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Bovine papilloma virus (BPV)-encoded E1 protein contains multiple activities required for BPV DNA replication

(DNA helicase/highly unwound superhelical DNA/origin binding)

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ABSTRACT **Replication of bovine papilloma virus (BPV) DNA requires two virus-encoded proteins, E1 and E2, while all other proteins are supplied by the host cell. Here, we describe the isolation of the E1 protein and show that it is a multifunctional protein. Purified E1 protein was required for the *in vitro* replication of BPV origin-containing DNA by extracts of mouse cells, as reported [Yang, L., Li, R., Mohr, I. J., Clark, R. & Botchan, M. R. (1991) *Nature (London)* 353, 628–632]. In addition, the E1 protein cosedimented with a number of other activities including (*i*) DNA helicase activity, (*ii*) BPV origin-containing DNA-specific binding activity, (*iii*) DNA-dependent ATPase activity, and (*iv*) BPV origin-specific unwinding of superhelical DNA. The E1 protein, acting as a helicase, moved in the 3′ → 5′ direction, like simian virus 40 (SV40) large tumor antigen, which plays a pivotal role in SV40 DNA replication. However, unlike the SV40 large tumor antigen, the helicase activity of E1 was stimulated 5-fold by the presence of a fork structure at the junction between single-stranded and double-stranded DNA and was supported efficiently by all eight nucleoside triphosphates. The E1-catalyzed ATPase activity required the presence of single-stranded or double-stranded DNAs.**

In cells transformed with bovine papilloma virus (BPV), the viral DNA exists as a free replicating plasmid at a constant copy number (1). The replication of BPV DNA *in vivo* was shown to depend completely upon two BPV-encoded proteins, the 48-kDa E2 protein and the 68-kDa E1 protein (2). In addition, the minimal origin sequence required to support BPV DNA replication has been identified (nt 7911–22 of BPV DNA) (3, 4). This sequence contains a binding site for the E1 protein and part of a sequence that acts as a binding site for the E2 protein.

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

We have isolated and characterized the two viral-encoded proteins required for this replication pathway, E1 and E2. In this report, we describe the biochemical properties of the purified BPV E1 protein. We show that this protein supports the replication of BPV ori$^+$ DNA *in vitro*, as reported (4). We also demonstrate that this protein possesses a number of different activities required for the replication of the BPV viral genome. These include a DNA helicase activity that moves in the 3′ → 5′ direction, a BPV ori$^+$ DNA-binding activity that is stimulated by ATP and $MgCl_2$, and the ability to unwind superhelical DNA leading to the production of highly unwound superhelical DNA (form U DNA). Thus the role of E1 in the BPV system is analogous to the role of large tumor antigen (T antigen) in the replication of simian virus 40 (SV40) DNA (5–7).

MATERIALS AND METHODS

Cell Cultures and Recombinant Baculoviruses. Growth conditions of the mouse FM3A cell line and the preparation of extracts were as described (4). Recombinant baculoviruses containing wild-type BPV E1 and E2 genes have been described (8). The E1 protein used here contained the hemagglutinin epitope of influenza virus (9) followed by a thrombin cleavage site. An oligonucleotide encoding the amino acid residues MYPYDVPDYASLGGPLPRGS was ligated in the *Nla* III site (nt 848) at the first methionine codon of the E1 gene. This increased the size of the E1 protein to ≈70 kDa.

Enzymes and DNA. Human single-stranded DNA-binding protein (SSB) and SV40 T antigen were prepared as described by Seo *et al.* (10) and by Mastrangelo *et al.* (11), respectively. Plasmid pUCOM was constructed by inserting a synthetic sequence containing the 64-bp minimal origin (nt 7911–22), described by Yang *et al.* (4), into the *Bam*HI restriction site of pUC19 plasmid. Plasmid pKSO, which contains the origin plus the E2 binding sites 11 and 12 (nt 7805–100), was a kind gift of M. Botchan (University of California, Berkeley). Plasmid DNAs were isolated by standard procedures (12) and were purified by two successive CsCl gradients. The substrates used to assay the nonspecific DNA helicase activity of E1 and its directionality were as described (13). In brief, two oligomers, a 52-mer (5′-CGAACAATTCAGCGGCTTTAACCGGACGCTCGACGCCATTAATAATGTTTTC-3′) complementary to φX174 DNA at nt 703–754 and a 73-mer containing the 52-mer and a 21-nt 5′ tail (5′-AATCATAGATAGCATCTCCGT-3′) that has no homology to the φX174 sequence, were synthesized. These oligomers were annealed to φX174 single-stranded circular DNA (sscDNA), and the 3′ ends were labeled with the Klenow fragment of DNA polymerase I. To determine the polarity of the helicase reaction, φX174 sscDNA hybridized with the 73-mer was cleaved with *Msp* I and then end-labeled as above. The difference in size between the two oligonucleotides located at the ends of the linearized single-stranded DNA (ssDNA) permitted the determination of the directionality of the helicase activity. Linear duplex DNAs used to assay the origin binding activity of the E1 protein were prepared by isolating the 232-bp

