Journal of Virology, May 1997, p. 3652–3665 Vol. 71, No. 5
0022-538X/97/$04.00+0
Copyright © 1997, American Society for Microbiology

A Papillomavirus E2 Phosphorylation Mutant Exhibits Normal Transient Replication and Transcription but Is Defective in Transformation and Plasmid Retention

CHRIS W. LEHMAN,[1]* DAVID S. KING,[2] AND MICHAEL R. BOTCHAN[1]

Department of Molecular and Cell Biology[1] and Howard Hughes Medical Institute,[2] University of California, Berkeley, California 94720-3204

Received 2 December 1996/Accepted 29 January 1997

Papillomavirus DNA persists in infected cells as a nuclear plasmid, causing epithelial lesions in many hosts, including humans. The viral protein E2 is required for both replication and transcription to facilitate this persistence. Bovine papillomavirus E2 protein is phosphorylated at two predominant sites. Phosphorylation of one of these sites (serine 301) inhibits replication of the genome. Using mass spectrometry and Edman sequencing, we have mapped additional phosphorylation sites in tryptic peptides to positions which lie primarily in the putatively unstructured hinge region of E2. Mutation of the major sites facilitates transformation in the absence of viral repressors and only has a minor effect on transformation when the repressors are present. Mutation of the major phosphorylation sites combined with one additional change at a newly discovered site (serine 235) blocks transformation. Transformation can be restored by mutating this residue to aspartic acid, mimicking a phosphorylated amino acid, suggesting that phosphorylation is key to the regulation. Transformation by the mutant genome can also be rescued by ectopic expression of the E2 enhancer protein, demonstrating a loss of function by the mutant protein and not a toxic defect. In transient assays, phosphorylation site mutants of E2 protein were normal for all viral functions tested, including replication, transcriptional activation and repression (by the overlapping mutant repressors), protein accumulation, and surprisingly, viral oncogene E5 promoter activation. While the mutant genome transiently replicated to high levels, stable replication was defective, suggesting that a function of E2 required for plasmid retention is regulated by phosphorylation.

Papillomaviruses induce benign epithelial lesions (papillomas and warts) in many mammalian species. Several human papillomavirus subtypes can produce lesions progressing to malignant carcinomas and are the leading causal agent for cervical cancer (52, 71). Bovine papillomavirus (BPV), which causes warts in cattle, has been a prototypic papillomavirus, with its transformation of mouse cells serving as a model for certain aspects of oncogenic transformation (12). In particular this virus has provided a system for genetic analysis of viral persistence and virus-cell interactions for plasmid replication.

Several viral proteins have been found to be required for plasmid retention and oncogenic transformation (9, 15, 32, 47, 69). Utilizing the mouse C127 cell transformation assay, E5, which is the smallest known oncogene, has been found to be the major oncogene in BPV, being both necessary (10, 53, 70) and sufficient (27) for such transformation. E5 protein induces cellular proliferation and abrogation of contact inhibition, resulting in oncogenic transformation. Other important transformation phenotypes require E6 and E7 proteins facilitating anchorage-independent growth in soft agar (41). Very different assays have been used for human papillomavirus but one can conclude that E5 retains its oncogenicity in the human viruses and that full transformation depends on E6 and E7 proteins (reviewed in reference 19).

The E2 open reading frame encodes a family of proteins which form the central regulatory system of the virus, controlling directly both viral gene expression and replication. E2 acts as a transcriptional activator by interacting with E2 binding sites, thereby enhancing transcription of all early viral transcripts (60). For example, E2 expression is autocatalytic, as the protein activates expression from several promoters that can produce an effective E2 mRNA (3, 57, 63). The transcription of viral promoter P4 (also known by the coordinate of a major start site P_{2443}) is stimulated by E2, producing a predominant unspliced mRNA encoding E2 and a spliced transcript which is the major message for E5 production (3, 57).

Viral transcription is down regulated by two shorter repressor forms of E2. These naturally occurring truncated forms of E2 share the C-terminal DNA binding domain with full-length E2 but lack the transcriptional activation domain. They therefore act as transcriptional (7, 23) and replicational (6, 30) repressors. The promoters for these repressors (P3 and P5) are themselves also controlled by E2. P5 has two E2 binding sites that cooperatively interact with Sp1 for activity (29, 58). By controlling the balance of viral activator and repressors, regulation of plasmid copy number is achieved. Genetic studies have shown that mutations that cripple E2C initiation lead to enhanced viral gene activity and higher stable plasmid copy number, while inactivation of both repressor proteins abolishes transformation and stable plasmid maintenance (24, 50). Moreover, the ratio of the repressors to the activator changes through the cell cycle (68), but how this ratio is achieved is not fully understood. It is interesting that the E2 binding sites within the E2C promoter have relative affinities more than 10-fold lower for E2 than do those in the upstream regulatory region (URR) that activate promoters for full-length E2 (28). Despite this difference, and the repressor's shorter half-life (20), repressors predominate in the steady state, suggesting that other cellular factors contribute to the balance of repressors and activator.

The E2 protein family also plays a key role in the regulation

* Corresponding author. Mailing address: Botchan Lab, 401 Barker Hall, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720-3204. Phone: (510) 642-5932. Fax: (510) 642-7000. E-mail: Lehman@mendel.berkeley.edu.

of viral DNA replication. E2 and the viral helicase E1 are both required for efficient plasmid replication in vivo (47, 62) and in vitro (67). E2 is thought to aid in E1 binding to the origin, thus helping in the efficiency and specificity of E1 assembly at the start site. Whereas E2 acts as an ancillary factor for replication activity in vitro, it is absolutely required in vivo for initiation of BPV DNA replication. This absolute requirement in vivo is likely due to E1 levels and to its affinity for the viral origin and competing cellular sites which titrate E1 away from its template (55, 67).

The critical roles that E2 plays in the viral life cycle and the numerous cellular proteins with which it interacts have provided impetus for many structure-function studies. The protein has been functionally divided into three domains by sequence analysis (14), mutagenesis (11, 35, 37, 45), and X-ray crystallography (17). These studies have defined separate protein folding regions and genetically distinct cassettes that have independent activity. An amino-terminal activation domain (critical for both replication and transcription functions of E2) has been separated from a carboxy-terminal DNA binding/dimerization domain by an internal, putatively unstructured hinge. The evidence that the hinge region is unstructured is based solely on the prevalence of prolines within this domain and its accessibility to proteases (11). Consistent with this view for an unstructured linker between two functional domains is the lack of conservation in this region among papillomaviruses despite conservation in the DNA binding and activation domains. Deletion analysis has shown that some hinge sequence (perhaps a critical length) is required for replication function (65). Although these sequences were largely dispensable for transient transcription when high levels of E2 protein were present, potentially important interactions with cellular transcription machinery may also be dependent on this domain (16, 48).

The BPV E2 protein is known to contain two phosphorylation sites which are located in the hinge region, serines 298 and 301, accounting for much of the phosphorylation (36). Consistent with the notion that the hinge plays a critical role in E2 activity was the finding that when phosphorylation of amino acid 301 is prevented by mutation to an alanine, the resulting genome replicates to high levels in both transient and stable assays (38). This finding implies that phosphorylation of this site negatively regulates some function of E2 required for replication in the cell. Down regulation of E2 replication activity by phosphorylation is consistent with its change in phosphorylation state through the cell cycle. E2 appears to be in an underphosphorylated state in S phase (43). In contrast to the enhanced replication phenotype, the oncogenic transformation efficiency and transcriptional activation of the genome containing phosphorylation site mutations appear normal. The specific replicational effect of the 301 mutation may be accounted for by the observation that E1 interacts with underphosphorylated E2 (33). However, in our laboratory, no biochemical activity has been found to be altered by E2 phosphorylation at the 298 and 301 sites (59), and the biochemical correlates of these modifications remains uncertain.

This history and the interesting effects of E2 modifications on replication activity led us to identify additional phosphorylation sites and to examine how mutation of these sites affects E2 function. In this study, three new phosphorylation sites have been mapped to the hinge region of E2 protein. One of these sites had no observable phenotype when it was mutated singly. However, in combination with mutations at the other major hinge phosphorylation sites, the mutant protein was severely crippled for function(s) required for viral plasmid retention and oncogenic transformation. Surprisingly normal E2 function was observed in all transient assays used. Taken together with prior results, our data suggest that phosphorylation can both positively and negatively regulate some as yet unidentified function(s) required for stable plasmid replication.

MATERIALS AND METHODS

Cell culture. Cells of the insect *Spodoptera frugiperda* Sf9 cells were maintained in suspension by using Grace's insect medium supplemented with 10% fetal calf serum (FCS), 0.33% Yeastolate (Difco), 0.33% lactalbumin (Difco), and 0.1% Pluronic F-68 (Gibco) at 26°C with shaking. Cells were infected at a density of 5×10^5 to 8×10^5 cells/ml with recombinant *Autographa californica* baculovirus expressing wild-type or mutant E2 protein at a multiplicity of infection of 3 to 5; the cells were harvested 48 h post infection. For metabolic labeling of protein, cells were washed with Tris-buffered saline (TBS) 42 to 48 h postinfection, starved in phosphate-free Ex-Cell 401 medium (JRH Biosciences) plus 10% dialyzed FCS, 0.17% Yeastolate, 0.17% lactalbumin, and 0.1% Pluronic F-68 for 2 to 3 h, and then labeled in suspension for 2 to 3 h with $H_3{}^{32}PO_4$ (1 mCi/ml; ICN) at 2×10^7 to 4×10^7 cells/ml with shaking at room temperature. When indicated, cells were treated for the last 30 min of the incubation with the phosphatase inhibitor okadaic acid (OA; Gibco or LC Services) at 310 nM. Cells were pelleted, washed with TBS, and frozen at −70°C until processed.

Mouse mammary tumor fibroblast (C127) cells were maintained in Dulbecco modified Eagle medium plus 10% FCS, supplemented with penicillin and streptomycin. Transfections were by electroporation using a Gene Pulser unit (Bio-Rad) at 290 V and 960 μF, using $\sim 5 \times 10^6$ cells in 250 to 270 μl. In all electroporation experiments, sample DNAs were cotransfected with 50 μg of sheared salmon sperm DNA as a carrier. The samples were split onto two to six plates. For mammalian cell metabolic labeling, C127 cells were washed with TBS 40 to 48 h posttransfection, starved for 2 h in phosphate-free Dulbecco modified Eagle medium plus 10% dialyzed FCS, and labeled in 1.5 ml per 10-cm-diameter plate with 1 mCi of $H_3{}^{32}PO_4$ per ml for 2 to 3 h with rocking at 37°C. When appropriate, neomycin-resistant (Neo^r) cells were selected with 1 mg of G418 (Gibco) per ml beginning 48 h posttransfection for 1 week, followed by maintenance at 0.5 mg of G418 per ml.

