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Biochemistry

Formation of the complex of bovine papillomavirus E1 and E2 proteins is modulated by E2 phosphorylation and depends upon sequences within the carboxyl terminus of E1

(protein interactions/regulation of DNA replication)

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ABSTRACT **The 68-kDa bovine papillomavirus (BPV) type 1 replication protein E1 and the 48-kDa transactivator protein E2 form a complex that specifically binds DNA [Mohr, I. J., Clark, R., Sun, S., Androphy, E. J., MacPherson, P. & Botchan, M. R. (1990) *Science* 250, 1694–1699]. We have confirmed this observation and shown that the E1–E2 complex binds to DNA fragments that contain the BPV plasmid maintenance sequence 1 and a site for the initiation of bidirectional BPV DNA synthesis. The E1 protein was found to bind preferentially to non- or underphosphorylated species of E2, suggesting that the phosphorylation state of E2 modulates the association of the two proteins. Replication-deficient E1 mutants with single amino acid substitutions and deletions in the carboxyl terminus failed to interact with E2, indicating that a region in the E1 carboxyl terminus is required for E1 to interact with E2. Our results suggest that the replication deficiency of some E1 mutants reflects their inability to associate with E2.**

Bovine papillomavirus (BPV) type 1 (BPV-1) provides an attractive system to study regulatory control circuits of eukaryotic DNA replication. The viral DNA replicates and maintains itself stably as a multicopy nuclear plasmid in transformed rodent cells (1, 2). Several viral early genes have been implicated in plasmid maintenance (3, 4), and recent studies indicated a direct requirement for the *E1* and *E2* gene products in the replication of BPV plasmids (5).

Genetic analysis suggested that the BPV *E1* open reading frame (ORF) encodes at least two trans-acting functions, a negative modulator M and a positive replication function R (6). Two *E1*-encoded phosphoproteins were identified: a 23-kDa protein that contains the E1 amino terminus and corresponds to E1 M and a full-length 68-kDa E1 protein that contains both the E1 M and the E1 R domain (7–9). The 68-kDa E1 protein binds ATP, and BPV genomes with a mutation in the ATP-binding domain are replication deficient (8, 9). This suggested a direct involvement of the 68-kDa E1 protein in the initiation of DNA synthesis. In addition, a repressor function for viral gene expression has been associated with E1 (10, 11).

Mutations in the BPV *E2* ORF have multiple effects on viral transcription, DNA replication, or both (12). Three site-specific DNA-binding proteins are encoded by *E2*: a 48-kDa full-length transactivator protein and two transcriptional repressor proteins (E2C and E8-E2), which lack the amino-terminal transactivation domain (13–18). Frameshift mutations throughout the *E2* ORF eliminated BPV plasmid replication (4, 19–21). In addition, a deletion removing most of the *E2* ORF (3, 22) led to replication-deficient BPV genomes (M.L. and M. Botchan, unpublished observation). Mutant BPV genomes lacking the E2C repressor function displayed an increased plasmid copy number (23). Furthermore, BPV genomes containing a temperature-sensitive E2 transactivator function were replication deficient at nonpermissive temperatures (24). Taken together, the genetic data suggest a direct involvement of the 48-kDa E2 protein in viral replication.

Regulatory cis-acting DNA sequences involved in BPV DNA synthesis have been localized to the BPV noncoding upstream regulatory region (URR). A plasmid-maintenance sequence, PMS-1, located between nucleotides (nt) 6945 and 7476 (22) overlaps a replication initiation region mapped by electron microscopy (25). Recently, two-dimensional DNA gel electrophoresis placed a site for bidirectional initiation of BPV DNA synthesis distal to PMS-1 to within a region around nt 7730 (26).

Formation of a complex between the BPV E1 and E2 proteins has been demonstrated (27). However, a functional analysis of the roles of this protein complex in DNA replication is difficult, because the intracellular abundance of the E1 and E2 proteins is low. Thus, we and others (9, 27, 28) have expressed these proteins in recombinant baculovirus/insect systems. In this report we describe studies probing the interaction between the E1 and E2 proteins. We investigated whether phosphorylation of the proteins could affect E1–E2 complex formation. Furthermore, we assayed replication-deficient E1 mutants for their ability to associate with E2.

