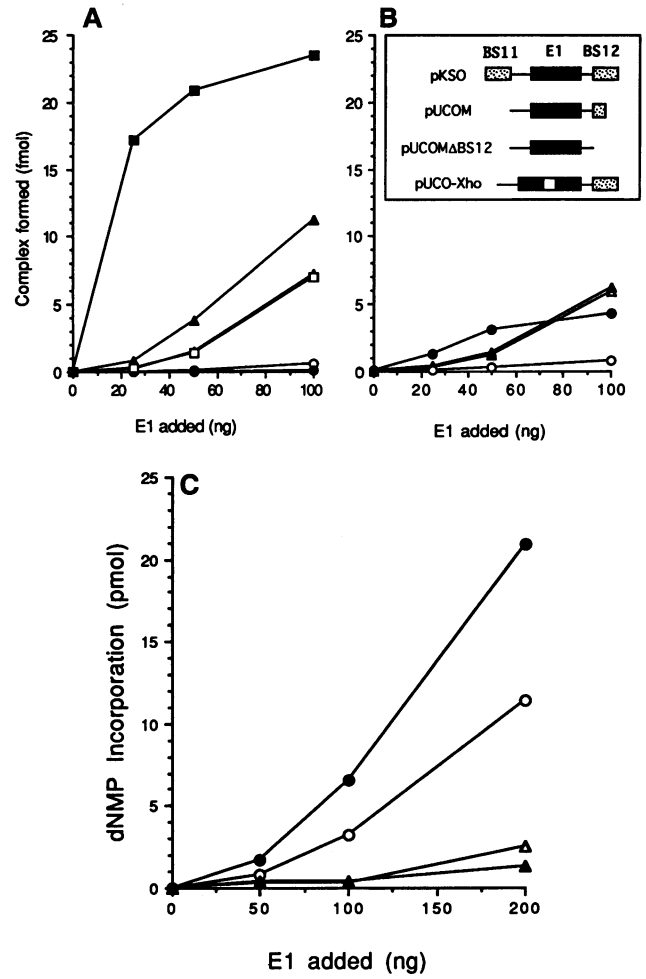

FIG. 2. Influence of the E2 protein on binding of E1 to ori$^+$ DNA. (*A*) Various concentrations of E1 were incubated at 37°C for 15 min with labeled fragments isolated from either pKSO (squares), pUCOM (triangles), or pUC19 (circles) DNA with (solid symbols) or without (open symbols) the E2 protein (60 ng). Reactions were carried out under footprinting conditions as described. A summary of the different BPV ori$^+$ DNA fragments used is shown in *B*. (*B*) The same reactions described in *A* were repeated with labeled DNA fragments from pUCOMΔBS12 (triangles) or pUCO-Xho (circles) DNA with (solid symbols) or without (open symbols) the E2 protein (60 ng). (*Inset*) E1 BS (solid box), E2 BS11 and -12 (stippled box), and *Xho* I linker insertion (open box). (*C*) Replication reactions were carried out with pKSO RFI DNA (circles) and pUCO-Xho RFI DNA (triangles) in the presence (solid symbols) or absence (open symbols) of E2 as described.

DNA, E2 reduced E1 binding to nonspecific DNA isolated from the pUC19 vector 3- to 4-fold (Fig. 2*A*).

The effect of E2 on the DNA replication reaction was also examined. Nucleotide incorporation in the presence of ori$^-$ DNA was also reproducibly reduced by E2 addition, whereas E2 stimulated ori$^+$ DNA replication 2-fold (Fig. 2*C*).

Stimulation of E1–ori$^+$ DNA complex formation by E2 in the presence of an E2 BS suggests that the E2 BS12, adjacent to the E1 BS, plays an important role in E1 binding to the origin. This was verified by using the pUCOMΔBS12 DNA fragment in which E2 BS12 was completely deleted. As shown in Fig. 2*B*, complex formation with this substrate was unaffected by the presence of E2. We also tested the pUCO-Xho DNA fragment, which contained an *Xho* I linker inserted in the middle of E1 BS in addition to the intact E2 BS12. In the absence of E2, the pUCO-Xho fragment hardly complexed with E1 (<0.5 fmol); however, E2 stimulated complex formation significantly, although the total complex formed was 3- to 4-fold lower than that observed with the pKSO fragment.