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Abbreviations: BPV, bovine papilloma virus; SV40, simian virus 40; T antigen, large tumor antigen; SSB, single-stranded DNA-binding protein; ori$^+$ DNA, origin-containing DNA; DTT, dithiothreitol; ssDNA, single-stranded DNA; sscDNA, single-stranded circular DNA; RFI, covalently closed circular; form U DNA, highly unwound superhelical DNA; ATP[γS], adenosine 5′-[γ-thio]triphosphate; AppNp, adenosine 5′-[β,γ-imido]triphosphate; AppCp, adenosine 5′-[β,γ-methylene]triphosphate.

*Eco*RI–*Pvu* II DNA fragment, 296-bp *Eco*RI–*Pvu* II DNA fragment, and 242-bp *Eco*RI–*Bam*HI DNA fragment by the restriction cleavage of pUC19, pUCOM, and pKSO, respectively. These fragments (2 pmol) were then dephosphorylated with calf intestine alkaline phosphatase (Pharmacia), labeled with T4 polynucleotide kinase (Pharmacia), and gel purified. They each possessed a specific activity of about 2000 cpm per fmol.

Replication Assays. Reactions (40 μl) were as described (14) except that mixtures contained 25 μM dNTPs, 30 mM creatine phosphate (di-Tris salt, pH 7.5), 150 μg of FM3A extract, and 0.24 μg of covalently closed circular (RFI) pKSO DNA. Incorporation was monitored by measuring acid-insoluble material, while products were analyzed as described (4). One unit of E1 activity was defined as the amount of enzyme that incorporated 1 nmol of dNTP after 90 min at 37°C under conditions described above.

Purification of the E1 Protein. Nuclear extracts of Sf9 cells, infected with recombinant baculovirus that expressed the hemagglutinin-tagged E1 protein, were prepared as described (15). The extract (1.2 mg/ml, 181 ml) was adjusted to 25 mM Tris·HCl, pH 7.5/6% (wt/vol) PEG 8000/1 M NaCl/10 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride, stirred on ice for 30 min, and centrifuged at 6000 × *g* for 1 hr. The supernatant (0.64 mg/ml, 230 ml) was directly loaded onto a hydroxylapatite (HTP; Bio-Rad) column (1.5 × 5.7 cm, 10 ml) equilibrated with buffer B [5 mM potassium phosphate, pH 7.5/10% (vol/vol) glycerol/1 mM dithiothreitol (DTT)/antipain (0.2 mg/liter)/leupeptin (0.1 mg/liter)/0.1 mM phenylmethylsulfonyl fluoride]. The column was developed with a linear gradient (150 ml) from 5 mM to 750 mM potassium phosphate in the same buffer. Fractions that supported BPV ori$^+$ DNA replication, eluting at 0.15 M potassium phosphate, were pooled and dialyzed 12 hr against 4 liters of buffer A [25 mM Tris·HCl, pH 7.5/10% glycerol/1 mM EDTA/1 mM DTT/antipain (0.2 mg/liter)/leupeptin (0.1 mg/liter)/0.1 mM phenylmethylsulfonyl fluoride] containing 0.2 M NaCl. The dialyzed fraction (1.23 mg/ml, 37.5 ml; total of 80.4 units; specific activity of 1.7 units/mg) was loaded onto a 1-ml FPLC Mono Q HR5/5 column (Pharmacia) after adjusting to 0.15 M NaCl with buffer A. The column was developed with a linear gradient (30 ml) of 0.15–0.5 M NaCl in buffer A. The replication activity and DNA-dependent ATPase activity, eluting at ≈0.32 M NaCl, were pooled and dialyzed for 9 hr against 2 liters of buffer C [25 mM potassium phosphate buffer, pH 7.5/10% glycerol/1 mM EDTA/1 mM DTT/antipain (0.2 mg/liter)/leupeptin (0.1 mg/liter)/0.1 mM phenylmethylsulfonyl fluoride] containing 0.15 M NaCl. The dialyzed fraction (1.4 mg/ml, 4.5 ml; total of 53.9 units; specific activity of 8.7 units/mg) was loaded onto a 1-ml FPLC Mono S HR5/5 column and eluted with a linear gradient (20 ml) of 0.15–0.65 M NaCl in buffer C. A single peak of replication activity coeluted with a single peak of DNA-dependent ATPase activity at ≈0.28 M NaCl. Half of the pooled fraction (0.6 mg/ml, 2.4 ml; total of 34.2 units; specific activity of 24.5 units/mg) was concentrated to 2.6 mg/ml using a Centriflo-CF25 membrane cone (Amicon) and then subjected to glycerol gradient sedimentation (5 ml, 15–35% in buffer A containing 0.5 M NaCl) for 28 hr at 45,000 rpm in a Beckman SW50.1 rotor. Fractions (12 drops) were collected from the bottom of the tube, aliquoted, and stored at −80°C. The combined glycerol gradient fractions (0.4 mg/ml, 1 ml) contained a total of 14 units and a specific activity of 32 units/mg. Samples freeze-thawed three times lost 50% of their activity as measured by ori$^+$ DNA binding activity.