MATERIALS AND METHODS

Cell Cultures. *Spodoptera frugiperda* cells (Sf9 cells) were grown in suspension cultures at 27°C in Grace's medium (GIBCO) containing 10% fetal bovine serum (GIBCO), 0.33% Yeastolate (Difco), and 0.33% lactalbumin hydrolysate (Difco) as described (29). Mouse C127 cell lines were grown in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal bovine serum.

Recombinant Baculoviruses. Wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and the transfer vector pVL941 (30) were provided by Ora Rosen (Sloan–Kettering Cancer Center). To construct AcNPV-E1, the BPV *Nru* I–*Stu* I restriction fragment (nt 838–3351), ligated to *Bam*HI linkers, was inserted into the unique *Bam*HI site of pVL941. To construct AcNPV-E2, the BPV *Ssp* I–*Bam*HI restriction fragment (nt 2520–4451) was inserted into pVL941 at its unique *Bam*HI site upon conversion of the BPV *Ssp* I site into a *Bam*HI site. Generation of recombinant viruses was as described (29).

Antisera. E1 antisera were raised in rabbits (Pocono Rabbit Farm, Canadensis, PA) against a bacterially expressed E1 protein containing E1 amino acids 220–605 (anti-E1). Anti-

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Abbreviations: BPV, bovine papillomavirus; BPV-1, BPV type 1; ORF, open reading frame; PMS-1, plasmid maintenance sequence 1; nt, nucleotide(s); AcNPV, *Autographa californica* nuclear polyhedrosis virus; SV40, simian virus 40; T antigen, SV40 large tumor antigen.

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sera against the amino terminus of E1 (1NA; ref. 9) and antisera containing E2 antibodies (anti-E2) were a gift from E. Androphy (Tufts University).

Radiolabeling and Preparation of Crude Extracts. For viral infections Sf9 cells ($3-4 \times 10^6$) were transferred from suspension cultures to 60-mm tissue culture dishes. Single infections used virus with a multiplicity of infection (moi) of 10; for coinfections an moi of 5 each of AcNPV-E1 and AcNPV-E2 was used. At 36–38 hr after infection, cells were starved for 1 hr in Grace's medium lacking either methionine or phosphate. Proteins were labeled for 6–8 hr with either [^{35}S]methionine (0.25 mCi/ml, >800 Ci/mmol, NEN; 1 Ci = 37 GBq) or with $^{32}P_i$ (1 mCi/ml, NEN). Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 150 μl of buffer A [20 mM Tris·HCl at pH 9.0, 0.3 M NaCl, 1 mM EDTA, 10% (vol/vol) glycerol, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin at 10 μg/ml, L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride (TLCK) at 10 μg/ml, and L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone (TPCK) at 30 μg/ml]. After incubation for 20 min on ice, nuclei were pelleted by centrifugation at 20,000 rpm in a TL100.3 rotor for 10 min at 4°C. The supernatants were neutralized by adding 75 μl of buffer B (100 mM Tris·HCl at pH 6.8, 0.3 M NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 1 mM dithiothreitol, and the protease inhibitors described above). Under these conditions the E2 protein was quantitatively recovered in the soluble supernatants, whereas 60–70% of E1 protein remained in the nuclear fraction (data not shown). The protein concentration of the supernatants was 5–7 mg/ml (31).

Immunoprecipitations. Ten to 20 μl of the extracts was incubated with 2 μl of E1 or E2 antibody (40–50 mg/ml) in 200 μl buffer C (20 mM Hepes/NaOH at pH 7.5, 0.15 M NaCl, 10% glycerol, 0.5% Nonidet P-40, and protease inhibitors as above) for 2 hr at 4°C. Immunocomplexes were collected by addition of 200 μl of a 10% staphylococcal protein A-Sepharose (Sigma) slurry equilibrated in buffer C. After incubation for 50 min beads were sedimented, washed four times with 1 ml of buffer C, incubated in 1 ml of buffer C plus 0.5 M NaCl for 10–15 min at 37°C, and washed once with buffer C. Proteins were dissociated in SDS sample buffer (32), boiled, and separated on SDS/polyacrylamide gels. Gels were fixed, dried, and subjected to autoradiography.