No effect of E1 protein on the binding of E2 to a duplex oligonucleotide that contained E2 BS10 was observed (data not shown). However, the possibility that E1 can affect E2 binding to an E2 BS adjacent to an E1 BS as is present in the ori$^+$ DNA remains to be explored.

Origin-Specific Unwinding Activity of the E1 Protein. Since the E1 protein binds specifically to the BPV origin and possesses DNA helicase activity (5), we examined whether the E1 protein unwinds ori$^+$ duplex DNA fragments. The E1 protein displaced both pUC19 (ori$^-$) and pKSO (ori$^+$) duplex DNA fragments in the presence of ATP and HSSB when no competitor DNA was added (Fig. 3 *A* and *B*, lanes 4). The unwinding reaction was dependent on the presence of ATP, HSSB, and the E1 protein (Fig. 3 *A* and *B*, lanes 1–3). However, the ori$^+$ pKSO fragment was unwound more efficiently (6-fold) than the ori$^-$ pUC19 fragment (Fig. 3 *A* and

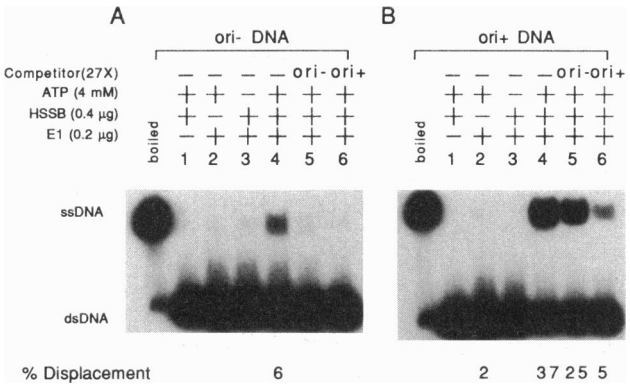

FIG. 3. Unwinding of BPV ori$^+$ DNA by the E1 protein. Unwinding reactions were carried out with ori$^-$ pUC19 fragments (*A*) and with ori$^+$ pKSO DNA fragments (*B*) as described. Additions or omissions are indicated above each lane. Numbers below indicate percentage input duplex fragment converted to single-stranded DNA (ssDNA). dsDNA, double-stranded DNA.

B, compare lanes 4). The E1-dependent unwinding of the pUC19 DNA fragment was completely abolished by the presence of a 27-fold molar excess of competitor DNA, either ori$^-$ or ori$^+$ DNA (Fig. 3*A*, lanes 5 and 6). In contrast, with the pKSO DNA fragment, the E1 protein still produced unwound single-stranded DNA in the presence of competitor ori$^-$ DNA, although the efficiency was slightly reduced (Fig. 3*B*, lane 5); the addition of a 27-fold molar excess of ori$^+$ DNA as competitor reduced labeled single-stranded DNA formation >7-fold. This demonstrated that the E1 protein alone catalyzes the origin-specific unwinding reaction in the absence of the E2 protein.

E2 Protein Stimulates the Origin-Specific Unwinding Activity of E1 Protein. The enhancement of E1 binding to the origin by the E2 protein influenced the unwinding of ori$^+$ DNA as well. In these experiments, unwinding reactions were carried out in the presence of 50-fold molar excess of the 1-kb DNA ladder (Bio-Rad) as competitor to prevent non-origin-dependent unwinding. The unwinding of both pKSO and pUCOMΔBS12 DNA fragments was increased in response to increasing amounts of E1 protein alone (Fig. 4 *A* and *B*, lanes 2–5). About 20% of the input DNA was displaced in the presence of 0.2 μg of E1 protein after 1.5 hr of incubation. When the E2 protein (60 ng) was included in the reaction, the unwinding of the pKSO DNA fragment, which contains E2 BS12, was increased 5- to 6-fold (Fig. 4*A*, lanes 6–9); in contrast, the E2 protein had no effect on the unwinding reaction with the pUCOMΔBS12 fragment, which lacks an E2 BS (Fig. 4*B*, lanes 6–9). In the presence of the E2 protein, the unwinding of the pUCOM DNA fragment, which contains the origin and half of E2 BS12 was stimulated to the same extent as displacement of the pKSO fragment (data not shown). However, the E2 protein did not affect the unwinding efficiency of ori$^-$ DNA (pUC19 DNA fragment) in the absence of competitor DNA (data not shown). This is consistent with the observation that the E2 protein did not affect the nonspecific DNA helicase activity of E1 (5).

