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Phosphorylation Sites of the E2 Transcriptional Regulatory Proteins of Bovine Papillomavirus Type 1

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The E2 open reading frame of bovine papillomavirus type 1 (BPV-1) encodes three transcriptional regulatory proteins. The full-length open reading frame encodes a protein of 410 amino acids which functions as a transcriptional transactivator. Two transcriptional repressor proteins, E2-TR and E8/E2, contain the C-terminal 249 and 204 amino acids, respectively. We have expressed both the full-length E2 protein and the E2-TR repressor protein in insect cells, by using recombinant baculoviruses, and in mammalian COS-1 cells, by using a chimeric simian virus 40/BPV-1 virus. Analysis of the E2 proteins revealed that both the transactivator and repressor forms are phosphorylated predominately on serine residues at similar sites in both expression systems. By a combination of peptide mapping and site-directed mutagenesis techniques, the serine residues at positions 298 and 301 were determined to be the major phosphorylation sites of the BPV-1 E2 proteins.

The E2 open reading frame (ORF) of bovine papillomavirus type 1 (BPV-1) is capable of encoding at least three polypeptides with transcriptional regulatory properties. The entire E2 ORF encodes a 410-amino-acid protein that functions as a transcriptional transactivator by binding to specific DNA enhancer sequences within the BPV-1 long control region and by stimulating transcription from several viral promoters (1, 31, 42, 43). The 3′ half of the ORF has been shown to encode two additional smaller polypeptides that function to suppress full-length E2-mediated transactivation (6, 20, 21). One of these polypeptides, E2-TR (transcriptional repressor), is expressed from the P_{3080} promoter and translated from an internal initiation codon in the E2 ORF (20). The second repressor, designated E8/E2, is encoded by a spliced message which links 11 amino acids from the E8 ORF to the 3′ portion of the E2 ORF via the splice acceptor site at nucleotide 3225 (6, 19, 21). Three E2-encoded proteins have been identified in BPV-1-transformed cells, with apparent molecular masses of 48, 31, and 28 kilodaltons, and mutational analyses have identified the largest to be the transactivator, the smallest to be the E8/E2 repressor, and the 31-kilodalton species to be the repressor expressed from the P_{3080} promoter (17, 19). All three E2-encoded polypeptides have been shown to be coexpressed in BPV-1-transformed cells at a ratio of 1 E2:10 E2-TR:3 E8/E2, thereby potentially explaining the low abundance of virus transcripts normally found in BPV-1-transformed cells (17).

All three E2 proteins share a common carboxy-terminal domain of approximately 100 amino acids in length which encodes the specific DNA-binding function (27, 32). An amino-terminal domain of about 220 amino acids, which is unique to the full-length transactivator polypeptide, encodes the transcriptional activation function (12, 14, 26). The common carboxy-terminal domain contains sequences which promote E2 dimer formation, and the E2 proteins bind DNA as dimers (9, 26, 33). Thus, it is possible that the repressor proteins function to inhibit E2-mediated transactivation either by competitive binding between the transactivator and repressor proteins for the E2 DNA binding sites in the viral genome or by the formation of potentially inactive heterodimers between transactivator and repressor species.

Phosphorylation is known to be an important posttranslational modification of cellular and viral regulatory proteins, including those involved in the control of transcription and the cell cycle (2, 5, 7, 8, 36, 47). In this study, we report the results of an examination of the phosphorylation state of BPV-1 E2-encoded proteins. Our data demonstrate that the E2 polypeptides are phosphorylated primarily on one or more serine residues at a site adjacent to the carboxy-terminal DNA-binding domain, which is common to all three E2 proteins. These results raise the possibility that posttranslational phosphorylation may be important for the function of the BPV-1 E2 proteins.