DNA-Binding Assays. Immunoprecipitation–DNA-binding assays (33) were performed as described (13). The plasmid pXS-SV40 contains the BPV *Xba* I–*Sma* I fragment (nt 6132–945) and the simian virus 40 (SV40) 311-base-pair (bp) *Eco*RII-G fragment cloned in a vector derived from pML-1 (34). Plasmid DNA (5 μg), cleaved with *Taq* I restriction endonuclease, was labeled with the Klenow fragment of DNA polymerase (Boehringer Mannheim) and [α-^{32}P]dCTP (50 μCi, >3000 Ci/mmol). Protein A-Sepharose beads containing antibody–antigen complexes were mixed with 20 ng of purified end-labeled DNA and a nonspecific competitor DNA (pML-1) at 10 μg/ml in 200 μl of buffer C and incubated for 1–2 hr at 4°C. The beads were washed five times with 1 ml of buffer C. Bound DNA fragments were dissociated from protein by incubating the pellets in 50 mM EDTA (pH 8.0) containing 1% SDS for 15 min at 65°C. Dissociated DNA, recovered by extraction with phenol/chloroform and precipitation with ethanol was separated on 2% agarose gels. The gels were dried and subjected to autoradiography.

Construction of E1 Mutants. Site-directed mutagenesis within the E1 ORF utilized the gapped duplex method (35). The E1 mutants described in this study are listed in Table 1. Mutants E1 FM17 and FM3 contain a 6-bp *Xho* I linker (CTCGAG) at *Alu* I sites between BPV nt 1998 and nt 1999 (FM17), and nt 2391 and nt 2392 (FM3). Mutant E1ΔFM17-FM3 contains an in-frame deletion between the *Xho* I sites of FM17 and FM3. Mutant E1 1137-1, provided by P. Lambert (University of Wisconsin, Madison, WI) contains a translational termination linker insertion at BPV nt 2113 (17). Restriction fragments containing E1 mutations were used to replace the corresponding wild-type fragments in pML BPV_{100}[3] and in pVL941-E1.

Table 1. Effect of E1 mutations on BPV replication and E2 binding

Mutant*	Amino acid change	Position and codon change†	Plasmid maintenance	E2 binding
E1 90 S-N	Ser → Asn	1117: AGT → AAT	−	+
E1 91 S-A	Ser → Ala	1119: TCG → GCG	−	+
E1 102 T-A	Thr → Ala	1152: ACT → GCT	−	+
E1 424 P-S	Pro → Ser	2118: CCA → TCA	−	−
E1 434 P-S	Pro → Ser	2148: CCT → TCT	−	−
E1 516 P-S	Pro → Ser	2394: CCA → TCA	−	−
E1 FM17	384-RA-385‡	1998-CTCGAG-1999§	−	−
E1 FM3	515-RA-516‡	2391-CTCGAG-2392§	−	−
E1ΔFM17-FM3	Δ385–515¶	Δ1999–2392	ND	−
E1 1137	Δ424–605¶	2113: TTL	−‖	−

TTL, translational termination linker; ND, not done.
*Numbers indicate positions of amino acid substitutions in E1.
†Positions of nucleotide change (underlined) in the *E1* gene.
‡Positions of amino acids between which two residues (Arg-Ala) were inserted.
§Nucleotide positions in the *E1* gene between which 6 base pairs (*Xho* I linker) were inserted.
¶Deletion of indicated amino acids and nucleotides.
‖Replication deficiency reported by Lambert and Howley (10).

RESULTS

BPV E1 Replication Protein Preferentially Associates with Underphosphorylated Species of the BPV E2 Transactivator. To study the role of the BPV E1–E2 protein complex in viral DNA replication, we expressed the full-length E1 and E2 polypeptides in insect cells by using recombinant baculoviruses. Fig. 1*A* shows that either E1 or E2 antibodies coprecipitated the 48-kDa E2 and the 68-kDa E1 polypeptides from extracts containing both proteins (lanes 3, 4, 9, and 10). In addition, consistent with the results by Mohr *et al.* (27), efficient complex formation was observed when extracts containing either protein were mixed *in vitro* (lanes 5, 6, 11, and 12).