Helicase and ATPase Assays. Both assays were performed as described (10). Helicase activity was measured in reaction mixtures (20 μl) containing 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 50 μg of bovine serum albumin, and 15 fmol of ^{32}P-labeled oligonucleotide annealed to φX174 sscDNA (2000 cpm/fmol). Reaction mixtures (20 μl) for the ATPase assay contained 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 50 μM ATP (10,000 cpm/pmol), 1 mM DTT, 50 μg of bovine serum albumin, and 100 ng of M13 sscDNA.

DNA Unwinding Assay. The unwinding of BPV ori$^+$ plasmids with the E1 protein was performed as described (16). Reactions were carried out using conditions described for the replication assay with 0.4 μg of human SSB, 400 units of topoisomerase I, and ATP as the only nucleotide.

Nitrocellulose Filter Binding and Gel Retardation Assays. 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, 0.2–0.5 μg of a 1-kb DNA ladder (Bio-Rad) as nonspecific competitor DNA, 30 fmol of the ori$^+$ restriction fragment from plasmid pKSO (2000 cpm/fmol), and E1 protein, as indicated, were assembled on ice and incubated for 15 min at 37°C. The mixtures were then passed through 0.45-μm nitrocellulose filters (Millipore) presoaked in wash buffer (25 mM potassium phosphate, pH 7.5/10% glycerol/0.1 M potassium glutamate/1 mM EDTA/0.5 mM DTT) after alkaline treatment as described (17). The filters were washed with three 1-ml portions of wash buffer, dried, and assayed for radioactivity. The same reaction mixture, as described above, was used for the gel retardation assay. After 15 min at 37°C, glutaraldehyde (0.2%) was added, and the reactions were analyzed by electrophoresis through 1.8% horizontal agarose gels in 0.5× TBE (45 mM Tris/45 mM boric acid/1 mM EDTA). Dried gels were subjected to autoradiography.

RESULTS

Purification of E1. The isolation of the BPV-encoded E1 protein from extracts of baculovirus-infected Sf9 cells is summarized in *Materials and Methods*. During purification, the activity of the E1 protein was monitored by its ability to support BPV ori$^+$ DNA replication in extracts of uninfected FM3A cells under the conditions described in *Materials and Methods*. Crude E1 extracts and the supernatant from the PEG/NaCl partition could not be assayed due to the presence of nuclease activity. Instead, the presence of the E1 protein was verified by immunologic procedures (8). The PEG/NaCl partition step was critical; without this, multiple E1 protein peaks were detected in a number of column chromatographic or glycerol gradient centrifugation steps, possibly due to the nonspecific association of E1 with nucleic acid. The overall purification cannot be estimated, but we surmise from SDS/polyacrylamide gel analysis that 5–10% of the soluble protein in crude extracts is the E1 protein. Thus, the E1 protein isolated using this procedure represents about 5% of the E1 present in the initial extract. SDS/PAGE analysis of the glycerol gradient fractions (the last purification step) indicated that the E1 preparation was ≈90% pure. The protein sedimented with a value of 4.3 S, which is consistent with E1 behaving as a monomer of 70 kDa (Fig. 1 *A* and *B*).