MATERIALS AND METHODS

Baculovirus expression system. DNA encoding both the full-length E2 polypeptide and the shorter E2-TR repressor were cloned into the baculovirus expression vector, pAC373 (44). *Bam*HI linkers were inserted into the *Hin*dIII site of $pTZE2_m$ and the *Eco*RI site of pTZE2-TR, which have been described previously (27). A *Bam*HI fragment from each plasmid was cloned into the *Bam*HI site of pAC373, resulting in pAC-E2 or pAC-E2-TR which encode E2 or E2-TR polypeptides, respectively. SF9 cells were cotransfected with 1 μg of *Autographa californica* nuclear polyhedrosis virus DNA and 2 μg of of the pAC373 constructs, as described previously (44). The supernatant was harvested 6 days posttransfection, and recombinant viruses were isolated by several rounds of plaque purification as described by Summers and Smith (44). Two plaque-purified virus isolates, vAC-E2 (full-length E2) and vAC-E2-TR (E2-TR repressor), were used in these studies.

SV40/BPV recombinant virus expression system. Plasmid, $pTZE2_{kz}$, was constructed from $pTZE2_g$ (27), which contains BPV-1 sequences from *Bst*EII (nucleotide 2406) to *Bam*HI (nucleotide 4451). Sequences from *Bst*EII to *Sph*I (nucleotide 2622) were replaced with synthetic oligonucleotides that provided a Kozak consensus initiation codon for

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the E2 ORF. (The ATG for the E2 ORF is at nucleotide 2606). The *Bst*EII-to-*Bst*XI (nucleotide 3889) fragment of pTZE2$_{kz}$ was substituted for the corresponding fragment in the simian virus 40 (SV40)/BPV-1 recombinant plasmid, pPAVA-1 (38), resulting in plasmid pSB-E2$_{kz}$. Virus particles were generated in CMT4 cells as described by Settleman and DiMaio (38).

Viral infection and immunoprecipitation. SF9 cells were infected with wild-type or recombinant baculoviruses, and COS-1 cells were infected with either SV40 or the SV40/BPV-1 recombinant viruses at a high multiplicity of infection. Infected cells were metabolically labeled at 40 to 48 h postinfection. Cells were initially starved for 2 h in either Grace insect medium or Dulbecco modified Eagle medium, both of which were deficient in either methionine and cysteine or phosphate. Cells were labeled with [^{35}S]cysteine (0.25 mCi/ml, >600 Ci/mmol) and [^{35}S]methionine (0.25 mCi/ml, >800 Ci/mmol) for 2 h or with $^{32}P_i$ (1 mCi/ml) for 5 h. Cell monolayers were washed three times in ice-cold phosphate-buffered saline and extracted with modified RIPA buffer (20 mM MOPS [morpholinepropanesulfonic acid], pH 7.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing protease inhibitors phenylmethylsulfonyl fluoride, L-1-chlor-3(4-tosylamido)-7-amino-2-heptanon-hydrochloride, L-1-chlor-3(4-tosylamido)-4-phenyl-2-butanon, leupeptin and aprotinin. The lysate was preincubated for 30 min with *Staphylococcus aureus* protein A (Pansorbin; Calbiochem-Behring) and clarified by centrifugation. Ten microliters of SRQE antiserum or 2 μl of RNGS antiserum was added to the lysate and incubated overnight at 4°C. SRQE antiserum was generated against a synthetic peptide representing amino acids 290 to 310 of the full-length BPV-1 E2 polypeptide (27). RNGS antisera was prepared to a synthetic peptide corresponding to amino acids 39 to 64 of the murine lck protein sequence (46). Immune complexes were collected on *S. aureus* protein A, washed five times with alternate washes of RIPA and RIPA-1 M NaCl. Proteins were eluted in SDS-sample buffer, boiled, and separated by electrophoresis on 10% SDS-polyacrylamide gels. Gels were fixed, treated with Enlightning; (Dupont, NEN Research Products), and autoradiographed.

Phosphoamino acid analysis. Flasks (150 cm^2) of SF9 cells were infected with recombinant baculoviruses, vAC-E2 and vACE2-TR, labeled with $^{32}P_i$ at 40 h postinfection, and immunoprecipitated with E2-specific antisera, as described above. The proteins were separated by SDS-polyacrylamide gel electrophoresis, and the gels were dried without fixing or fluorography. E2 proteins were electroeluted from gel slices and recovered by trichloroacetic acid precipitation. The pellets were dissolved in 5.7 M hydrochloride (constant boil) and incubated for 1 h at 110°C. The hydrolyzed amino acids were lyophilized, washed several times in water, and separated by two-dimensional thin-layer electrophoresis. Radioactive spots were identified by reference to ninhydrin-detected phosphoamino acid standards.