Both polypeptides are expressed as nuclear phosphoproteins in mammalian cells (8, 9, 36). Furthermore, mutations at the major phosphorylation sites in E2 (36) lead to an increase in BPV plasmid copy number (A. A. McBride and P. M. Howley, personal communication). For these reasons we determined whether phosphorylation of either protein could affect E1–E2 complex formation. Sf9 cells, singly infected with AcNPV-E1 or AcNPV-E2 or coinfected with both viruses, were labeled with $^{32}P_i$. In contrast to the results described above (Fig. 1*A*, lanes 3–6), anti-E1 precipitated only ^{32}P-labeled E1 and did not coprecipitate ^{32}P-labeled E2 along with E1 from extracts derived from E1–E2 coinfection (Fig. 1*B*, lane 3) or from a mixture of E1- and E2-containing extracts (Fig. 1*B*, lanes 4 and 5). In these experiments unlabeled E2 protein was, however, visualized by immunoblotting with E2 antibody. In addition, anti-E1 efficiently coprecipitated [^{35}S]methionine-labeled E2 when mixed with ^{32}P-labeled E1 (data not shown). Fig. 1*B* (lanes 8–10) demonstrates that anti-E2, however, did coprecipitate ^{32}P-labeled E1 protein with E2 from these extracts. Moreover, alkaline phosphatase treatment of immunoprecipitated ^{32}P-labeled E2 stimulated the binding of ^{32}P-labeled E1 protein to E2 2- to 3-fold (data not shown). E1 antibody will precipitate the

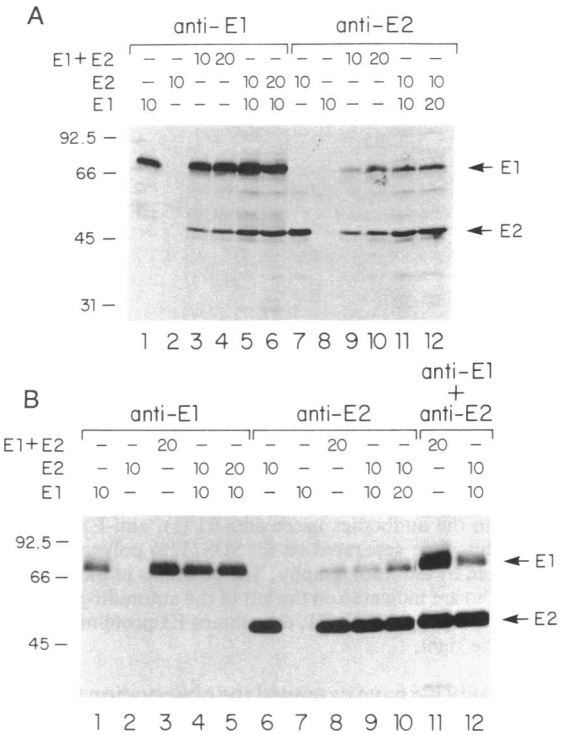

FIG. 1. Formation of the complex between the BPV E1 and E2 proteins. Crude Sf9 cell extracts (5–7 mg of protein per ml) derived from single infections (E1 or E2) or double infections (E1+E2) were incubated with antibodies to E1 or E2. Precipitated proteins were separated on SDS/10% polyacrylamide gels and visualized by autoradiography. The numbers above the lanes in the rows labeled E1, E2, and E1+E2 refer to the amounts (μl) of the extracts used. The positions of molecular mass markers (kDa) are indicated on the left, and the positions of E1 and E2 proteins are indicated on the right of the autoradiograms. (*A*) Immunoprecipitation of [^{35}S]methionine-labeled proteins. (*B*) Immunoprecipitation of ^{32}P-labeled proteins.

entire pool of E1 but will coprecipitate only E2 that is bound to E1. Likewise, anti-E2 will precipitate free and bound, phosphorylated and underphosphorylated, E2, but will coprecipitate only that fraction of E1 complexed to E2. Thus, the detection of labeled E1 in lanes 8–10 was due to its association with nonphosphorylated E2 present in the mixture of precipitated E2 proteins.