Protein purification. E2 protein was purified by DNA oligo affinity chromatography (21), using three copies of a double-stranded E2 binding site (biotin-CTC(AA$\underline{ACC}$GTCTTC$\underline{GGT}$GC)$_3$ and its complement bound to streptavidin-agarose) in a method similar to that described elsewhere (28). Briefly, frozen Sf9 cell pellets from baculovirus infections were thawed and mixed in 4 pellet volumes of lysis buffer (25 mM HEPES [pH 7.4], 300 mM KCl, 5 mM EDTA, 20% glycerol, 0.3% Triton X-100, 50 mM NaF, 5 mM $NaHPO_4$, 1 mM dithiothreitol [DTT]) plus protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.7 μg of pepstatin per ml, 10 μg of leupeptin per ml) for 30 min on ice; diluted with 10 volumes dilution buffer (lysis buffer with no KCl, Triton X-100, NaF, or $NaHPO_4$); mixed with 45 μg of sheared salmon sperm DNA per mg of protein and incubated on ice 10 min; and spun at $100{,}000 \times g$ for 20 min at 4°C. The supernatant was loaded on a 1-ml oligo affinity column with a Superose 6B precolumn preequilibrated with Z buffer (25 mM HEPES [pH 7.4], 100 mM KCl, 5 mM EDTA, 20% glycerol 0.1% Triton X-100, 1 mM DTT, protease inhibitors). The oligo column was washed with 20 column volumes of Z buffer followed by 20 column volumes of Z buffer minus Triton X-100. Bound E2 protein was eluted with Z buffer minus Triton X-100 plus 1 M KCl, and column fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Proteins were detected by silver staining, with fractions containing purified E2 pooled and dialyzed against 500 ml of 20 mM $KHPO_4$ (pH 7.5)–100 mM K glutamate–1 mM EDTA–10% glycerol–1 mM DTT. Purified ^{32}P-labeled E2 was used directly without dialysis. Various baculovirus E2 expression vectors (wild type, A3, A4, and A5) were constructed as described below, and the resulting proteins were purified by oligo affinity chromatography.

Peptide analysis. Tryptic peptide mapping was performed as follows. Fifty micrograms of unlabeled OA-treated A3 protein and $\sim 10^6$ cpm of ^{32}P-labeled, OA-treated A3 protein were mixed and precipitated for 1 h on ice at a final concentration of 15% trichloroacetic acid. The pellet was washed with 70% ethanol and suspended in 50 μl of 50 mM NH_4 bicarbonate. The partially dissolved pellet was digested at 37°C with 15 μg of sequencing-grade trypsin (Boehringer Mannheim) for 20 to 24 h followed by 10 μg of fresh trypsin for an additional 20 to 24 h. High-pressure liquid chromatography (HPLC) was performed on a 1- by 150-mm reversed-phase C_{18} column (Michrom Bioresources Inc.) with samples loaded in 5% acetonitrile–0.1% trifluoroacetic acid. Elution was with a 5 to 50% acetonitrile gradient at 60 μl/min over 35 min, with protein monitored by absorbance at 210 nm; individual peaks were manually collected, and ^{32}P signal was detected by Cerenkov counting. Fractions containing ^{32}P were injected into a Hewlett-Packard model 5989A quadrupole mass spectrometer equipped with a model 59987A electrospray ion source. Hewlett-Packard software facilitated charge state assignment and deconvolution of the data yielding peptide masses. The predicted masses were determined with MacBioSpec 1.0.1 software (PE Sciex Instruments), using averaged masses. All peptides were considered in making assignments, including those not predicted to be generated by consensus trypsin cleavage sites. Peptides whose sequences predict masses

within 2 Da of the measured masses (and +80, 160, or 240 Da for phosphorylated peptides) were included, but only those within 1 Da are presented (see Table 1).

Protein analysis. For mass analysis, full-length protein was purified by oligo affinity chromatography, further purified by HPLC on a reversed-phase C_{18} column, and injected into the mass spectrometer. E2 protein that was metabolically labeled in C127 cells was immunoprecipitated from lysates after a 30-min incubation at 4°C in 1 ml of radioimmunoprecipitation assay buffer (150 mM Tris-HCl [pH 7.5], 150 mM NaCl, 50 mM NaF, 5 mM $NaHPO_4$, 1 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, protease inhibitors) per 10-cm-diameter plate. Monoclonal antibody B202, which was previously epitope mapped to bind amino acids 284 to 310 within E2 protein (1), was precipitated by binding to protein A-agarose beads. The immunoprecipitated material was separated by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). Total E2 protein was detected by using antibody B202 and protein A-horseradish peroxidase (Amersham) and developed by using enhanced chemiluminescence (NEN). Protein levels were quantitated by scanning several X-ray film exposures with the signal integrated by using NIH Image software. This quantitation was possible because the signals were within 30% of each other and within the linear range as established by standards in another blot. Subsequent to Western blotting, the membranes were washed with TBS and dried, and the ^{32}P-E2 was detected by autoradiography (quantitated with a Fuji phosphorimager). E5 protein was immunoprecipitated from cells transfected with *Bam*HI-cleaved pMLNeoBPV. After selection with G418, individual drug-resistant colonies were picked and amplified. Each of three 10-cm-diameter nearly confluent plates were lysed with 0.6 ml of E5 radioimmunoprecipitation assay buffer (20 mM morpholinepropanesulfonic acid [pH 7.0], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, protease inhibitors) as described above except that E5 peptide antiserum (kindly provided by Dan DiMaio) was used, followed by Western blotting with the E5 antibody (54).

Amino acid analysis. For phosphoamino acid analysis, radiolabeled E2 protein was trichloroacetic acid precipitated, and the pellet was washed with 70% ethanol and boiled in 5.7 M HCl for 1 h, followed by lyophilization. The pellet was dissolved in pH 1.9 buffer (7.8% acetic acid, 2.5% formic acid), mixed with 0.5 μg of each unlabeled phosphoamino acid reference, and separated by two-dimensional thin-layer electrophoresis, using the pH 3.5 buffer (5% acetic acid, 0.5% pyridine) for the second dimension as described elsewhere (56). The amino acid standards were visualized by ninhydrin staining; ^{32}P was detected by autoradiography and phosphorimager analysis. Edman degradation sequencing was performed on HPLC fractions containing ^{32}P, using an Applied Biosystems model 477A sequencer equipped with a model 120A analyzer.

Plasmid construction. The origin-containing plasmid pKSO (67); cytomegalovirus (CMV) promoter-driven E1, E2, E2C, and E8/E2 expression constructs pCGE1, pCGE2, pCGE2C (62), and pCGE8/E2, respectively (7); the BPV genome pMLBPV, Neo^r genome, and vectors pNeoBPV and pNeo5 (32); the repressorless BPV genome pMLBPV E2C$^-$, E8/E2$^-$ (also called p1474-1 [24]); and the chloramphenicol acetyltransferase (CAT) reporter constructs pBPVE5-CAT (46) and pBPVE2-CAT (also called p805-88 [58]) have been described. The luciferase reporter construct pE2RE1tkLuc (25) was made by replacing the *Bgl*II-*Pfl*MI fragment containing the CAT gene from pE2RE1tk-CAT with the *Bgl*II-*Pfl*MI fragment of pT81 containing the luciferase coding sequence (42). pE2RE1tk-CAT was made by ligation of the *Hin*dIII-to-blunted *Nde*I sites of the vector fragment of pBLCAT (31) to the *Sma*I-to-*Hin*dIII fragment of UR229. UR229 is composed of BPV positions 6958 to 7831 containing the E2 response element 1 (E2RE1) region ligated to the *Bam*HI and blunted *Hin*cII sites in the pUC18 polylinker.

Site-directed mutagenesis was by the *ung dut* method (51). pGC2 is a small cloning vector into which was cloned the 5′ end of E2, creating pGC2HXE2, which was used for mutagenesis. Specific individual sequence changes are as indicated in Table 2. Mutations were confirmed by restriction site polymorphisms and sequencing and were transferred within a *Sph*I-to-*Asp*718 restriction fragment to pCGE2, within a *Ppu*MI-to-*Bln*I fragment to pCGE2C and pCGE8/E2, or within a *Bln*I-to-*Asp*718 fragment to pMLBPV. The A3 mutations were derived from p550 (38) and cloned into each construct within an *Asp*718-to-*Bst*XI fragment.

To eliminate the possibility of other mutations as the cause of the A4 phenotype, the A4 mutation in the BPV genome was marker rescued by using restriction fragments which had in common either a *Bln*I-to-*Asp*718 fragment or an *Asp*718-to-*Bst*XI fragment. Both strands of the *Bln*I-to-*Asp*718 fragment were sequenced and found to contain the single point mutation at nucleotide 3310 coding for alanine at amino acid 235. One strand of the *Asp*718-to-*Bst*XI fragment was sequenced and found to contain the three introduced point mutations coding for A3. These 235 and A3 mutations within a *Bln*I-to-*Bst*XI fragment were also sufficient to poison the transformation by an otherwise wild-type viral genome.

Replication and oncogenic transformation assays. Plasmids pCGE1, pCGE2 (with or without mutations), and pKSO were cotransfected into C127 cells, plated onto 10-cm-diameter dishes, and harvested at the indicated time points by a modified Hirt method (18). Briefly, plates were washed with phosphate-buffered saline and lysed with 1 ml of lysis buffer with rocking for 5 min; 0.25 ml of 5 M NaCl was added; the components were mixed for 10 min on ice and spun for 10 min. The Hirt supernatant was further purified by extraction with 0.75 ml of phenol-chloroform-isoamyl alcohol (25:24:1), the aqueous phase was isopropanol precipitated, and the pellet was washed with 70% ethanol. The purified DNA was digested with *Eco*RI, creating a linear DNA and methylation-sensitive *Dpn*I, and analyzed by Southern blotting using a random prime-labeled pKSO probe. Analysis of intact viral DNA replication was similar except that only the *Bam*HI-cleaved pMLBPV DNA was transfected, and random prime-labeled BPV was used as a probe. Replicated, *Dpn*I-resistant DNA was detected by autoradiography and quantitated by phosphorimager (Molecular Dynamics or Fuji) analysis. Transformation assays were performed with 1 μg of *Bam*HI-cleaved pMLBPV DNA. Two weeks posttransfection, the cells were fixed with glutaraldehyde and stained with methylene blue.

Gene expression assays. Reporter constructs (pE2RE1tkLuc, pURR-Luc, pBPVE2CAT, or pBPVE5CAT) were cotransfected with an E2 source (pCGE2 or pMLBPV) into C127 cells. Samples were harvested at the times indicated by washing the plates with phosphate-buffered saline and the addition of the appropriate reporter lysis buffer. For CAT activity, 0.6 ml of CAT lysis buffer (25 mM Bicine [pH 7.8], 2 mM CDTA [Sigma], 0.5% Tween 20) was used per 10-cm-diameter plate, and the plates were frozen at −70°C until assayed. Protein levels were determined in the extracts (clarified by brief centrifugation) by the Bradford assay (Bio-Rad). Equivalent amounts of protein were examined for CAT activities. CAT assays used 25 to 100 μg of extract in 120 mM Tris-HCl (pH 7.8)–0.8 mM acetyl coenzyme A (Calbiochem)–20 μM D-threo-[dichloroacetyl-1-^{14}C]chloramphenicol (Amersham) in 135 μl for 2 to 20 h (adjusted to retain linearity). Acetylated forms of chloramphenicol were isolated and separated by thin-layer chromatography as described previously (51), detected by autoradiography, and quantitated by phosphorimager analysis. For luciferase activity, 0.4 ml of luciferase lysis buffer (25 mM Tris-phosphate [pH 7.8], 2 mM CDTA, 10% glycerol, 1% Triton X-100, 2 mM DTT) was used per 6-cm-diameter plate to extract the enzyme. Extracts were collected after a 15-min room temperature lysis followed by clarification by brief centrifugation. Luciferase assays used 20 μl of extract plus 100 μl of reaction buffer (19 mM Tricine [pH 7.8], 1 mM Mg carbonate, 2.5 mM $MgSO_4$, 0.1 mM EDTA, 33 mM DTT, 270 μM coenzyme A, 470 μM luciferin, 540 μM ATP), with the light signal integrated over 15 s by using a Turner luminometer.