The gene coding for the E1 protein was engineered to contain the hemagglutinin epitope of influenza virus (9). However, we have failed to elute active preparations of E1 from hemagglutinin monoclonal antibodies covalently linked to protein A-Sepharose columns. In our hands, only low levels of E1 protein could be eluted from such columns.

The Purified E1 Preparation Supports DNA Replication. The *in vitro* replication of pKSO DNA, which contains the BPV origin, by extracts of FM3A cells was examined. In the presence of 0.2 μg or more of E1 protein, the replication reaction showed no dependency on the E2 protein (data not shown). Analysis of the glycerol gradient fractions indicated that the major 70-kDa band sedimented coincidentally with the replication activity (Fig. 1). The replication reaction resulted in products (analyzed by neutral agarose gel elec-

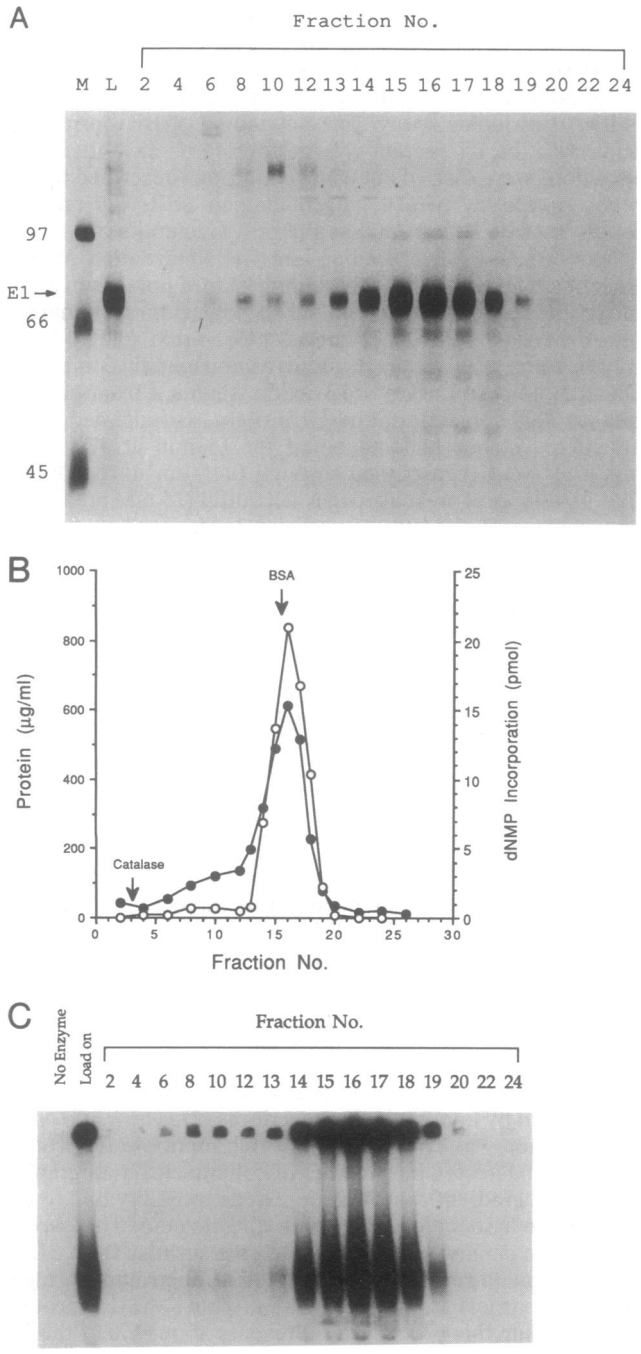

FIG. 1. Glycerol gradient centrifugation of the E1 protein. Fractions (0.2 ml) were collected and analyzed for both protein and BPV ori$^+$ replication activity. Protein markers, bovine serum albumin (4.4 S) and catalase (11.2 S), were run in a parallel gradient, and their sedimentation was measured using the Bradford (18) protein procedure. The peaks of their distributions are indicated. (*A*) SDS/10% PAGE gel of the glycerol gradient fractions (1 μl) was carried out as described by Laemmli (19), and the gel was silver-stained. Lane M, molecular size markers (in kDa); lane L, 0.4 μg of the Mono S fraction, the starting material used for the glycerol gradient step. (*B*) Determination of the protein concentration (●) and replication activity (○) across the glycerol gradient. In the replication assay, reactions contained 1 μl of the glycerol gradient fractions and were incubated at 37°C for 90 min in the standard replication mixtures. (*C*) Autoradiogram of the replication products formed.