Binding of the E1–E2 Complex to DNA. All *E2*-encoded polypeptides bind to specific short palindromic sequences (5′-ACCN$_6$GGT-3′) (13, 37). Seventeen different binding sites on BPV DNA were identified (38). Of these, 12 are clustered in the viral upstream regulatory region. In contrast, E1 from crude extracts displayed nonspecific binding to DNA (9, 28) and weak specific binding when partially purified (27). Therefore we examined the DNA-binding properties of E1, E2, and E1–E2 immunocomplexes, using the McKay assay (33). Fig. 2*A* demonstrates that E1, precipitated with amino- or carboxyl-terminal-specific E1 antibodies, did bind to all DNA fragments present in the reaction mixture, with no apparent specificity. When E1 extracts were incubated with anti-E2, or when antibody was omitted, no DNA binding was observed (data not shown). In keeping with earlier findings (27), E2 specifically bound to the 317-bp *Taq* I BPV DNA fragment containing E2 binding sites 5–10 (Fig. 2*B*, lanes 3 and 4). When E1+E2-containing extracts were subjected to precipitation with E1 antibodies, the 317- and 569-bp *Taq* I BPV DNA fragments were bound with equal affinity (Fig. 2*B*, lanes 6–8). Furthermore, the nonspecific binding of E1 to DNA appeared to be greatly reduced in the presence of E2. These results confirm (27) that the E1–E2 complex, precipitated with E1 antibodies, binds DNA with the specificity of E2.

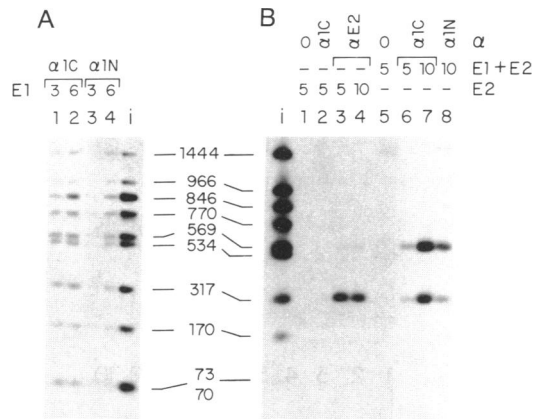

FIG. 2. Unspecific DNA binding of E1 and specific DNA binding of the E1–E2 complex. Different amounts (μl) of crude extracts (E1, E2, E1+E2) indicated by the numbers above the lanes were immunoprecipitated with E1 antibodies (αIC), antiserum INA (αIN, which is specific for the amino terminus of E1), or anti-E2 (αE2). In lanes marked 0 antibody was omitted. DNA-binding reaction mixtures containing ^{32}P-labeled *Taq* I fragments of pXS-SV40 DNA were assembled and processed as described in *Materials and Methods*. Lanes marked i contained the mixture of input DNA fragments, and their sizes (nt) are indicated. (*A*) Binding of E1 to DNA. (*B*) Binding of E2 (lanes 3 and 4) and of E1–E2 (lanes 6–8) to DNA.

However, the 569-bp fragment containing E2 binding sites 1 and 2 was retained with higher affinity by E1–E2 than by E2 alone.

A Region in the Carboxyl-Terminal Part of E1 Is Required for E1–E2 Interaction. An explanation for the loss of function of most replication-deficient E1 mutants has remained elusive. A failure of certain E1 mutants to interact with E2 could perhaps account for loss of E1 replication functions.

Therefore, the ability of various E1 mutants to associate with E2 was tested. The results are summarized in Table 1. All mutants were replication deficient in C127 cells (Fig. 3 and data not shown) and their phenotypes will be described elsewhere. The mutants E1 90 S-N, E1 91 S-A, and E1 102 T-A contain single amino acid substitutions in the amino terminus of E1. The mutants E1 424 P-S, E1 434 P-S, E1 516 P-S, and E1 FM3 have lesions in the carboxyl terminus of E1. Fig. 4*A* demonstrates that anti-E1 coprecipitated E2 along with the amino-terminal mutant E1 proteins (lanes 4–6) as readily as with wild-type E1 (lane 3). Similarly, anti-E2 coprecipitated these mutant E1 proteins together with E2 (Fig. 4*C*, lane 4, and data not shown). In contrast, the association of E2 with the mutant proteins E1 424 P-S, E1 434 P-S, E1 516 P-S, and E1 FM3 was abolished or greatly diminished when extracts derived from the respective coinfections were incubated with anti-E1 (Fig. 4*B*, lanes 6–9) or with anti-E2. (Fig. 4C, lanes 5–8).