RESULTS

Phosphate mapping of baculovirus-expressed E2 protein. In mapping the major phosphorylation sites, McBride and coworkers noticed seven spots in their two-dimensional gel analysis of full-length E2 (36). Residual labeling of E2 protein, mutant for the two major sites (serines 298 and 301), was also seen. These results suggest that there are up to six different phosphorylation sites on E2, including the two previously identified residues. To simplify the mapping of these additional positions, protein mutant at the predetermined sites was used. This form of E2 is called A3 because amino acids 298, 301, and 290 were mutated to alanines. Because many phosphorylation sites may be only transiently phosphorylated, the cells producing E2 protein were incubated with a phosphatase inhibitor during the labeling period to prevent removal of the phosphate groups by cellular enzymes. Many precedents are available for the functional significance of sites which are only transiently phosphorylated. A specific example relating to viral replicational control is found in simian virus 40 T antigen, in which an apparently minor phosphorylation site has dramatic effects on its activity (40).

Insect cells were infected with recombinant baculovirus expressing the A3 protein. At 48 h postinfection, the cells were treated with the phosphatase inhibitor OA and harvested 30 min later. The concentration of OA used here (310 nM) should strongly inhibit protein phosphatase 2A (PP2A) with a 50% inhibitory concentration [IC_{50}] of ~1 nM, to a lesser extent inhibit PP1 (IC_{50}, 10 to 15 nM), but not inhibit PP2B (IC_{50}, 5 μmM) or PP2C (IC_{50}, >10 μM) (4, 8). A parallel smaller-scale infection was metabolically labeled with phosphate and also treated with OA. E2 proteins from each infection were purified separately by binding to an oligo affinity column containing multimerized E2 binding sites, and the radiolabeled material was used as a tracer for phosphorylated amino acids and peptides. As shown in Fig. 1A, homogeneous protein with respect to mass and phosphate incorporation was obtained and used as the starting material for mapping the phosphorylation sites.

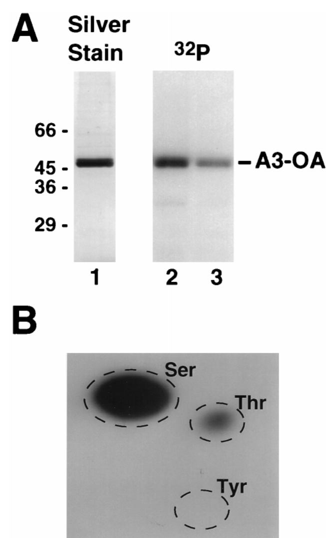

FIG. 1. E2 protein purified from baculovirus was used for mapping phosphorylated residues. (A) The proteins were separated on a 12% gel by SDS-PAGE. Lane 1 shows A3 protein which was purified from cells treated with OA and silver stained. Lanes 2 and 3 show an autoradiogram of two eluted fractions of ^{32}P-labeled protein from DNA affinity chromatography which were pooled and provided the tracer for subsequent procedures. (B) Metabolically labeled A3 protein was hydrolyzed to its constituent amino acids, which were separated as described in Materials and Methods. The positions of phosphoamino acid standards were detected by staining and are indicated by the dotted lines on this autoradiogram. Sizes are indicated in kilodaltons.

A fraction of the phosphate-labeled E2 protein was hydrolyzed to its constituent amino acids, which were then separated by two-dimensional thin-layer electrophoresis (Fig. 1B). About 98% of the phosphorylated material comigrated with a ninhydrin-stained phosphoserine standard. The remaining 2% comigrated with a phosphothreonine standard (no phosphotyrosine was apparent). Therefore the effort to identify modified sites was concentrated on the 36 serine residues in E2.

Purified labeled and unlabeled proteins were mixed, precipitated, and trypsin digested into manageably sized peptides for identification of phosphopeptides. These tryptic peptides were separated on a reversed-phase HPLC column, and protein absorbance and radioactivity were measured (Fig. 2A). Five major peaks containing phosphate were obtained. Individual fractions containing ^{32}P-peptides were injected into an electrospray ionization mass spectrometer to determine their precise masses (for a review see reference 34). To identify peptides corresponding to each mass species, the masses of all possible E2 peptides were determined by computer analysis and compared to the observed values. Generally an unambiguous mass assignment within 1 Da of that observed could be made. One example of the results obtained with the peak eluted at about retention time 14 min is shown in Fig. 2B. From the mass spectrometry, one can deduce that the charge states produced by electrospray for this example derive from a single homogeneous peptide species with a molecular mass of 1,765.06 Da. This mass uniquely fits an E2 peptide, residues 275 to 291, with a single phosphate group. A summary of the phosphopeptides identified by this analysis is shown in Table 1.

Interestingly, one phosphate-containing species mapped by mass spectrometry contained two peptides linked together by a disulfide linkage (Table 1). This species was composed of residues 227 to 247 of the hinge region linked to residues 340 to 342 of the DNA binding domain. As the material was not reduced prior to proteolysis, one expects retention of existing disulfide bonds after trypsin digestion and HPLC separation. The assignment of a disulfide between these two peptides can be made with some confidence for several reasons. First, the HPLC fraction contains a peptide with a mass consistent to this linkage within 0.4 to 0.6 Da in two different preparations. In a subsequent experiment using another vector (A4 [Table 1]), the mass of this material drops by 16 Da when amino acid 235 is mutated to alanine as predicted by the mass change for such a protein. Finally, the Edman sequence data match the predicted sequence for this peptide. Because this is the primary species containing this peptide, it is unlikely that this disulfide bond was formed by random mixing after proteolysis. The presence of a disulfide bond between these regions of the protein suggests close proximity between the DNA binding domain and the hinge in its tertiary structure and may be relevant to the effects of redox potential on DNA binding (39).

Several other radioactive fractions were also subjected to Edman protein sequencing to confirm the peptide identifications made by mass spectrometry. In addition to confirming a particular mass assignment, the sequence data could also be used in some cases to further refine the assignment of a phosphorylated position to a particular serine within a peptide. In this sequencing method, phosphorylated residues are seen as a blank (or reduced signal for partially phosphorylated sites) for that cycle. For example (Fig. 2C), a tryptic peptide starting at amino acid 275 begins with three serines. The first two sites yielded a strong signal eluting at the position predicted for serine, while the third residue yielded a much lower signal demonstrating that residue 277 is phosphorylated.

The data shown in Fig. 2 demonstrate that the major phosphorylated position in A3 protein purified from baculovirus was serine 277. This can be inferred from the fact that it was the major phosphate-containing peak of the five (containing 30% of the total counts per minute) and was found to be homogeneous both by mass spectrometry and thin-layer chromatography (Fig. 2B and reference 26). Other phosphate peaks were found to contain a mix of peptides by these methods. Table 1 summarizes the mass spectrometry and sequencing data obtained with several recombinant E2 expression vectors. The positions cluster predominantly in the putatively unstructured hinge region, with some also present in the amino-terminal activation domain. Figure 3 summarizes the three major sites of phosphorylation mapped in this study and the two positions previously mapped (36). Only those sites identified both by mass spectrometry and sequencing are indicated here. The other peptides shown in Table 1 were identified by only one or the other method, and therefore the confidence in their assignment is decreased. It is possible that other peptides not identified here are also phosphorylated to a lesser degree or were so hydrophilic that they were not separated by the reversed-phase column.

Serine-to-alanine mutations at codons in the E2 gene were introduced into vectors to block phosphorylation at the mapped positions. In some cases, the serines were also mutated to aspartic acid in order to mimic phosphorylation of that amino acid (Table 2 shows the mutations made and the nomenclature used). These mutations were introduced singly and in sets into baculovirus and mammalian cell expression constructs.

We also measured the phosphate levels of intact E2 protein (i.e., without proteolysis). Full-length E2 protein was purified from insect cells expressing wild-type and mutant A4 (E2 protein with alanines replacing serines at positions 235, 290, 298, and 301) protein with and without prior treatment with phosphatase inhibitor (Fig. 2D). These preparations were further

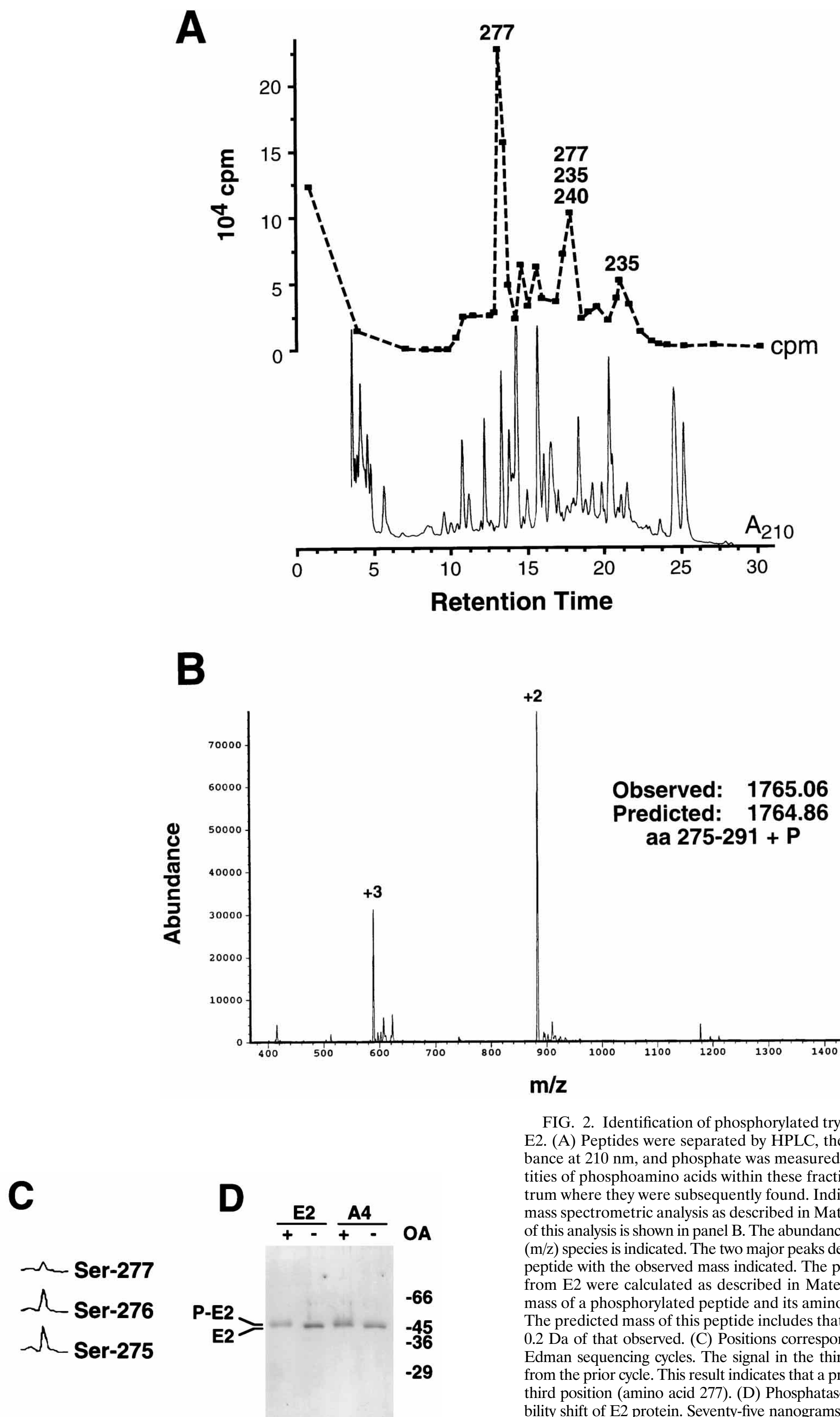

FIG. 2. Identification of phosphorylated tryptic fragments and amino acids in E2. (A) Peptides were separated by HPLC, the protein was measured by absorbance at 210 nm, and phosphate was measured by Cerenkov counting. The identities of phosphoamino acids within these fractions are indicated above the spectrum where they were subsequently found. Individual fractions were subjected to mass spectrometric analysis as described in Materials and Methods. One example of this analysis is shown in panel B. The abundance of particular mass-to-charge ratio (m/z) species is indicated. The two major peaks derive from charge states of the same peptide with the observed mass indicated. The possible masses of peptides derived from E2 were calculated as described in Materials and Methods; the predicted mass of a phosphorylated peptide and its amino acid (aa) coordinates are shown. The predicted mass of this peptide includes that for one phosphate and was within 0.2 Da of that observed. (C) Positions corresponding to serine in three sequential Edman sequencing cycles. The signal in the third cycle may result from carryover from the prior cycle. This result indicates that a predominant phosphoserine is at this third position (amino acid 277). (D) Phosphatase inhibitor treatment causes a mobility shift of E2 protein. Seventy-five nanograms of wild-type or A4 mutant protein purified from cells untreated (−) or treated (+) with OA was subjected to SDS-PAGE followed by silver staining. The more slowly migrating heterogeneous species are indicated here as P-E2. Mass spectrometry is consistent with the notion that this shift is due to hyperphosphorylation. Sizes are indicated in kilodaltons.