trophoresis) similar to those described by Yang *et al.* (4) (Fig. 1*C*). Alkaline gel electrophoresis analysis of the products formed in the reaction revealed that the labeled products varied in size from 150 nt to full-length DNA (data not shown). When DNA lacking the BPV origin (pUC19 RFI) was used in place of pKSO RFI DNA, the incorporation was reduced 7- to 10-fold (data not shown).

Origin Binding Activity of the E1 Protein. The E1 protein selectively binds to DNA containing the BPV minimal origin (Fig. 2). Two assays were used to examine this reaction. One involved nitrocellulose binding (Fig. 2*B*), whereas the other measured the retardation of migration of an ori$^+$ DNA fragment from pKSO upon gel electrophoresis (Fig. 2). Ori$^+$ DNA fragments isolated from pUCOM gave similar results (data not shown). In both cases, Mg^{2+} and ATP were essential for maximum complex formation, and DNA fragments lacking the ori$^+$ sequence from pUC19 did not form complexes (Fig. 2*A*, lanes a and b). As shown in Fig. 2, these activities cosedimented with the major 70-kDa protein band and replication activity. Fraction 16 of the glycerol gradient contained the peak of activity in all of these assays.

ssDNA-Dependent ATPase Activities. Analysis of the glycerol gradient fractions of E1 for ATPase activity revealed the presence of ssDNA (M13)-dependent ATPase activities (Fig. 3*A*) that cosedimented with the replication and origin binding activities. Double-stranded DNA, pUC19 RFI or RFIII, also supported ATP hydrolysis with an efficiency of 20% of that observed with M13 ssDNA (data not shown). In the absence of nucleic acid, virtually no nucleotide hydrolysis was observed (Fig. 3*A*).

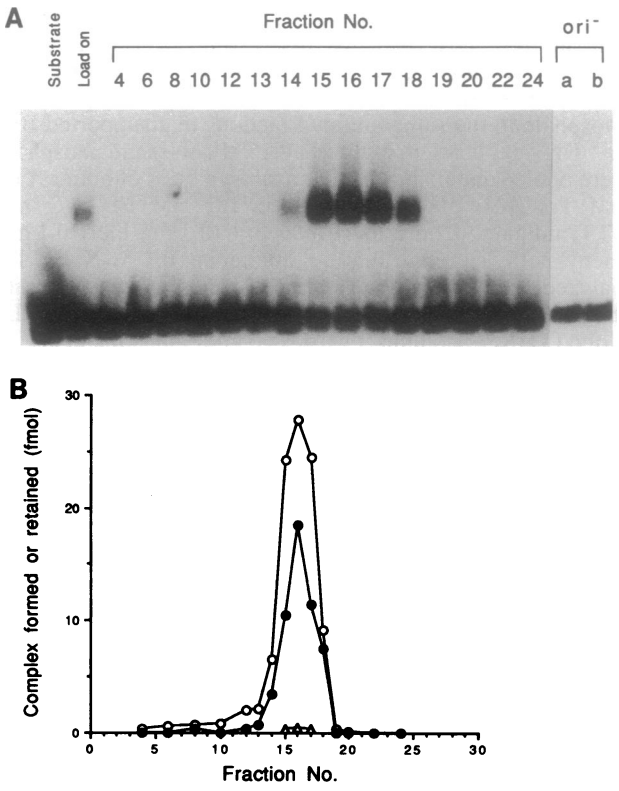

FIG. 2. Analysis of glycerol gradient fractions for origin-binding activities. The same glycerol gradient fractions as described in Fig. 1 were assayed for origin-binding activity by using the nitrocellulose filter-binding and gel retardation assays. (*A*) Autoradiogram of a gel using the retardation assay. Lanes a and b, pUC19 restriction fragment, which lacks the 64-bp minimal origin of BPV (ori$^-$), was used. Lane a contained no E1 protein, whereas lane b contained 0.2 μg of the glycerol gradient fraction 16. (*B*) Quantitation of the nitrocellulose filter binding (○) and the gel retardation (●) assays. For both assays, reactions contained 0.2 μl of glycerol gradient fractions in reaction mixtures described in *Materials and Methods*. Reaction mixtures (△) containing the pUC19 restriction fragment (ori$^-$) were examined with glycerol gradient fractions 15–17 using the nitrocellulose filter-binding assay.