V8 mapping. Analysis of proteins by limited proteolysis with *S. aureus* V8 protease (Pierce Chemical Co.) has been described previously (4). Briefly, dried gel slices containing radiolabeled E2 proteins were inserted into the wells of a second SDS-polyacrylamide gel and overlaid with 30% glycerol–0.25 M Tris, pH 6.8. Various concentrations of V8 protease in 5% glycerol–0.25 M Tris (pH 6.8)–0.25% bromophenol blue were layered on top, and the samples were run into the separating gel. Electrophoresis was interrupted for 20 min to allow digestion and then was resumed.

Cyanogen bromide cleavage. ^{32}P-labeled E2 proteins were electroeluted from gel slices, trichloroacetic acid precipitated, and cleaved with cyanogen bromide (50 mg/ml in 70% formic acid) for 1 h at room temperature. The samples were lyophilized and washed in water, and the resulting polypeptides were resolved on a 20% SDS-polyacrylamide gel.

Cleavage with endoproteinase LysC. E2 proteins were electroeluted from gel slices, trichloroacetic acid precipitated, and dissolved in 25 mM Tris (pH 8.5)–1 mM EDTA–0.1% SDS. Proteins were digested with 5 μg of endoproteinase LysC (Boehringer Mannheim Biochemicals; sequencing grade) for 10 h at 35°C and analyzed by SDS-polyacrylamide gel electrophoresis.

Construction of E2 mutants. Double-stranded oligonucleotides of coding sequence 5′CGTAACGGTTCGGAAGTAC GAGATCCCCTAGTAACTTATGAGGGATCACTTCCTC CTGCT3′ containing the appropriate sticky ends were cloned between the *Dra*II (nucleotide 3259) and *Kpn*I (nucleotide 3460) sites or between the *Kpn*I (nucleotide 3460) and *Sty*I (nucleotide 3535) sites within the E2 ORF in pTZE2$_{kz}$. This resulted in plasmids pTZE2$_{kz}$RNGS2 and pTZE2$_{kz}$RNGS4, respectively. This oligonucleotide encodes the synthetic epitope, RNGSEVRDPLVTYEGSLPPA. The *Bst*EII-to-*Bst*XI fragment of pPAVA-1 was replaced with the corresponding fragment containing the E2 ORF from pTZE2$_{kz}$RNGS2 and pTZE2$_{kz}$RNGS4 to generate pSBE2$_{kz}$ RNGS2 and pSBE2$_{kz}$RNGS4. Synthetic oligonucleotides were synthesized containing sequences between the *Kpn*I (nucleotide 3460) and *Sty*I (nucleotide 3535) sites of the E2 ORF. Oligonucleotides encoding either wild-type or specifically mutated E2 sequences were cloned between the *Kpn*I and *Sty*I sites in the plasmid pTZE2$_{kz}$ to generate pTZE2$_{kz}$ A290, pTZE2$_{kz}$A298, pTZE2$_{kz}$A301, and pTZE2$_{kz}$AAA. The specific mutations in each codon were as follows: Ser-290 to Ala-290, TCA to GCA; Ser-298 to Ala-298, TCG to GCG; Ser-301 to Ala-301, TCC to GCC. The mutations were verified by restriction analysis and DNA sequencing, and the *Bst*EII-to-*Bst*XI fragments containing the specific E2 mutations were substituted for the corresponding wild-type fragment in pPAVA-1. Recombinant viruses encoding the mutated E2 proteins were generated as described above and were designated vSB-E2$_{kz}$A290, vSB-E2$_{kz}$A298, vSB-E2$_{kz}$ A301, and vSB-E2$_{kz}$AAA.