To further explore this finding two E1 deletion mutants were tested in this assay. Mutant E1 1137 (17) is expected to express an E1 protein lacking 182 amino acids from the carboxyl terminus. Mutant E1ΔFM17-FM3 has undergone a deletion removing 131 amino acids between residues 384 and 516 of the BPV E1 protein, including the positions of the carboxyl-terminal E1 point mutations described above. Infection of AcNPV-E1 1137 and AcNPV-E1ΔFM17-FM3 resulted in the expression of E1 polypeptides with apparent molecular masses of approximately 50–52 kDa (Fig. 5, lane 6) and 56 kDa (Fig. 5, lane 10), respectively. However, as demonstrated in Fig. 5, the interaction of the mutant protein E1 1137 with E2 was severely impaired (lanes 7 and 8) and no association of E1ΔFM17-FM3 with E2 could be seen (lanes 11 and 12). The results presented in Figs. 4 and 5 suggest that residues located between positions 384 and 516 in the carboxyl terminus of E1 are essential for E1–E2 interaction.

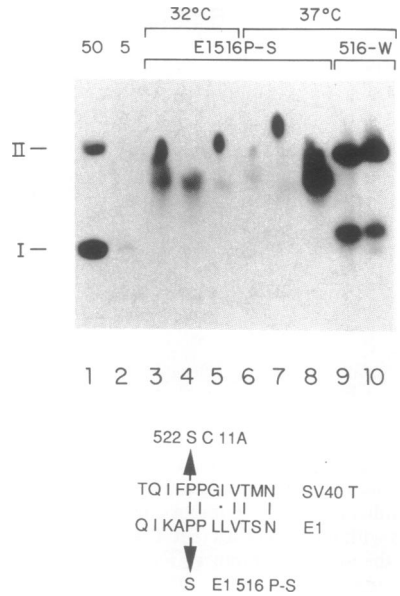

FIG. 3. Replication deficiency of BPV genomes containing the mutation E1 516 P-S. Uncut genomic DNA prepared from stably transformed C127 cell lines was subjected to Southern blot analysis as previously described (3). Lanes 3–8 show the DNA analysis from six cell lines independently transformed by pMLBPV 516 P-S. Lanes 9 and 10 show the analysis from two cell lines derived upon transfection with a pMLBPV plasmid, 516-W, in which the mutation at position 516 in pMLBPV 516 P-S was replaced by a small restriction fragment containing wild-type (W) sequences. None of the 516 P-S cell lines grown either at 32°C (lanes 3–5) or at 37°C (lanes 6–8) contained free BPV plasmid DNA. In contrast, the 516-W cell lines (lanes 9 and 10) did contain supercoiled (replicative form I, RFI) and nicked circular (replicative form II, RFII) plasmid DNA. Lanes 1 and 2 contained 50 and 5 copies of the input plasmid DNA, respectively. The migration of RFI and RFII DNA is indicated on the left. Conserved amino acid sequences between E1 and SV40 large tumor (T) antigen are shown at the bottom. The proline-to-serine change in BPV E1 at residue 516 is indicated. The corresponding change at position 522 in SV40 T antigen gives rise to the mutant C11A (39) deficient in helicase activity (40).