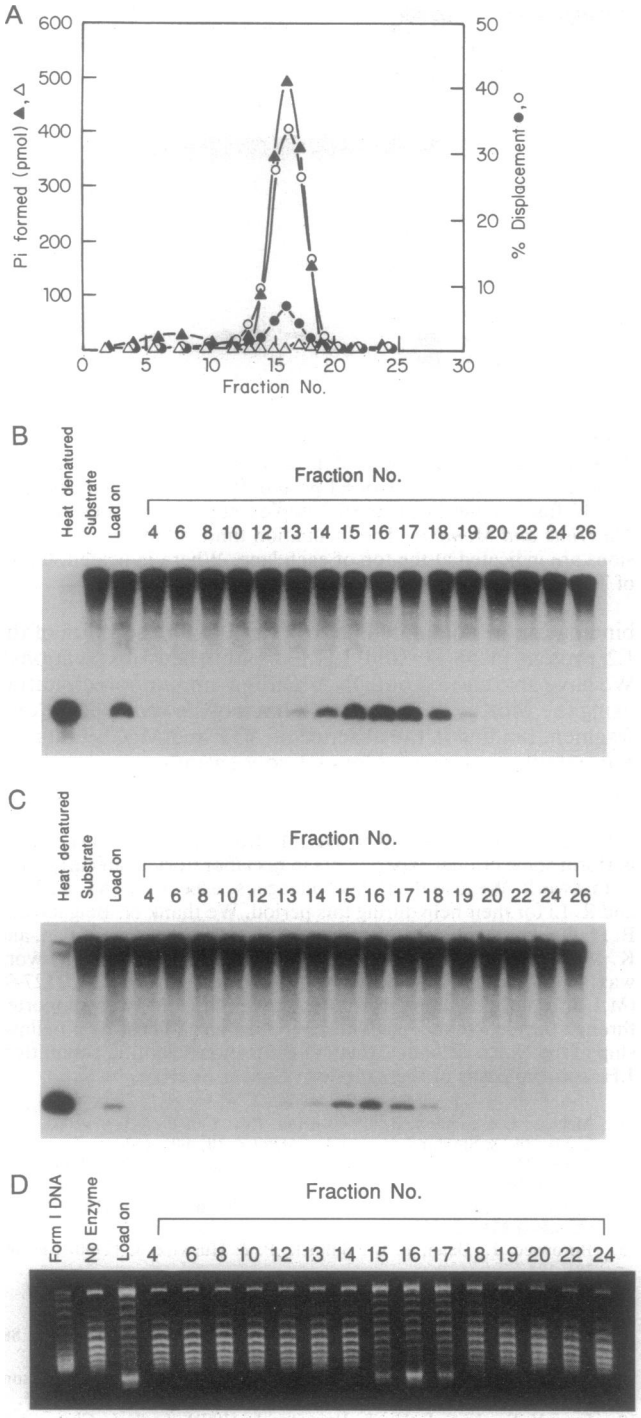

FIG. 3. Analysis of glycerol gradient fractions for ATPase, nonspecific DNA helicase, and unwinding activity with ori+ RFI DNA. The same glycerol gradient fractions as described in Fig. 1 were analyzed for ATPase, nonspecific DNA helicase, and unwinding activity using BPV ori+ RFI DNA. (*A*) Quantitation of ATPase and nonspecific DNA helicase activities measured with glycerol gradient fractions. ATPase activity was measured in the presence (▲) and in the absence (△) of ssDNA. Reaction mixtures contained 0.2 μl of the glycerol gradient fractions and were incubated for 1 hr at 37°C as described in *Materials and Methods*. Nonspecific DNA helicase activity was detected in the glycerol gradient fractions with the 5′-tailed (○) and nontailed (●) substrates shown in *B* and *C*, respectively. (*B*) Autoradiogram of the helicase activity with the 5′-tailed substrate. Reactions contained 0.2 μl of glycerol gradient fractions and 15 fmol of the 5′-tailed substrate and were incubated for 15 min at 37°C. Load on, 80 ng of the Mono S fraction, the starting material for the glycerol gradient step. (*C*) The reactions as in *B* were repeated with the nontailed DNA substrate. (*D*) The unwinding assay with BPV ori+ RFI DNA was carried out with 1 μl of each glycerol gradient fraction and other ingredients described in *Materials and Methods*. Load on, 0.4 μg of the Mono S fraction.