Two-dimensional electrophoresis. Two-dimensional electrophoresis was carried out as described by O'Farrell (34). E2 polypeptides were immunoprecipitated and washed as described above, and the *S. aureus* protein A immune complexes were washed twice in distilled water. Polypeptides were eluted into sample buffer (9 M urea, 2% Nonidet P-40, 2% ampholines (pH 3 to 10), 5% β-mercaptoethanol, 0.3% SDS). Polypeptides were separated in the first dimension by isoelectric focusing and in the second by electrophoresis on a 10% SDS-polyacrylamide gel. Polypeptide species were identified with reference to carbamylated isoelectric protein standards (Pharmacia, Inc.).

RESULTS

Expression of BPV-1 E2 polypeptides. The level of E2 proteins detected in BPV-1-transformed cells is below that required for extensive analysis of posttranslational modifications (17) and, thus, for these studies the BPV-1 E2 polypeptides were overexpressed in two different cell culture systems. Both the full-length E2 polypeptide and the shorter E2-TR repressor polypeptide were expressed in SF9 insect cells, using a recombinant baculovirus, and in addi-

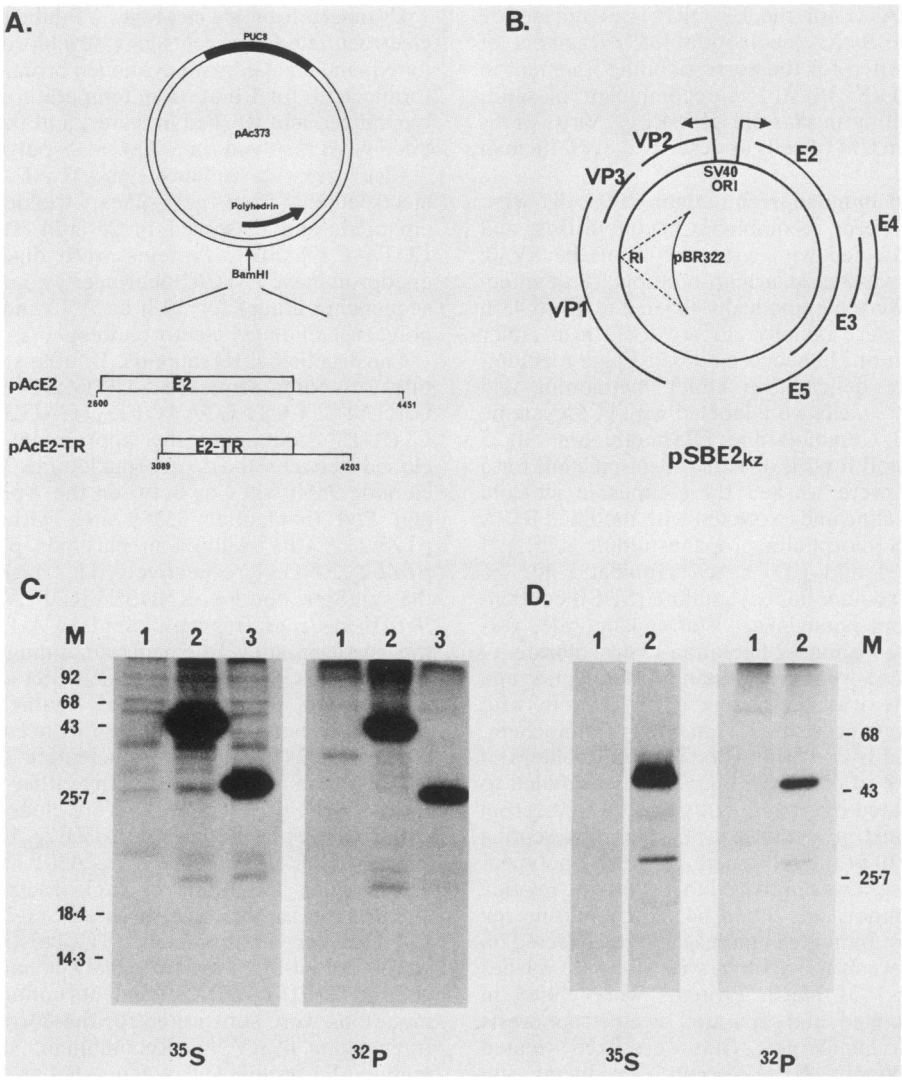

FIG. 1. (A) Structure of the expression vectors, pAcE2 and pAcE2-TR, which were used to generate recombinant baculoviruses, vAcE2 and vAcE2-TR, respectively. (B) Map of the recombinant virus plasmid, $pSBE2_{kz}$, which contains BPV-1 sequences which encode the E2, E3, E4, and E5 ORFs cloned downstream from the SV40 early promoter. The plasmid also contains the SV40 late region and origin of replication. The pBR322 vector sequences can be removed by cleavage with *Eco*RI. (C) Gel electrophoretic analysis of expression of BPV-1 E2 polypeptides in SF9 cells. SF9 cells were infected with either wild-type *Autographa californica* nuclear polyhedrosis virus (lanes 1) or with recombinant viruses vAcE2 (lanes 2) and vAcE2-TR (lanes 3). At 40 h postinfection, the cells were metabolically labeled with either [^{35}S]methionine and [^{35}S]cysteine (left panel) or $^{32}P_i$ (right panel). E2 