Unwinding of Supercoiled ori$^+$ DNA. The E1 protein specifically unwinds circular duplex BPV ori$^+$ DNA, generating form U DNA in the presence of a SSB, topoisomerase I, and ATP. As shown in Fig. 4*C*, a rapidly migrating form U DNA product was formed by the E1 protein. Formation of form U required the BPV origin, ATP, and HSSB. Adenosine 5′-[γ-thio]triphosphate did not support this reaction (Fig. 4*C*). The origin specificity was observed only in the presence of competitor DNA such as pSVLD plasmid or phage λ DNA. In the absence of competitor DNA, both ori$^+$ pKSO plasmid and ori$^-$ pUCO-Xho plasmid were unwound efficiently (30%

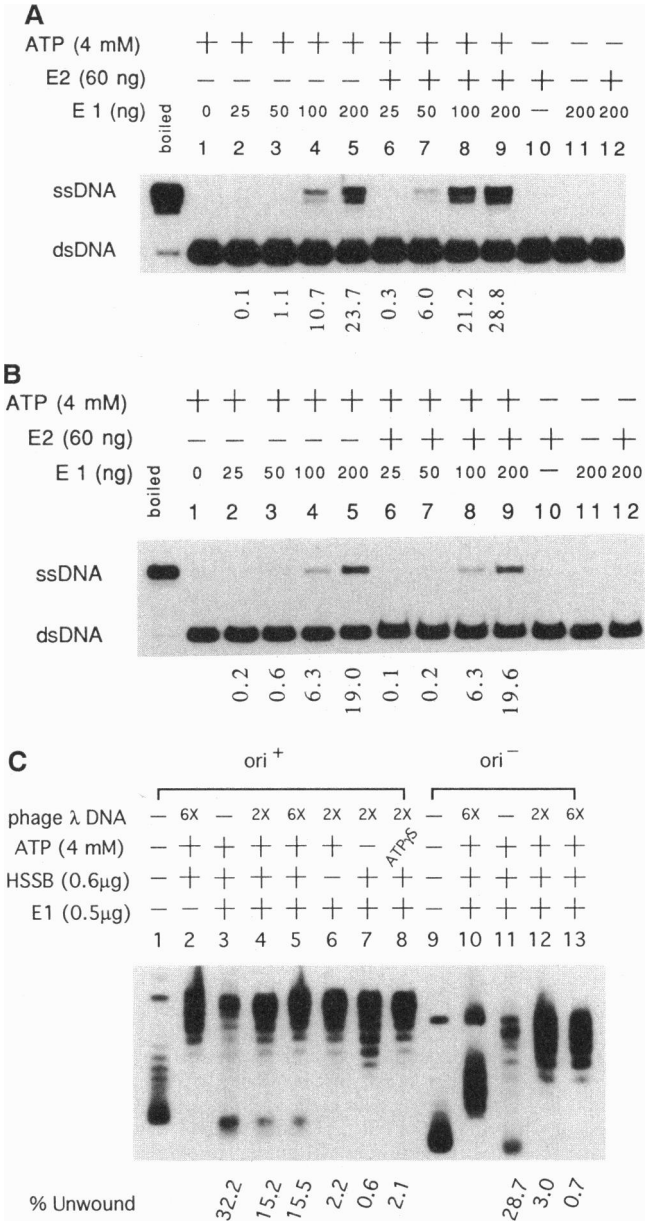

FIG. 4. Influence of E2 protein on E1 catalyzed unwinding of ori$^+$ DNA. (*A*) Increasing amounts of E1 in the absence and presence of the E2 protein (60 ng) were incubated with ^{32}P-labeled pKSO DNA fragment. Additions and omissions are indicated above each lane. Numbers below indicate percentage duplex DNA fragment converted to single-stranded DNA (ssDNA). Background values, which were normally 2–3% of the input substrate, have been subtracted. (*B*) The same experiment described in *A* was repeated with a pUCOMΔBS12 DNA fragment. (*C*) Unwinding assays with ori$^+$ (pKSO) and ori$^-$ (pUCO-Xho) plasmids were carried out as described. Each reaction mixture contained 100 ng of RFI DNA. After 30 min of incubation at 37°C, products were electrophoresed on a 1.7% agarose gel containing chloroquine (1.5 μg/ml) and photographed after staining with ethidium bromide. Additions or omissions are indicated above each lane. The concentration of adenosine 5′-[γ-thio]triphosphate used was 4 mM. Numbers below represent percentage input DNA converted to form U measured by phosphoimage analysis of an autoradiogram obtained by the Southern blot hybridization technique (13). dsDNA, double-stranded DNA.