The E1 Protein Contains DNA Helicase Activity. The E1 protein contains a weak but significant sequence homology to the SV40 T antigen (20). The presence of DNA-dependent ATPase activity suggested that this protein might also contain DNA helicase activity. To measure DNA helicase activity, two substrates were used; one contained a 5′-tailed substrate, which was displaced more efficiently (>5-fold) than the untailed substrate (Fig. 3 *A–C*). The DNA helicase activity cosedimented with the ssDNA-dependent ATPase activity and the other activities described above.

The Unwinding of Supercoiled ori+ DNA. The ability of the E1 protein to specifically bind DNA containing the core origin together with the intrinsic DNA helicase activity suggested that the E1 protein might generate highly unwound DNA, form U, from circular duplex BPV ori+ DNA in the presence of an SSB, topoisomerase I, and ATP. As shown in Fig. 3*D*, a rapidly migrating highly unwound DNA product was formed by the glycerol gradient fractions containing the E1 protein. This activity also peaked at the same position in the gradient as did the activities described above. The formation of the highly unwound DNA product required the BPV origin, ATP, $MgCl_2$, and human SSB (data not shown).

Requirements for Helicase Activity of E1. The DNA helicase activity of E1 required $MgCl_2$ and a hydrolyzable form of ATP (Table 1). Neither adenosine 5′-[γ-thio]triphosphate (ATP[γS]), adenosine 5′-[β,γ-imido]triphosphate (AppNp), adenosine 5′-[β,γ-methylene]triphosphate (AppCp), or ADP substituted for ATP. In the presence of 4 mM ATP, DNA helicase activity was hardly affected by the addition of equimolar amounts of ATP[γS], ADP, AppCp, or AppNp. All of the rNTPs and dNTPs examined efficiently substituted for ATP in the helicase reaction at either 0.5 mM or 4 mM (Table 1). The displacement reaction was unaffected by the addition of an ATP-regenerating system or by the addition of 0.05 and 0.1 M NaCl. This is in contrast to the origin-binding activity of E1, which was inhibited >90% by the presence of 0.1 M NaCl (data not shown). The addition of E2 did not affect the helicase activity of E1 (Table 1), whereas E2 stimulated origin-specific binding and unwinding reactions (data not shown).

Determination of Directionality of E1 Helicase Activity. The directionality of the movement of the E1 protein was determined. With the substrate used (Fig. 4), displacement of the 45-mer results from translocation of the helicase in the 3′ → 5′ direction, whereas release of the 32-mer results from 5′ → 3′ movement of the helicase. The 45-mer was exclusively displaced by the E1 protein (Fig. 4, lanes 1–6) and by SV40 T antigen (Fig. 4, lanes 7 and 8). The rate of displacement of the 32-mer by the E1 helicase activity was negligible compared to that of the 45-mer. The release of the DNA fragment by the E1 protein required ATP (Fig. 4, lane 6). These results demonstrated that the E1 protein moves in the 3′ → 5′ direction, like SV40 T antigen, suggesting that E1 moves at the replication fork along the leading-strand template.