polypeptides were immunoprecipitated with the E2-specific antisera SRQE and analyzed by SDS-polyacrylamide gel electrophoresis. M, Protein molecular mass standards given in kilodaltons. (D) Gel electrophoretic analysis of expression of BPV-1 E2 polypeptides in COS-1 cells. COS-1 cells were either mock infected (lanes 1) or infected with the recombinant virus, $vSBE2_{kz}$ (lanes 2). At 40 h postinfection, cells were metabolically labeled with either [^{35}S]methionine and [^{35}S]cysteine (left panel) or with $^{32}P_i$ (right panel). E2 polypeptides were immunoprecipitated with the E2-specific antisera SRQE and analyzed by SDS-polyacrylamide gel electrophoresis. M, Protein molecular mass standards.

tion, the full-length E2 protein was synthesized in monkey COS-1 cells by an SV40/BPV-1 recombinant virus.

For the baculovirus expression vector, the full-length E2 gene was derived from a plasmid, $pTZE2_m$, in which the initiation codon for the ORF was replaced by a Kozak consensus ATG to facilitate translation efficiency (27). The E2-TR gene was derived from a plasmid, pTZE2-TR, that contains the C-terminal two-thirds of the E2 ORF and which expresses the E2-TR polypeptide from an initiation codon at nucleotide 3091 of the BPV-1 genome. Both genes were cloned into the baculovirus expression vector, pAC373, as described in Materials and Methods and as shown in Fig. 1A. The vector, pAC373, contains the baculovirus AcMNPV polyhedrin gene with a single *Bam*HI cloning site approximately 50 base pairs downstream from the transcriptional start site and 8 base pairs upstream from the polyhedrin translation initiation codon. Foreign genes inserted into this site can be expressed in insect cells from the polyhedrin promoter. The E2-expressing vectors, designated pAcE2 and pAcE2-TR, were cotransfected into SF9 insect cells with wild-type AcMNPV DNA. Recombinant viruses that express the E2 proteins result from homologous recombination between the polyhedrin sequences in the vector and viral DNAs and were purified by several rounds of plaque