TABLE 1. Phosphorylated tryptic peptides

Peptide(s) (Ser,Thr)[a]	Protein[b]	Retention time[c] (min)	Mass (Da)			Edman sequence[d]
			Predicted mass	Observed mass	Δ	
50–84 (3S,T)	A5	18	3,955.55 + 1P	3,954.81	−0.74	Not in seq
		18	4,035.53 + 2P	4,034.87	−0.66	
59–101 (5S,2T)	A5	22	5,228.55 + 3P	5,227.98	−0.57	ND
173–208 (6S,3T)	A4	25	4,204.21 + 2P	4,204.81	+0.60	Not in seq
		24	4,284.19 + 3P	4,284.20	+0.01	
180–193 (2S,2T)	A5	13	1,748.73 + 1P	1,749.37	+0.64	ND
227–247 + 340–342 Disulfide (235A) (2S)	A4	19	2,501.90	2,502.33	+0.43	EAEPAQPVASLLGsPA(C)GPI(R)
		19	2,581.88 + 1P	2,582.27	+0.39	
227–258 (235A) (2S)	A4	19	3,228.69	3,228.57	−0.12	EAEPAQPVASLLGsPA(C)GPI(R)
		19	3,308.67 + 1P	3,309.34	+0.67	
227–247 + 340–342 Disulfide (3S)	A3	17.5	2,517.90	2,517.28	+0.62	EAEPAQPVsSLLG(S)P
		17.5	2,597.88 + 1P	2,597.28	+0.60	
259–274 (2S)	A4	16	1,659.83	1,659.78	−0.05	ND
		16	1,739.81 + 1P	1,739.57	−0.24	
275–291 (3S,2T)	A3	13.5	1,764.86 + 1P	1,765.06	+0.20	SSsTPV
	A4	14.5	1,764.86 + 1P	1,764.76	−0.10	SSsTPVQGTVPV(D)LAA(R)
275–311 (3S,4T)	A4	18.5	3,964.23	3,964.12	−0.09	ND
		18.5	4,044.21 + 1P	4,043.92	−0.29	
312–322 (2T)	A5	14	1,300.70	1,301.50	+0.80	ND
		14	1,380.68 + 1P	1,381.65	+0.97	

[a] Phosphopeptides indicated are those identified by mass spectrometric analysis.
[b] The protein indicated represents the E2 mutant which was used in that tryptic mapping experiment (see Materials and Methods and Table 2).
[c] HPLC retention time on a reversed-phase C_{18} column.
[d] Indicated in the single amino acid code. ND, not done; Not in seq, peptide not found in the analysis; lowercase amino acid, very low amino acid signal found in that cycle; amino acid in parentheses, amino acid not found. Phosphorylated amino acids, carboxy-terminal residues, and cysteines are not expected to be observed.

purified individually by reversed-phase HPLC, and the resulting protein peaks were injected directly into the mass spectrometer. Table 3 presents the results of this analysis. Masses consistent with an amino terminally acetylated E2 protein and multiply phosphorylated forms were observed. The primary sequence of the amino terminus of E2 does indeed predict an acetylated methionine that should be retained in the steady-state pool. Specifically, glutamic acid and threonine near the amino terminus are predictors of acetylated methionines which are not removed (2). As expected, OA treatment resulted in a shift to more highly phosphorylated forms in both the wild-type

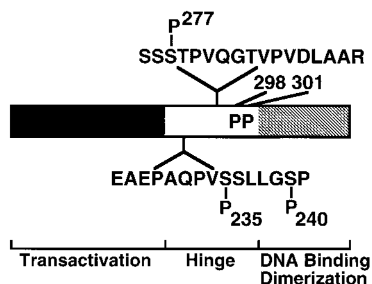

FIG. 3. Summary of the phosphorylated positions in E2. E2 is represented with the three domains indicated: the amino-terminal activation domain; the carboxy-terminal DNA binding and dimerization domain; and the unstructured hinge. The two previously identified phosphoserines at positions 298 and 301 are indicated as P in the body of the protein. The phosphopeptide amino acid sequences and newly identified sites are shown. Only those which could be unambiguously identified by both mass spectrometric and sequence data are shown. Additional sites were found by only one of the methods and are not indicated here (see Table 1).

TABLE 2. E2 mutations

Amino acid position[a]	Nucleotide change[b]	Sequence coordinate(s)[b]
62A	T→G	2791
62D	T→G, C→A	2791, 2792
171A	T→G	3118
181A	A→G, G→C	3148, 3149
187A	T→G	3166
199A	T→G	3202
203A	T→G	3214
235A	T→G	3310
235D	T→G, C→A	3310, 3311
240A	T→G	3325
271A	T→G	3418
277A	T→G	3436
277D	T→G, C→A	3436, 3437

[a] Amino acid position indicates the serine in E2 which was mutated to alanine (A) or aspartic acid (D). Nomenclature used for each mutation within E2 protein or the genome: A3 = S→A at 290, 298, and 301; A4 = S→A at 235, 290, 298, and 301; A5 = S→A at 235, 277, 290, 298, and 301; A3-xxx = A3 plus an additional S→A mutation at the indicated position.
[b] Change at the indicated sequence coordinates of BPV-1.

TABLE 3. Phosphorylation of E2 protein in Sf9 cells

Protein	Phosphorylation state[a]	Mass (Da)	
		Observed mass[b]	Δ from prediction[c]
E2	+0 P	45,497	+5
	+1 P	45,574	+2
	+2 P	45,659	+7
	+3 P	45,766	+34
	+4 P	45,817	+5
E2 + OA[d]	+2 P	45,652	0
	+3 P	45,737	+5
	+4 P	45,813	+1
	+5 P	45,903	+11
	+6 P	45,972	0
E2A4	+0 P	45,452	+24
	+1 P	45,518	+10
	+2 P	45,599	+11
E2A4 + OA	+1 P	45,525	+17
	+2 P	45,604	+16
	+3 P	45,676	+8

[a] Identified phosphate number determined from the observed mass.
[b] Measured by mass spectrometry.
[c] The mass of amino-terminally acetylated full-length E2 protein is predicted to be 45,492 Da; E2A4 mutations alter the predicted mass to 45,428 Da. The amino acid sequence predicts that the amino-terminal methionine would be retained and acetylated, resulting in the addition of 42.04 Da. Each phosphate group adds 79.98 Da to the predicted mass.
[d] OA treatment of Sf9 cells prior to protein purification.

and mutant proteins. Wild-type protein was found in many forms whose masses predict that each contains zero to four phosphates. OA treatment increased the phosphate number by an average of two phosphates to two to six. A4 protein went from zero to two phosphates to one to three phosphates with OA treatment. The mean numbers of phosphates on these proteins were two and four for the wild type and one and two for the mutant (without and with OA treatment, respectively). Thus, mutation of the serines at amino acid positions 235, 290, 298, and 301 resulted in a decrease in the level of phosphorylation by one to three sites, consistent with the assignments. The data also shows that the protein was heterogeneous and contains multiply phosphorylated species. Even with these four potential sites mutated, there are at least two additional phosphorylation sites beyond the major one mapped here (at Ser 277). Other positions identified here, and shown in Table 1, may correspond to this residual phosphorylation.

To study the relevance of these modifications to viral function in mammalian cells, the extents of phosphorylation of E2 and mutants of E2 were examined in mouse cells. The proteins were expressed in mammalian cells, metabolically labeled, purified by immunoprecipitation, and separated by SDS-PAGE. Protein levels were measured by Western blot analysis, thus controlling for potential differences in final yield. Figure 4 shows the results with the ^{32}P signal (top) and the total protein levels (bottom). The specific activity of each protein was calculated by dividing the quantitated ^{32}P by the protein levels as described in Materials and Methods. Consistent with results of McBride and coworkers (36), A3 protein contained less phosphate than wild-type protein (about 40% of the wild-type level in this experiment). Consistent with the mapping studies reported above, mutation of the serine at position 277 reduced the level of E2 phosphate labeling. Moreover, mutation of the 235 residue decreased the phosphorylation by nearly 50%. This result, when compared to the data from Sf9 cells, indicates that the frequency of occupancy of a phosphate at 235 and 277 may be different in the two systems though the same sites are utilized. Finally, in a protein referred to as A5, five sites (amino acids 235, 277, 290, 298, and 301) were mutated; the residual incorporation of phosphate was only about 10% of the wild-type level and may be ascribed to phosphorylation of residue 240. This result suggests that all major E2 phosphorylation sites in C127 cells have been found. This result does not preclude the possibility that other minor pools of phosphorylated E2 have significant physiological significance.

Oncogenic transformation by mutant BPV genomes. To examine the in vivo functions of phosphorylation, mutants which selectively block phosphorylation were tested in assays for E2's diverse activities. We initially examined the effects of these mutations on transformation which requires direct E2 function for viral replication and transcription. Our focus was on residues 235 and 277, the new sites found to be phosphorylated as described above. Each of these mutations was introduced into full-length BPV genomes either singly or in combination with the A3 mutations. Figure 5A shows a typical transformation assay with wild-type and mutant genomes A3, 235, and A4. The single mutation at 235 had no effect, whereas A3 resulted in a small reduction in oncogenic transformation. In contrast, the combination of these mutations in the genome A4 nearly eliminated transformation (2 to 5% of the wild-type level).

The data summarized in Fig. 5C represents the accumulated results of 12 separate transfections using seven different wild-type, four different A4, and five different A3 DNA preparations. Moreover, we could show by marker rescue and marker poisoning experiments into A4 and wild-type genomes, respectively (see Materials and Methods), that the single mutations by themselves, in contrast to the double mutants, were without phenotype. We also extended and confirmed our observations by using the calcium phosphate precipitation transfection method in C127 cells and electroporation of NIH 3T3 cells (26). In addition, similar data were obtained when the cleaved pMLBPV DNA was religated prior to transfection as performed by other authors (38).

We were curious to investigate the transformation phenotypes of the A4 and A3 mutants in the absence of virus-encoded repressors. Study of such a genetic interaction between E2 phosphorylation and the repressors is warranted by the fact that this region of the E2 hinge is also incorporated into the repressor proteins. Furthermore, previously reported observations showed that at least one form of the repressors is required for viral oncogenic transformation of C127 cells (24, 64). The A4 and A3 mutations were combined with mutations

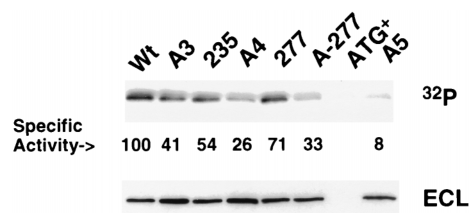

FIG. 4. Phosphorylation of E2 in mammalian cells. C127 cells were transfected with 10 μg of E2 expression constructs containing the mutation indicated (ATG$^+$ is the vector control). After metabolic labeling, the protein was immunoprecipitated with an E2 monoclonal antibody and separated by SDS-PAGE. Total E2 protein was detected by Western blotting (ECL); phosphorylation was detected on the same membrane by autoradiography (^{32}P). Bands were quantitated as described in Materials and Methods. The specific activity is indicated for each mutant protein and is expressed as a percent of wild-type (Wt) activity.