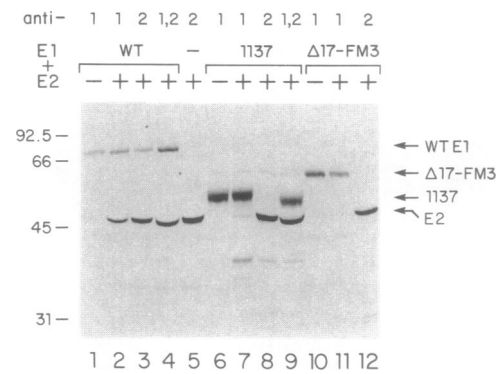

FIG. 5. Effect of deletions in E1 on E2–E1 association. Immunoprecipitations of [^{35}S]methionine-labeled proteins were carried out with extracts derived from single infections (lanes 1, 5, 6, and 10) or from coinfections of E2 plus wild-type (WT) E1 (lanes 2–4), E1 1137 (lanes 7–9), or E1ΔFM17-FM3 (lanes 11 and 12). Numbers above the lanes refer to the antibodies used: anti-E1 (1), anti-E2 (2), or both (1,2). Proteins were separated on an SDS/12% polyacrylamide gel and visualized by autoradiography. The positions of molecular mass markers (kDa) are indicated on the left of the autoradiogram, and the positions of wild-type (WT) E1, the mutant E1 proteins, and E2 are shown on the right.

DISCUSSION

The BPV E1 replication protein and *E2*-encoded transactivator play pivotal roles in viral DNA replication and the control of viral gene expression.

In this study we have extended the observation (27) that the 68-kDa E1 and 48-kDa E2 proteins associate when expressed in insect Sf9 cells. We found that E1 antibodies could not coprecipitate ^{32}P-labeled E2, whereas E2 antibodies did coprecipitate ^{32}P-labeled E1 (Fig. 1*B*). Furthermore, binding of ^{32}P-labeled E1 to E2 was stimulated 2- to 3-fold when ^{32}P-labeled immunoprecipitated E2 protein was treated with alkaline phosphatase prior to incubation with extracts containing E1 (data not shown). While we cannot rule out that a minor fraction of E1, not well recognized by the antibody used (anti-E1), binds efficiently to phosphorylated E2, the most likely interpretation is that E1 preferentially associates with non- or underphosphorylated E2. It is widely appreciated that phosphorylation states affect the function of proteins involved in transcription, replication, and cell cycle control. For example, the replication functions of the SV40 large T antigen are regulated by its phosphorylation state (41). Thus, the ratio of phosphorylated to nonphosphorylated E2 may be an important negative control for E1–E2 interaction and for viral replication. Alternatively, we cannot ex-

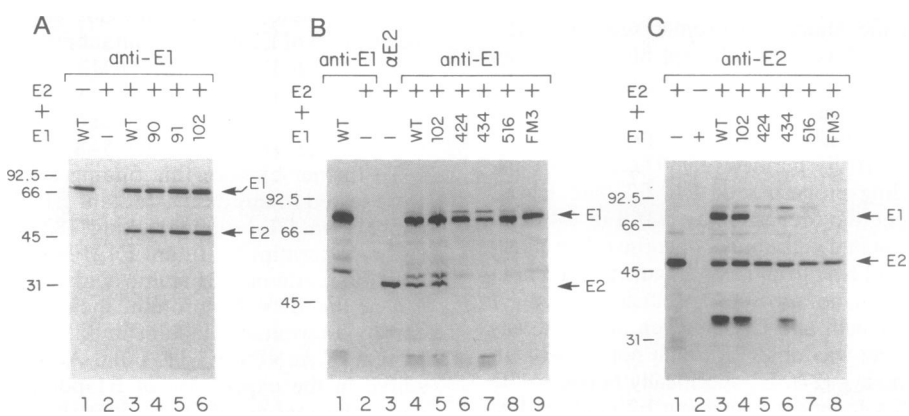

FIG. 4. Effect of E1 mutations on formation of E1–E2 complex. Immunoprecipitations of [^{35}S]methionine-labeled proteins derived from single infections (lanes 1 and 2 in *A* and *C*; lanes 1–3 in *B*) or from double infections (all other lanes) were carried out with the antibodies indicated. Precipitated proteins were separated on SDS/10% polyacrylamide gels and visualized by autoradiography. The positions of molecular mass markers (kDa) are indicated on the left and the positions of E1 and E2 are shown on the right of each autoradiogram. (*A*) Complex formation of E2 with wild-type E1 (WT, lane 3) and mutants E1 90 S-N (90, lane 4), E1 91 S-A (91, lane 5), and E1 102 T-A (102, lane 6). (*B* and *C*) Failure of complex formation between E2 and E1 mutants E1 424 P-S (424), E1 434 P-S (434), E1 516 P-S (516), and E1 FM3 (FM3) upon precipitation with anti-E1 antibody (*B*, lanes 6–9) or with anti-E2 antibody (*C*, lanes 5–8).

clude that a specific conformation of the E2 protein, which prevents its phosphorylation, mediates E2–E1 complex formation. Further studies are needed that determine the ratio of nonphosphorylated to phosphorylated E2 protein in the presence and absence of E1.