of total input DNA) by the E1 protein. However, form U production from the ori$^-$ plasmid was abolished with increasing concentrations of competitor DNA, whereas form U produced from ori$^+$ DNA was partially reduced by 2- to 6-fold increases in competitor DNA (Fig. 4*C*, lanes 4, 5, 12, and 13).

This suggests that the E1/DNA ratio used influences origin specificity. At high ratios, binding, unwinding, and replication occur with DNA lacking the BPV origin (data not shown).

DISCUSSION

We have shown that the E1 protein binds specifically to BPV ori$^+$ DNA. These results are in accord with the reports of Wilson and Ludes-Meyers (21) and Ustav *et al.* (3) that the E1 protein specifically binds to the BPV origin. Footprinting studies (4) also indicated that purified E1 protein protected DNA sequences centered about the 18-bp palindrome region at the origin. In all of these experiments, however, ATP was not added. As shown in Fig. 1, the presence of ATP significantly affected the binding of E1 to ori$^+$ DNA, especially under conditions that support BPV DNA replication. It is likely that ATP binding to E1 induces conformational changes in the protein that affect its stable binding to the origin. These observations are strikingly similar to those made with SV40 T antigen (22). The complex formed in the absence of either $MgCl_2$ or ATP was less stable (3- to 4-fold) than the complex formed in the presence of ATP and $MgCl_2$ (data not shown).

At low levels of E1 protein, origin binding was stimulated up to 40-fold by the E2 protein. This is consistent with the observation that the E2 protein enhanced E1 DNA binding activity nearly 10-fold, as measured by DNA footprinting assays (4). To observe this effect, DNA containing both the E1 BS and at least half of the E2 BS12 was required. Similar requirements have been reported for replication of BPV DNA *in vivo* in short-term transfection assays (3). These results suggest that the E2 protein plays a key role in targeting the E1 protein to the BPV origin through E2 BS adjacent to the inverted repeats that define the E1 BS. This process is probably mediated by both protein–DNA (E1/E2–DNA) and protein–protein (E1–E2) interactions around the origin sequence. Half of the inverted repeats of the E1 BS plus the E2 BS12 (pUCO-Xho DNA) supported E1 complex formation in the presence of the E2 protein; the E1 protein alone formed only low levels of a complex with this DNA. The sigmoidal nature of the nucleoprotein complex formation and the ability of E1 to complex with the half-inverted repeat of the E1 BS in the presence of the E2 BS12 suggest that E1 may be assembled on both sites of the inverted repeat sequence. An E1 multimer, interacting with E2 protein complexed to E2 BS12, may facilitate the assembly of other E1 multimers on the second half of the inverted repeats. This observation is reminiscent of the interaction of SV40 T antigen with the SV40 origin (12). In the SV40 system, hexamers are assembled on each half site of the palindromic DNA sequence at the SV40 core origin and subsequently direct initiation of DNA synthesis.

Consistent with the observation that E2 stimulated E1 binding to the origin, E2 enhanced the origin-specific unwinding activity catalyzed by the E1 protein. The stimulation also required the presence of E2 BS12; unwinding of pUCOMΔBS12 DNA, which lacks both E2 BS11 and -12, was unaffected by the presence of E2 protein. We have not observed a stimulation of form U production by the addition of the E2 protein. These studies have been hampered by the observation that form U products, formed in the absence of E2, were reduced upon incubation with the E2 protein, suggesting the presence of an endonuclease in our E2 preparation (data not shown).

We have shown here that BPV origin binding and origin-specific unwinding activities reside with the E1 protein, while the E2 protein acts as an auxiliary factor that enhances these E1 activities. However, the detailed structure and nature of the complex assembled at the BPV origin remains to be elucidated.

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