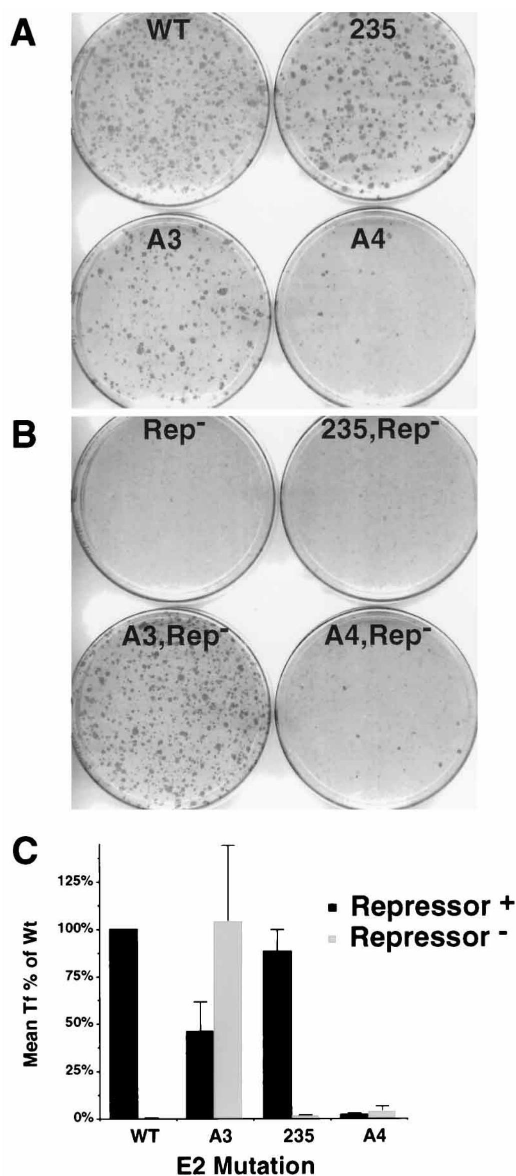

FIG. 5. Transformation defects manifested by BPV genomes containing phosphorylation site mutations. (A) Single or multiple amino acid changes were introduced into the genome. One microgram of *Bam*HI-cleaved pMLBPV was introduced into C127 cells by electroporation. After 2 weeks, the cells on each plate were fixed and stained. (B) Additional mutations which eliminated the splice site for E8/E2 and the initiating methionine for E2C were added to these mutant genomes to isolate E2 activator from the repressor functions. The stained plates shown are from the same experiment as shown in panel A. (C) Several experiments were quantitated, and the mean percentage of wild-type (Wt) transformation and standard deviations were plotted. Wild-type DNA yielded approximately 1,000 transformants per plate (corresponding to 2,000 to 8,000 transformants/μg of BPV DNA). The solid bars represent genomes expressing repressors; shaded bars represent those defective for repressors. Each mean value was derived from 5 to 11 separate transfection experiments.

which block expression of both forms of the repressors by changes at the E8/E2 splice donor and alteration of the methionine start codon of E2C. The data shown in Fig. 5B confirm the requirements for repressors in the wild-type genome (compare the wild type to Rep$^-$ in Fig. 5). Interestingly, the A3,Rep$^-$ mutant transformed cells at frequencies close to the wild type frequency, while the A4,Rep$^-$ mutant did not transform cells. This result shows that the phosphorylation of residues 298 and/or 301 enhance the requirement for repressors and that when these phosphorylation sites are mutant, repressors are not obligatory. Moreover, the A4 E2 allele is distinct in this regard. This result also clearly shows that the A4 mutant genome produces a full-length E2 gene that has a phenotype different from that of the A3 allele, as the differences between A4,Rep$^-$ and A3,Rep$^-$ cannot be ascribed to differences in the repressors.

Mutations at other positions in E2 together with or without the A3 mutations (62A, 62D, 240A, 271A, 277A, and 277D) were all found to transform at levels similar to the wild-type level (26). Mutations of residues 235 and 277 together resulted in no decrease in transformation, but when both of these changes were combined with A3 (resulting in the genome termed A5), transformation was as low as for the A4 mutant. These results suggest that transformation is blocked only when a particular combination of serines cannot be phosphorylated. To test this hypothesis, we mutated the E2 alanine codon at position 235 in the A4 genome to an aspartic acid. This change mimics by charge and somewhat by shape a phosphorylated serine residue. As anticipated by a phosphorylation requirement, when the alanine at position 235 in the A4 genome was mutated to an aspartic acid, transformation was restored to that of A3 (Fig. 6A). This result supports the notion that phosphorylation of one of these sites mutated in A4 within the hinge is critical for transformation. It further suggests either that there is synergy in function between the two or that either one or the other regions (235 or 301) must be phosphorylated for transforming function.

We next asked if the A4 mutant phenotype was due to a loss of E2 function or a gain of some dominant negative function. To address this, additional E2 protein was supplied by cotransfecting an E2 expression construct. Figure 6B shows that the transformation function of the A4 genome can be rescued in *trans* by additional ectopic expression of either wild-type or A4 E2 protein. The A4 viral genome could be brought to wild-type levels of transformation (about 1,000 transformants per 0.5 μg of genomic DNA) by expression of either forms of the CMV-driven E2 protein. The wild-type genome, in contrast, was enhanced five- to sixfold in transformation induction by this additional E2 protein. We do not understand why the two genomes reached different levels of transformation, but the data are consistent with an early requirement for high levels of E2 expression in an establishment phase and the fact that the expression constructs do not persist or express at high levels in later times. In any case, these results are consistent with a loss of E2 function in the A4 genome and the conclusion that the A4 protein is not toxic. Other experiments using cotransfection of the Neo^r gene further address this issue (see below). The 235D mutation, which retains the single point mutation from 235A in addition to a second adjacent base change, transforms to wild-type levels by itself, which argues against a *cis* transformation defect. These experiments in sum demonstrate that both the A3 and A4 mutations resulted in distinct changes in some aspect of E2 protein function. To determine which activity was altered by these mutations, each individual function of E2 protein was examined.

Transient replication. Replication of complete genomes or a minimal-origin-containing plasmid was tested in transient cell-based assays. When the minimal plasmid was used, expression constructs providing E1 and various E2 mutants were cotransfected. BPV genomes will generate transcripts making full-length E2 and the shorter repressor forms (which contain the hinge mutations including 235, 298, and 301). We initially examined the activation of replication by these mutants in the

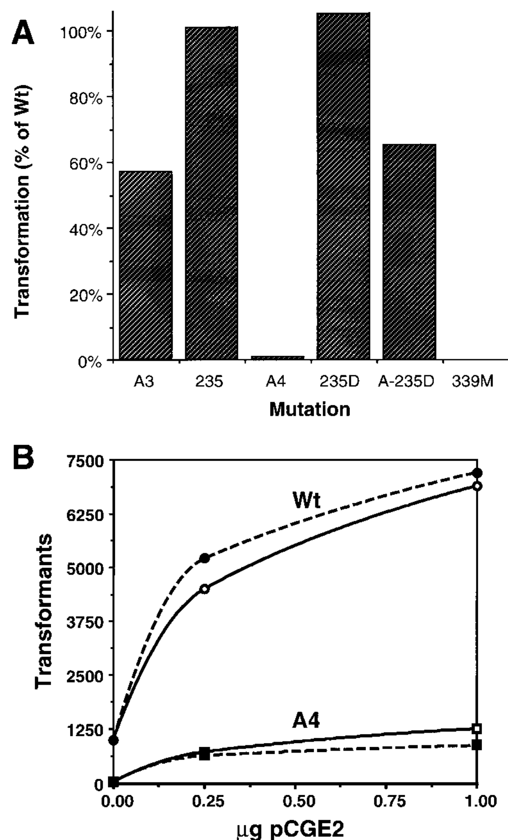

FIG. 6. Complementation of the A4 transformation defect by mimicking a phosphorylation site or by ectopic expression of E2 protein. (A) Mutation of amino acid 235 to an aspartic acid (235D) mimics phosphorylation and rescued transformation. The indicated mutations in pMLBPV were assayed for transformation as in Fig. 5, with the level of transformation in this representative experiment expressed as a percentage of the wild-type (Wt) level. Similar results were obtained in three independent transfections. 339M is a mutation in the DNA binding domain which blocks DNA binding. (B) Transformation was restored by the addition of E2 protein. One microgram of pMLBPV DNA with or without the A4 mutation was transfected into C127 cells with increasing amounts of wild-type or A4 E2 expression construct (pCGE2) and split to two plates. The lower two curves correspond to transformants obtained with the A4 genome, while the upper two curves those obtained with the wild-type BPV DNA. The dotted lines represent addition of the A4 mutant, while the solid lines represent the wild-type E2 expression construct. The number of transformants is plotted against added pCGE2.

context of the genome with its full complement of *cis* regulatory sites and the complete family of E2 proteins.

Consistent with previous results (38), the A3 genome replicated to higher levels than the wild type (Fig. 7A). Introduction of any other mutation (62A, 62D, 171A, 181A, 187A, 199A, 203A, 235A, 235D, 240A, 271A, 277A, or 277D) into the viral DNA did not change the replicational activities of the resulting genome (Fig. 7A and reference 26). Combination of any of these individual mutations with A3 also resulted in genomes which replicated to higher levels than the wild type. In other words, A3, and primarily amino acid 301 (38), was the sole site responsible for modulating transient replication activity of E2 by phosphorylation.

Because replication of the genome effectively integrates E2 replicational and transcriptional activities (since E2 regulates it own and E1's synthesis), we also tested these mutations by using the minimal origin plasmid pKSO. In these experiments, E1 and E2 expression should be equivalent in each sample and the repressors will be absent so that the only variable is the E2 mutation. Another mutation (339M) that is defective for DNA binding served as a negative control. Consistent with the genome replication experiments, any E2 protein which contained the A3 mutations facilitated higher levels of replication compared with wild-type protein (Fig. 7B and reference 26). This result demonstrates that the intrinsic transient replication activity of A4 and A3 protein was higher than wild-type activity. However, these results did not explain either the lack of transformation by A4 or the facilitation of transformation by A3 when repressors are absent.

Transcriptional activation and repression. To examine the transcriptional functions of E2, we tested the activation of expression of a luciferase reporter gene via a thymidine kinase promoter and the E2RE1 enhancer (see Materials and Methods). Figure 8A shows that transcriptional activation by E2 protein derived from A3 and A4 genomes activated transcription to higher levels than the wild type or 235 mutation alone. In other words, the transcriptional activation by A3 and A4 genomes paralleled their replication activity (see also reference 38).