We do not know at present whether phosphorylation of E1 influences the complex formation. The 68-kDa E1 protein contains multiple phosphorylation sites that can be localized to a 24-kDa amino-terminal and a 16-kDa carboxyl-terminal peptide (K. Zuklys and M.L., unpublished observation). From the data shown in Fig. 1*B* (lanes 8–10) we conclude that ^{32}P-labeled E1 protein was complexed to nonphosphorylated E2 present in the mixture of precipitated E2 protein.

Consistent with the results obtained by Mohr *et al.* (27), the E1–E2 complex binds specifically to BPV DNA fragments containing E2-binding sites. However, the E1–E2 complex was found to bind with equal efficiency to the 569- and 317-bp BPV fragments. The 569-bp fragment contains E2-binding sites 1 and 2 and PMS-1 (22); the 317-bp fragment contains E2-binding sites 5–10, including the transcriptional enhancer element E2RE1 (E2 response element 1; ref. 42) and a site for bidirectional initiation of BPV DNA synthesis (26). The strong affinity of the E1–E2 complex to the 569-bp fragment may be due to conformational changes of E2 or E1 leading to altered interactions of E2, E1, or both with DNA. It was reported that DNA loops can be formed between E2-bound sites that are separated over distance (43). Thus, it is intriguing to speculate that binding of E1–E2 to different genetic replication elements *in vivo* may induce their juxtaposition and thus allow for a multicomponent origin of replication to be formed.

When a series of replication-deficient E1 mutants were assayed for E2 interaction, our studies showed that a region in the carboxyl terminus of E1 is necessary for complex formation. (*i*) The amino-terminal E1 mutants E1 90 S-N, E1 91 S-A, and E1 T102 T-A efficiently formed a complex with E2 (Fig. 4*A*). These mutations affect serine and threonine residues clustered within the 24-kDa amino-terminal E1 peptide, which contains multiple phosphorylation sites. Clearly, the loss of replication function(s) of these mutants must affect mechanisms that do not involve E2 interaction. (*ii*) The E1 mutants 424 P-S, 434 P-S, 516 P-S, and FM3 all failed to form a complex with E2 (Fig. 4 *B* and *C*). The 434 P-S mutant is deficient in ATP binding (8), and the location of E1 516 P-S corresponds to the SV40 T antigen mutation C11A (Fig. 3; ref. 39), which resulted in a helicase-deficient T antigen (40). It is possible that multiple functional domains overlap each other in the carboxyl terminus of the E1 protein, in a manner analogous to T antigen (41). (*iii*) Our results are substantiated by the failure of two E1 deletion mutants, E1 1137 and E1ΔFM17-FM3, to interact with E2 (Fig. 5). The replication-deficient E1 mutants that failed to complex with E2 were also unable to take part in the specific DNA interactions described above (data not shown). Together, our results are consistent with the conclusion that a domain in E1 including residues 384–515 is necessary for E1–E2 interaction. Whether this region alone is also sufficient for complex formation requires further investigation.

The results obtained with mutant E1 1137 may also provide insight into the role of E1 as repressor for viral transcription. This mutant displayed enhanced transformation and transcription activity when compared with wild-type BPV (10, 17). In C127 cells transformed with E1 mutants deficient in E2 binding, higher amounts of free E2 transactivator might accumulate than in a wild-type background. This in turn might lead to an increase in transcriptional activity in the mutant background.

The complex formation of the BPV E1 replication protein and the *E2*-encoded transactivator raises many questions about initiation of BPV DNA synthesis, its control, and the coordinated regulation between viral replication and transcription. Characterization of the biochemical and structural properties of the E1–E2 complex in combination with an *in vitro* replication system should allow a functional analysis of both subunits and the identification of cellular factors that participate in regulated BPV replication.

Note Added in Proof. Recent experiments demonstrated that an E1 polypeptide containing residues 220–605 and lacking the amino terminus efficiently associated with the E2 protein (M.L., unpublished observation).

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