DISCUSSION

The results presented here define the biochemical properties of the BPV E1 protein. This protein has been purified using conventional steps to nearly 90% purity. The purified E1 preparation supported the replication of BPV DNA containing the minimal origin sequence in the presence of extracts of FM3A cells, as described by Yang *et al.* (4). Under the conditions that we used, the addition of the E2 protein was not essential for replication (Fig. 1 *B* and *C*). The BPV replication activity cosedimented with DNA-dependent

Table 1. Requirements for DNA helicase activity of E1

Addition or omission*	Amount added	% activity
None		100†
Omit $MgCl_2$ or ATP		<1
Add NaCl	0.05 or 0.1 M	116 or 112
Add ATP[γS]	4 mM	73
Add AppNp	4 mM	81
Add AppCp	4 mM	113
Add ADP	4 mM	77
Add E2 protein	30 or 60 ng	101 or 104
Add creatine phosphate and creatine phosphokinase	20 mM and 1 μg	110
Omit ATP and add ATP[γS], AppNp, AppCp, or ADP	4 mM	<1
Omit ATP, add other rNTPs or dNTPs	0.5 mM	61–82
	4 mM	58–116

*Addition to or omission from the complete reaction mixture, which contained 15 fmol of the partial duplex φX174 sscDNA substrate containing a 5′ tail (73-mer), 0.1 μg of E1 (glycerol gradient fraction), and 2 mM ATP in the standard reaction mixture described in *Materials and Methods*.

†In these experiments, the 100% value represents the displacement of 3 fmol of DNA substrate.

ATPase activity, DNA helicase activity, origin-binding activity, and the ability to unwind closed circular duplex DNA containing the BPV origin, leading to the production of form U DNA. As shown in Fig. 3*D*, the distribution of topoisomers formed in the presence of ATP and the E1 protein was altered, indicating that the E1 protein can also untwist BPV ori$^+$ DNA. Further proof that these activities are intrinsic to the E1 protein was obtained. Both DNA-dependent ATPase and replication activities copurified during the isolation of the E1 protein. In addition, polyclonal antibodies prepared from rabbits immunized with the carboxyl region of the E1 protein, cloned and isolated from an *E. coli* expression system, decreased the mobility of the E1 BPV ori$^+$ DNA complex (data not shown).

These observations indicate that the E1 protein is a multifunctional protein containing all of the activities previously described for the SV40 and polyoma T antigens. In fact, there are some regions of sequence homology between T antigen and the E1 protein. These include the ATP-binding and the helicase domains, which were first noted by Clertant and Seif (20). Though these multifunctional proteins possess marked similarities in their activities, there are notable differences. Most prominent is the role of the E2 protein, which acts as an auxiliary protein that has been reported to activate the origin binding and the replication activities of the E1 protein. There is no counterpart of the E2 protein that acts in conjunction with SV40 or polyoma T antigens. The other striking feature is that in the case of the T antigens ssDNA stimulates nucleoside triphosphatase activity 2- to 4-fold, whereas with the BPV E1 protein, virtually no ATPase activity was detected in the absence of ssDNA. The significance of this difference remains to be explored.

The results presented here 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 of the origin. Yang *et al.* (4) also showed that the E2 protein enhanced the DNA binding activity of the E1 protein nearly 10-fold. In all of these experiments, ATP was not added. We have also observed that at low levels of E1 protein, origin binding can be stimulated up to 40-fold by the addition of the E2 protein (Y.-S.S., and J.H., unpublished observations). We have also carried out DNA binding–immunoprecipitation using the McKay assay (15) and have observed specific ori$^+$ fragment binding in the absence of ATP and $MgCl_2$ (data not presented). However, at low E1 concentrations, origin binding can be stimulated markedly by $MgCl_2$ and ATP.

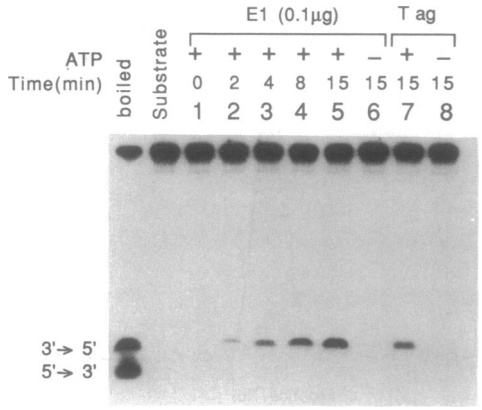

FIG. 4. The E1 protein unwinds duplex DNA in the 3′ → 5′ direction. The kinetic analysis of the DNA displacement reaction was carried out by using 0.1 μg of E1 protein (glycerol gradient fraction) and 15 fmol of the linear partial duplex substrate as described in *Materials and Methods*. The incubation time, additions, and omissions are indicated at the top of each lane. Where indicated, 0.2 μg of T antigen (T ag) was used.

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