To eliminate the complication derived from differences in replication as well as possible differences in repressor function, we also tested transcription function by using protein derived from CMV-driven E2 expression constructs. All of the E2 phosphorylation site mutations reported here (62A, 62D, 171A, 181A, 187A, 199A, 203A, 235A, 235D, 240A, 271A, 277A, and 277D with and without A3) activated transcription to wild-type levels (Fig. 8B and reference 26). Similar results were obtained in assays using a URR-luciferase reporter which contains the entire URR of BPV linked directly to the luciferase open

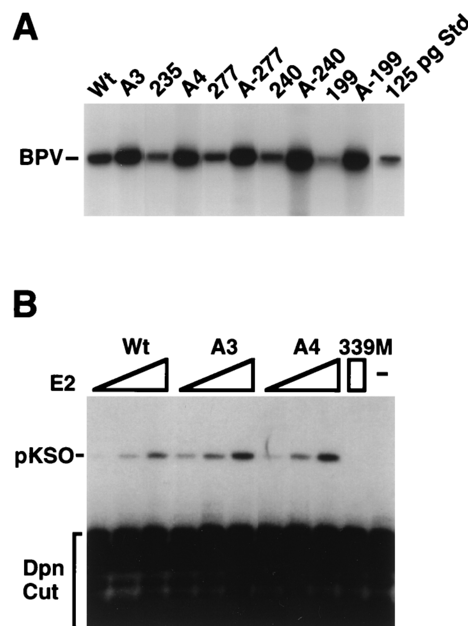

FIG. 7. E2 proteins mutant for serine 301 facilitate replication of origin-containing DNA to high levels. (A) One microgram of pMLBPV DNA with the indicated mutation was transfected into C127 cells, and low M_r-DNA was harvested at day 6 as described in Materials and Methods. *Dpn*I-resistant linear genome-length DNA was detected by Southern analysis. A linear 7.95-kb BPV standard (Std) representing 125 pg is also included. Wt, wild type. (B) The minimal origin-containing plasmid pKSO was used to test E2 replication function. Two micrograms of pKSO, 1 μg of pCGE1, and 0, 0.4, 1, or 2.5 μg of pCGE2 (either wild type or harboring the indicated mutation) were cotransfected. Samples were harvested at day 6 and subjected to Southern blotting. The positions of replicated *Dpn*I-resistant pKSO and the unreplicated cleaved material are indicated.

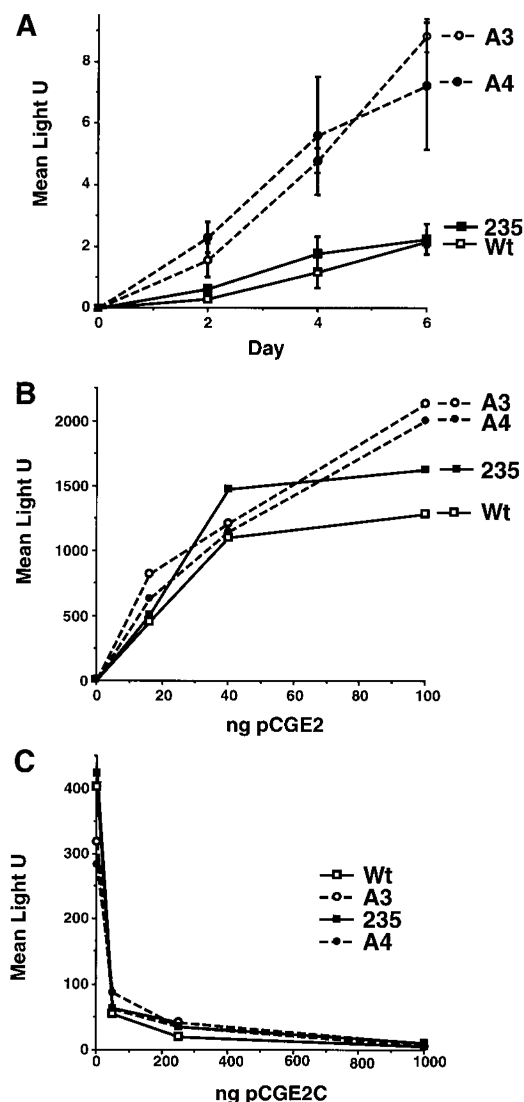

FIG. 8. Transcriptional activation and repression are unaffected by any of the phosphorylation site mutations. (A) Transcriptional activation driven by the balance of full-length E2 activator and the shorter repressors expressed from the genome. One microgram of pMLBPV (released from the prokaryotic vector) was cotransfected with 1.5 μg of the luciferase reporter construct pE2RE1tkLuc, and luciferase activity was measured at the day indicated. Light activity and standard deviation are plotted for each genome, with four independent DNA preparations used for the wild type (Wt), three used for A3, two used for 235, and three used for A4 (duplicates tested for each sample). (B) Transcriptional activation driven by E2 expression constructs. Five micrograms of pE2RE1tkLuc was cotransfected with 0, 16, 40, or 100 ng of pCGE2, and luciferase activity was measured on day 2. Three DNA samples were used for the wild type and A3, one was used for 235, and two were used for A4; the means are plotted. (C) Repression of transcription by E2C. Forty nanograms of pCGE2, 2.5 μg of pE2RE1tkLuc, and 0, 50, 250, or 1,000 ng of pCGE2C were cotransfected, and luciferase activity was measured on day 2.

reading frame (26). Transcriptional activation was also tested in CV-1 cells, and similar wild-type levels were obtained with all of these mutants. Thus, the intrinsic transactivation function was unaffected by these mutations.

Because these mutations are also present in the repressor forms of E2, they were introduced into expression constructs which make either E2C or E8/E2. Wild-type or mutant E2 constructs were cotransfected with their conspecific (bearing the same mutation) repressor construct and the reporter plasmid. Repression of transcription was measured at increasing repressor concentrations with a constant activator concentration. Figure 8C shows that each E2C mutant can repress transcriptional activation like the wild-type repressor. The same result was obtained for mutant E8/E2 expression constructs (26).

These results suggest that E2 transcription function in transient assays is not regulated by phosphorylation. However, it is possible that only a particular promoter within the proper viral sequence context is differentially activated. Because E5 protein is both necessary and sufficient for oncogenic transformation by BPV in the assay used here, we sought to test whether expression of the major transcript encoding E5 was defective in the A4 genomes. To facilitate these experiments, we made use of constructs which have the CAT gene fused in frame with either E2 or E5 (46). Both of these proteins are translated from mRNAs made predominantly from the P4 promoter (3, 57).

Figure 9 shows that each of these E2 mutant proteins provided by the CMV expression vector activated the P4 promoter. The reporter used has all of the viral *cis* elements for transcription and replication control and expresses E1, but it does not express E2. Because these reporter constructs can be replicated and A3 and A4 were better at activating replication, the transcriptional signal was higher when the vectors expressing these proteins were used. Quantitatively lower but qualitatively similar results were obtained with this reporter when mutant genomes were the source for E2 and when pE5CAT was used as the reporter construct (26). This normal activation of the viral oncogene promoter was surprising considering that A4 genomes do not transform.

Stable plasmid maintenance and transformation. These aggregate results demonstrated that the A4 genome was not defective for any known viral function required for transient replication or transcription. Moreover, in direct assays probing the functions of the A4 allele of E2, we could not detect a defect in its known activities. However, transformation requires long-term viral protein function and viral persistence. Thus, the next logical step was to determine if the defective genome persisted and expressed the oncogene E5 throughout the maintenance phase.

As the A4 genome was defective for morphological transformation, our only approach to probe the state of long-term viral functions was to cotransfect the genomes with the Neo^r gene and to select for drug-resistant colonies. The cells which had not received these DNAs were killed by G418 selection, and the remaining drug-resistant cells were amplified. By mea-

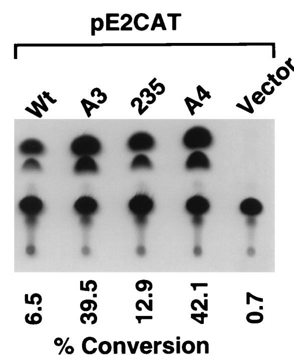

FIG. 9. Activation of the P4 promoter by E2 and E2 phosphorylation site mutants. Two micrograms of the reporter pBPVE2CAT was cotransfected with 100 ng of the indicated pCGE2 expression construct. CAT activity was measured at day 3, and the percentage of chloramphenicol acetylated is shown.

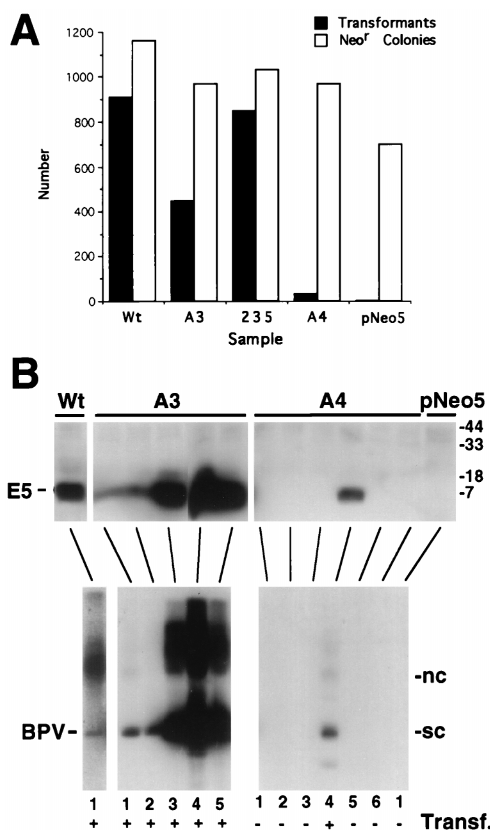

FIG. 10. The A4 mutant genome does not stably replicate itself or express E5. The Neo^r gene and pMLBPV mutants (released from the prokaryotic vector) were cotransfected, and drug resistant colonies were selected. (A) The number of Neo^r colonies and oncogenically transformed foci were quantitated at 2 and 4 weeks, respectively, and plotted. Wt, wild type. (B) Individual drug-resistant colonies were picked and amplified for analysis of E5 protein expression and plasmid DNA at 3 weeks. The upper panel shows E5 protein which was immunoprecipitated from extracts derived from individual clones, fractionated by SDS-PAGE (15% gel), and detected by Western blotting. The positions of protein M_r markers (in kilodaltons) and E5 protein are indicated. The lower panel shows uncut low-M_r DNA revealed by Southern blotting. The positions of supercoiled (sc) and nicked circular (nc) BPV DNA are indicated. The wild-type sample was from a representative clone and is from a 4-week exposure; all other lanes are from a 20-h exposure. A4 samples 1, 2, 3, 5, and 6 showed no indication of BPV DNA even on the 4-week exposure (see also Materials and Methods). Below each lane, the eventual oncogenic state of the clones is indicated. Transf., oncogenic transformation.

suring the number of Neo^r colonies and comparing the number of foci induced by the wild type, we obtained an indication of the cotransfection efficiency. Figure 10A shows that for wild-type genomes, the fraction of Neo^r colonies which proceeded to eventual morphological transformation was near unity. Furthermore, for wild-type BPV DNA, only a small fraction of the stained Neo^r colonies appeared morphologically flat (~25%) on the plates examined after 4 weeks. These results are consistent with the notion that BPV transformation penetrates with a slower kinetics than does neomycin resistance. In addition, when 12 Neo^r colonies that were morphologically flat were picked and expanded, all had BPV plasmids and eventually became oncogenically transformed. Thus, the cotransfection index was near 100%. Importantly, the number of drug-resistant colonies measured was the same when either the A4 mutant or the wild-type genome was cotransfected with the drug resistance marker. This provides further support for the contention that the A4 gene product was not toxic or inducing growth arrest (for example, inducing apoptosis or a cytostatic factor) in these cells. This is true because we assume that the cotransformation index with the A4 genome is equivalent to that measured for the wild-type DNA. Accordingly, if the A4 mutant was toxic or cytostatic, we would predict at least a fourfold reduction in Neo^r colonies. On the other hand, the number of Neo^r colonies which eventually became transformed by A4 was, as anticipated, extremely low and equivalent to that measured previously without the Neo^r gene (compare Fig. 5C and 10A).

Individual drug-resistant colonies were isolated after 2 weeks of selection and amplified for analysis of E5 protein expression and the state of the viral DNA. Figure 10B shows that E5 was expressed from all of the A3 clones but only one of six clones obtained from the A4 cotransfection experiment. This result indicates that despite the similar transient replication and P4 promoter activities of these two viruses, A4 was defective for some function required for stable E5 expression. The eventual oncogenic transformation paralleled the E5 expression patterns of all of these clones. That is, all of the A3 and wild-type clones became visibly morphologically transformed and did not show contact inhibition, whereas only one A4 clone (clone 4) became so transformed.

Low-M_r DNA was extracted from parallel plates from each of these clones and analyzed for plasmid viral DNA. The lower panel of Fig. 10B shows that when a particular clone expressed E5, it also contained episomal viral DNA. All of the A3 clones had relatively high amounts of plasmid DNA, and few if any showed bands indicative of integrated or rearranged DNA. Further, all clones cotransfected with wild-type BPV DNA expressed E5 protein and had plasmid DNA, though at lower levels than for A3 (26). All of the drug-resistant clones derived from A3 and wild-type cotransfections contained unit-length BPV plasmids. In contrast, only one of the six Neo^r clones picked from the A4 sample, and the one which also expressed E5, showed any detectable plasmid DNA. The sensitivity of this analysis indicated that the BPV DNA in these A4 clones dropped to below the detection limit, which was less than one copy per cell. Furthermore, these clones contained no detectable BPV sequence in total genomic DNA, indicating that the viral DNA was lost and not integrated (26). The sample derived from clone 4 also contained some lower-M_r material which may be a deleted or rearranged form of the BPV DNA. The presence of deleted genomes in the A4 sample and their absence in A3 clones suggests that deletion or another type of mutation in the A4 genome is obligate to allow for transformation. Consistent with this view, we found that when transformed foci were selected from the A4 cotransfection experiment, 80% of the clones contained rearrangements in the detected plasmids (26).

DISCUSSION

We have mapped new phosphorylation sites in the hinge region of the BPV-1 E2 protein. These positions were phosphorylated both in insect and mammalian cells; apparently all of the predominant sites modified in mammalian cells have now been identified, as an E2 mutant at five serines (the A5 protein) incorporated less than 10% of the phosphate incorporated by the wild-type protein. The new sites were mapped to three amino acids, 235, 240, and 277, in the protein; however, indications for minor phosphorylation sites in baculovirus-expressed E2 protein were observed in the analysis of the mass spectometric data. All of these sites guided our attempts to correlate functional significance of phosphorylation in the viral transformation and replication processes occurring in

mammalian cells. By site-directed mutagenesis of the viral genome, we found evidence for the functions of phosphorylation only at amino acid position 235.

While the single mutation at serine 235 had no phenotype on its own, when it was combined with E2 mutations at the previously identified phosphorylation sites, the resulting A4 genome was severely blocked for oncogenic transformation and plasmid retention. Every transient assay that was performed with the mutant E2 allele, either incorporated into the full-length activator or repressor forms in isolated expression constructs or in the intact viral genome, showed that the effected genes functioned at least as well as the wild type. Thus, it seems reasonable to suggest that the defect exposes some function of E2 critical at later stages in the establishment of the stable transformed state. Several lines of evidence demonstrate that the block to stable establishment was not due to a toxic or cytostatic effect on transfected cells. First, addition of extra E2A4 protein in *trans* restored transformation by the mutant genome. Therefore, this mutation results in a loss of function which can be overcome by an excess of protein. Second, no reduction in Neo^r colonies compared to the wild type was observed when the Neo^r gene was cotransfected with A4 genome. Third, in the short-term transient assays (protein expression, replication, and transcriptional activation), the A4 protein yielded activities similar to those of the A3 protein with no apparent toxicity.

That phosphorylation is directly required at this 235 site is substantiated by the fact that the A4 genome could be rescued for transformation by mutation of the mutant codon to an aspartic acid triplet. These data are also not compatible with the 235 mutation creating a *cis* defect, as the aspartic acid mutation maintains an altered sequence at this position. We conclude that phosphorylation either at position 235 or at positions 298 and 301 is required for stable viral functions in transformation and that loss of such capacity at both regions of the hinge results in a loss of E2 function.

Amino acid sequences of the E2 hinge region of papillomaviruses are not in general conserved, although the region always contains clusters of PEST sequences (14). PEST sequences were so named for their richness in proline, glutamic acid, serine, and threonine amino acids and for their propensity to target the degradation of a protein in which they are found (49). Moreover phosphorylation in the PEST region may also be critical for determining rates of degradation (61). The kinases that might exert this control are generally not clearly delineated, and for E2 proteins, this is again the case. While residues 298 and 301 are embedded in a context that fits consensus sites for cyclin-dependent kinases, mitogen-activated protein kinases, and casein kinases I or II, amino acid 235 does not closely fit any known kinase recognition site.

One model that seems to be consistent with most of our data and the foregoing discussion would be that the lack of transformation by the A4 genome is caused by decreased protein stability. It is possible that BPV E2 protein contains a region within the hinge which requires phosphorylation of one of several amino acids for stability or for degradation. If phosphorylation of all of these sites is blocked (as in the A4 mutation), or if the phosphates are removed by a phosphatase (such as after S phase), then the protein might be subjected to more rapid turnover. This putative instability of mutant E2 proteins may become apparent only at the low, limiting levels of E2 that are expressed at the stages of transformation when plasmid copy number reaches homeostasis. This hypothesis is required, as we see no differences in protein accumulation in transient assays (e.g., Fig. 4). If this model is correct, then the capacity for recognition or degradation of the underphosphorylated protein must be limited in the cell, with the system being overwhelmed by transient high-level expression of E2. Similar suggestions were made recently to account for why certain mutant E2 alleles appeared to be destabilized in a one-vector expression system whereas analogous mutants in other systems showed no such effect (13).

These ideas could also explain the curious finding reported here concerning the requirements for repressor proteins in BPV transformation. While wild-type genomes need at least one form of the repressors (24, 64), we found that the A3 genome did not display such a requirement. If repressors are normally required to down regulate the activity of E2, certain mutations in E2 itself that destabilize its activity would suppress this requirement. Furthermore, pushing such a speculation, we might suggest that the A4 mutation goes too far in this destabilization and leads to a null E2 phenotype.

These notions are conservative in that we do not need to postulate some new unidentified activity for the E2 protein distinct from their known roles in regulating viral replication and transcription. However, E2 may simply effect expression of cellular genes, as it does viral genes, required for stable plasmid maintenance or transformation. Indeed, Brokaw and coworkers recently reported mutations in the E2 activation domain which despite normal transient replication and transcription functions also failed to transform C127 cells (5). In these cases, the stable replication activities of the mutant genomes have not been tested. If they are also found to be defective for plasmid retention, it would be interesting to determine if the mutations are within the same complementation group for E2 function as in the phosphorylation site mutant identified here.

We must also consider the possibility that E2 does have novel activities for which we at present have no biochemical assays. For example, the activator may play some role in plasmid segregation, and this activity is dependent on phosphorylation of the hinge region. This defect may not become apparent early on but only after many rounds of replication and cellular division occur. If at each cell division segregation mechanisms (which are poorly understood) are defective, viral DNA may be lost or revertants may be generated at a higher frequency. Some indirect arguments for a requirement for E2 in nuclear retention or partitioning was suggested by Piirsoo et al., as these authors found that replication of a minimal origin plasmid with weak E2 binding sites was insufficient for stable plasmid maintenance, in contrast to the plasmid's behavior in transient assays (44). The suggestion was made, paralleling similar ideas in the Epstein-Barr virus oriP field (22, 66), that multiple E2 binding sites are required to sequester E2 protein in *cis* for nuclear retention or segregation functions. Along these lines, perhaps cooperative E2 binding to multiple sites in the URR for such purposes may rely on E2 phosphorylation.

The A4 genome transforms cells at a low level, and we have been able to select these few transformants. We have observed predominantly rearranged genomes from A4 transfections but not from wild-type or A3 transfections, suggesting that these rearrangements facilitated suppression of the A4 transformation defect. Clearly, mutation of the defective viral DNA could suppress the E2 defect caused by mutations in the phosphorylation sites of the hinge. We are currently examining these genomes with the hope that such suppressors will yield clues to the nature of E2 function uncovered by the phosphorylation mutants described.

ACKNOWLEDGMENTS

Thanks go to Tim Nottoli, Shaw Sun, and Robert Mendoza for preliminary studies on E2 phosphorylation and to Sharleen Zhou for

Edman degradation sequencing of peptides. Technical assistance was provided by Sun Y. Kim, and pE2RE1tkLuc was made by Jeff Lee. Plasmid p550 was kindly provided by Alison McBride, the E5 antibody was a generous gift from Dan DiMaio, and p805-88 and pBPVE5-CAT were generously provided by Barbara Spalholz. Many thanks go to members of the Botchan lab for reagents and helpful discussions. The manuscript was improved by suggestions from Sandra J. Lehman.

C.W.L. was supported in part by NIH postdoctoral fellowship CA09041 to the Cancer Research Lab, University of California, Berkeley. This work was supported by Public Health Service grants CA42414 and CA30490 from the National Institutes of Health to M.R.B.

REFERENCES

1. **Androphy, E. J.** 1989. Personal communication.
2. **Arfin, S. M., and R. A. Bradshaw.** 1988. Cotranslational processing and protein turnover in eukaryotic cells. Biochemistry **27:**7979–7984.
3. **Baker, C. C., and P. M. Howley.** 1987. Differential promoter utilization by the bovine papillomavirus in transformed cells and productively infected wart tissues. EMBO J. **6:**1027–1035.
4. **Bialojan, C., and A. Takai.** 1988. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. Biochem. J. **256:**283–290.
5. **Brokaw, J. L., M. Blanco, and A. A. McBride.** 1996. Amino acids critical for the functions of the bovine papillomavirus type 1 E2 transactivator. J. Virol. **70:**23–29.
6. **Chiang, C. M., G. Dong, T. R. Broker, and L. T. Chow.** 1992. Control of human papillomavirus type 11 origin of replication by the E2 family of transcription regulatory proteins. J. Virol. **66:**5224–5231.
7. **Choe, J., P. Vaillancourt, A. Stenlund, and M. Botchan.** 1989. Bovine papillomavirus type 1 encodes two forms of a transcriptional repressor: structural and functional analysis of new viral cDNAs. J. Virol. **63:**1743–1755.
8. **Cohen, P., S. Klumpp, and D. L. Schelling.** 1989. An improved procedure for identifying and quantitating protein phosphatases in mammalian tissues. FEBS Lett. **250:**596–600.
9. **DiMaio, D.** 1986. Nonsense mutation in open reading frame E2 of bovine papillomavirus DNA. J. Virol. **57:**475–480.
10. **DiMaio, D., D. Guralski, and J. T. Schiller.** 1986. Translation of open reading frame E5 of bovine papillomavirus is required for its transforming activity. Proc. Natl. Acad. Sci. USA **83:**1797–1801.
11. **Dostatni, N., F. Thierry, and M. Yaniv.** 1988. A dimer of BPV-1 E2 protein containing a protease resistant core interacts with its DNA target. EMBO J. **7:**3807–3816.
12. **Dvoretzky, I., R. Shober, S. K. Chattopadhyay, and D. Lowy.** 1980. A quantitative in vitro focus assay for bovine papilloma virus. Virology **103:**369–375.
13. **Ferguson, M. K., and M. R. Botchan.** 1996. Genetic analysis of the activation domain of bovine papillomavirus protein E2: its role in transcription and replication. J. Virol. **70:**4193–4199.
14. **Giri, I., and M. Yaniv.** 1988. Structural and mutational analysis of E2 *trans*-activating proteins of papillomaviruses reveals three distinct functional domains. EMBO J. **7:**2823–2829.
15. **Groff, D. E., and W. D. Lancaster.** 1986. Genetic analysis of the 3′ early region transformation and replication functions of bovine papillomavirus type 1. Virology **150:**221–230.
16. **Ham, J., G. Steger, and M. Yaniv.** 1994. Cooperativity in vivo between the E2 transactivator and the TATA box binding protein depends on core promoter structure. EMBO J. **13:**147–157.
17. **Hegde, R. S., S. R. Grossman, L. A. Laimins, and P. B. Sigler.** 1992. Crystal structure at 1.7 A of the bovine papillomavirus-1 E2 DNA-binding domain bound to its DNA target. Nature **359:**505–512.
18. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26:**365–369.
19. **Hoppe-Seyler, F., and K. Butz.** 1994. Cellular control of human papillomavirus oncogene transcription. Mol. Carcinog. **10:**134–141.
20. **Hubbert, N. L., J. T. Schiller, D. R. Lowy, and E. J. Androphy.** 1988. Bovine papilloma virus-transformed cells contain multiple E2 proteins. Proc. Natl. Acad. Sci. USA. **85:**5864–5868.
21. **Kadonaga, J. T., and R. Tjian.** 1986. Affinity purification of sequence-specific DNA binding proteins. Proc. Natl. Acad. Sci. USA **83:**5889–5893.
22. **Krysan, P. J., S. B. Haase, and M. P. Calos.** 1989. Isolation of human sequences that replicate autonomously in human cells. Mol. Cell. Biol. **9:**1026–1033.
23. **Lambert, P. F., N. L. Hubbert, P. M. Howley, and J. T. Schiller.** 1989. Genetic assignment of multiple E2 gene products in bovine papillomavirus-transformed cells. J. Virol. **63:**3151–3154.
24. **Lambert, P. F., B. C. Monk, and P. M. Howley.** 1990. Phenotypic analysis of bovine papillomavirus type 1 E2 repressor mutants. J. Virol. **64:**950–956.
25. **Lee, J., and M. R. Botchan.** 1996. Unpublished data.
26. **Lehman, C. W., and M. R. Botchan.** 1996. Unpublished data.
27. **Leptak, C., S. Ramon y Cajal, R. Kulke, D. R. Riese, B. H. Horwitz, G. P. Dotto, and D. DiMaio.** 1991. Tumorigenic transformation of mouse keratinocytes by the E5 genes of human papillomavirus type 16 and bovine papillomavirus type 1. J. Virol. **65:**7078–7083.
28. **Li, R., J. Knight, G. Bream, A. Stenlund, and M. Botchan.** 1989. Specific recognition nucleotides and their DNA context determine the affinity of E2 protein for 17 binding sites in the BPV-1 genome. Genes Dev. **3:**510–526.
29. **Li, R., J. D. Knight, S. P. Jackson, R. Tjian, and M. R. Botchan.** 1991. Direct interaction between Sp1 and the BPV enhancer E2 protein mediates synergistic activation of transcription. Cell **65:**493–505.
30. **Lim, D., M. Gossen, C. W. Lehman, and M. R. Botchan.** 1996. Unpublished data.
31. **Luckow, B., and G. Schutz.** 1987. CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. Nucleic Acids Res. **15:**5490.
32. **Lusky, M., and M. R. Botchan.** 1984. Characterization of the bovine papilloma virus plasmid maintenance sequences. Cell **36:**391–401.
33. **Lusky, M., and E. Fontane.** 1991. 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. Proc. Natl. Acad. Sci. USA **88:**6363–6367.
34. **Mann, M., and M. Wilm.** 1995. Electrospray mass spectrometry for protein characterization. Trends Biochem. Sci. **20:**219–224.
35. **McBride, A., R. Schlegel, and P. M. Howley.** 1988. The carboxy-terminal domain shared by the bovine papillomavirus E2 transaction and repressor proteins contains a specific DNA binding activity. EMBO J. **7:**533–539.
36. **McBride, A. A., J. B. Bolen, and P. M. Howley.** 1989. Phosphorylation sites of the E2 transcriptional regulatory proteins of bovine papillomavirus type 1. J. Virol. **63:**5076–5085.
37. **McBride, A. A., J. C. Byrne, and P. M. Howley.** 1989. E2 polypeptides encoded by bovine papillomavirus 1 form dimers through the carboxy-terminal DNA-binding domain: transactivation is mediated through the amino-terminal domain. Proc. Natl. Acad. Sci. USA **86:**510–514.
38. **McBride, A. A., and P. M. Howley.** 1991. Bovine papillomavirus with a mutation in the E2 serine 301 phosphorylation site replicates at a high copy number. J. Virol. **65:**6528–6534.
39. **McBride, A. A., R. D. Klausner, and P. M. Howley.** 1992. Conserved cysteine residue in the DNA-binding domain of the bovine papillomavirus type 1 E2 protein confers redox regulation of the DNA-binding activity in vitro. Proc. Natl. Acad. Sci. USA **89:**7531–7535.
40. **McVey, D., L. Brizuela, I. Mohr, D. R. Marshak, Y. Gluzman, and D. Beach.** 1989. Phosphorylation of large tumour antigen by cdc2 stimulates SV40 DNA replication. Nature **341:**503–507.
41. **Neary, K., and D. DiMaio.** 1989. Open reading frames E6 and E7 of bovine papillomavirus type 1 are both required for full transformation of mouse C127 cells. J. Virol. **63:**259–266.
42. **Nordeen, S. K.** 1988. Luciferase reporter gene vectors for analysis of promoters and enhancers. BioTechniques **6:**454–458.
43. **Nottoli, T.** 1992. Ph.D. thesis. University of California, Berkeley, Calif.
44. **Piirsoo, M., E. Ustav, T. Mandel, A. Stenlund, and M. Ustav.** 1996. *Cis* and *trans* requirements for stable episomal maintenance of the BPV-1 replicator. EMBO J. **15:**1–11.
45. **Prakash, S. S., S. R. Grossman, R. B. Pepinsky, L. A. Laimins, and E. J. Androphy.** 1992. Amino acids necessary for DNA contact and dimerization imply novel motifs in the papillomavirus E2 trans-activator. Genes Dev. **6:**105–116.
46. **Prakash, S. S., B. H. Horwitz, T. Zibello, J. Settleman, and D. DiMaio.** 1988. Bovine papillomavirus E2 gene regulates expression of the viral E5 transforming gene. J. Virol. **63:**3608–3613.
47. **Rabson, M. S., C. Yee, Y. C. Yang, and P. M. Howley.** 1986. Bovine papillomavirus type 1 3′ early region transformation and plasmid maintenance functions. J. Virol. **60:**626–634.
48. **Rank, N. M., and P. F. Lambert.** 1995. Bovine papillomavirus type 1 E2 transcriptional regulators directly bind two cellular transcription factors, TFIID and TFIIB. J. Virol. **69:**6323–6334.
49. **Rechsteiner, M.** 1990. PEST sequences are signals for rapid intracellular proteolysis. Semin. Cell Biol. **1:**433–440.
50. **Reise, D. J. I., J. Settleman, K. Neary, and D. DiMaio.** 1990. Bovine papillomavirus E2 repressor mutant displays a high copy number phenotype and enhanced transforming activity. J. Virol. **64:**944–949.
51. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
52. **Schiffman, M. H., H. M. Bauer, R. N. Hoover, A. G. Glass, D. M. Cadell, B. B. Rush, D. R. Scott, M. E. Sherman, R. J. Kurman, S. Wacholder, et al.** 1993. Epidemiologic evidence showing that human papillomavirus infection causes most cervical intraepithelial neoplasia. J. Natl. Cancer Inst. **85:**958–964.
53. **Schiller, J. T., W. C. Vass, K. H. Vousden, and D. R. Lowy.** 1986. E5 open reading frame of bovine papillomavirus type 1 encodes a transforming gene. J. Virol. **57:**1–6.
54. **Schlegel, R., M. Wade-Glass, M. S. Rabson, and Y.-C. Yang.** 1986. The E5 transforming gene of bovine papillomavirus encodes a small, hydrophobic polypeptide. Science **233:**464–467.
55. **Sedman, J., and A. Stenlund.** 1995. Co-operative interaction between the

initiator E1 and the transcriptional activator E2 is required for replicator specific DNA replication of bovine papillomavirus *in vivo* and *in vitro*. EMBO J. **14:**6218–6228.
56. **Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart.** 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. Cell **20:**807–816.
57. **Spalholz, B. A.** 1993. Importance of the bovine papillomavirus P_{2443} promoter in the regulation of E2 and E5 expression. J. Virol. **67:**6278–6284.
58. **Spalholz, B. A., S. B. Vande Pol, and P. M. Howley.** 1991. Characterization of the *cis* elements involved in basal and E2-transactivated expression of the bovine papillomavirus P_{2443} promoter. J. Virol. **65:**743–753.
59. **Sun, S., C. W. Lehman, E. Fouts, and M. B. Botchan.** 1996. Unpublished data.
60. **Szymanski, P., and A. Stenlund.** 1991. Regulation of early gene expression from the bovine papillomavirus genome in transiently transfected C127 cells. J. Virol. **65:**5710–5720.
61. **Tyers, M., G. Tokiwa, R. Nash, and B. Futcher.** 1992. The Cln3-Cdc28 kinase complex of S. cerevisiae is regulated by proteolysis and phosphorylation. EMBO J. **11:**1773–1784.
62. **Ustav, M., and A. Stenlund.** 1991. Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. EMBO J. **10:**449–457.
63. **Vaillancourt, P., T. Nottoli, J. Choe, and M. R. Botchan.** 1990. The E2 transactivator of bovine papillomavirus type 1 is expressed from multiple early promoters. J. Virol. **64:**3927–3937.
64. **Vande Pol, S. B., and P. M. Howley.** 1995. Negative regulation of the bovine papillomavirus E5, E6, and E7 oncogenes by the viral E1 and E2 genes. J. Virol. **69:**395–402.
65. **Winokur, P. L., and A. A. McBride.** 1992. Separation of the transcriptional activation and replication functions of the bovine papillomavirus-1 E2 protein. EMBO J. **11:**4111–4118.
66. **Wysokenski, D. A., and J. L. Yates.** 1989. Multiple EBNA1-binding sites are required to form an EBNA1-dependent enhancer and to activate a minimal replicative origin within oriP of Epstein-Barr virus. J. Virol. **63:**2657–2666.
67. **Yang, L., R. Li, I. J. Mohr, R. Clark, and M. R. Botchan.** 1991. Activation of BPV-1 replication *in vitro* by the transcription factor E2. Nature **353:**628–633.
68. **Yang, L., I. Mohr, R. Li, T. Nottoli, S. Sun, and M. Botchan.** 1991. The transcription factor E2 regulates BPV-1 DNA replication *in vitro* by direct protein-protein interaction. Cold Spring Harbor Symp. Quant. Biol. **56:**335–346.
69. **Yang, Y., B. Spalholz, M. Rabson, and P. Howley.** 1985. Dissociation of transforming and trans-activation functions for bovine papillomavirus type 1. Nature **318:**575–577.
70. **Yang, Y.-C., H. Okayama, and P. M. Howley.** 1985. Bovine papillomavirus contains multiple transforming genes. Proc. Natl. Acad. Sci. USA **82:**1030–1034.
71. **Zur Hausen, H.** 1991. Human papillomaviruses in the pathogenesis of anogenital cancer. Virology **184:**9–13.