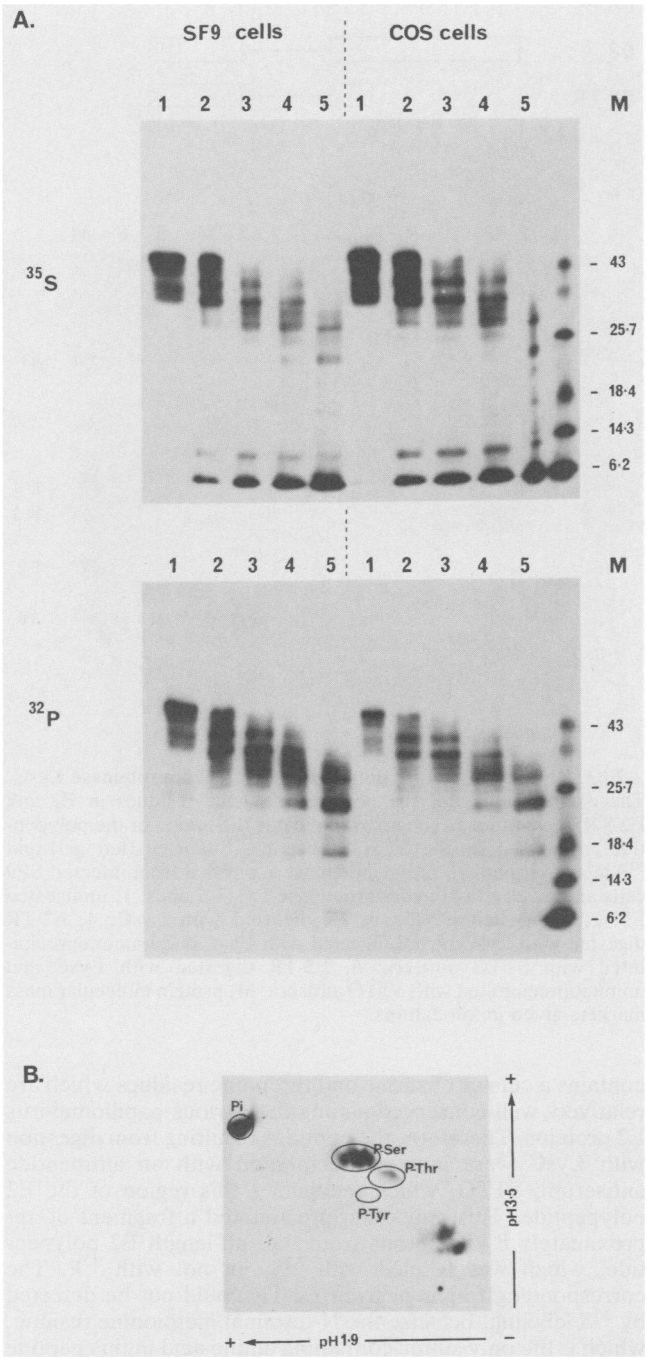

FIG. 2. (A) Digestion of the full-length E2 polypeptide with *S. aureus* V8 protease. ^{35}S-labeled (top panel) and ^{32}P-labeled (bottom panel) E2 polypeptides were immunoprecipitated from both SF9 (left halves) and COS-1 (right halves) cells and were purified on a SDS-polyacrylamide gel. Gel slices containing E2 proteins were inserted into a second gel and overlaid with increasing amounts of V8 protease. Proteins were electrophoresed into the stacking gel and incubated for 20 min before electrophoresis was resumed. The amounts (in nanograms) of V8 protease were as follows: lane 1, 0; lane 2, 40; lane 3, 200; lane 4, 800; lane 5, 4,000. M, Molecular mass markers given in kilodaltons. (B) Phosphoamino acid analysis. ^{32}P-labeled E2 protein was purified from SF9 cells and acid hydrolyzed, and the resulting amino acids were separated by two-dimensional thin-layer electrophoresis. Amino acids were separated in the first dimension in buffer with a pH of 1.9, and in the second they were separated in buffer with a pH of 3.5. The position of ninhydrin-stained phosphoamino acid standards are outlined.

purification. The E2 proteins expressed from the recombinant viruses could be detected by immunoprecipitation and were able to bind specifically to the E2 DNA recognition site, $ACCN_6GGT$ (data not shown).

The SV40/BPV-1 recombinant virus was first developed by Settleman and DiMaio and contains the BPV-1 E2-to-E5 region downstream of the SV40 early promoter in place of the SV40 early region (38). The SV40 replication origin is still intact, and when the virus is transfected into cells which express the SV40 early region, such as COS-1 cells, virion particles are produced. The original SV40/BPV-1 recombinant virus developed by Settleman and DiMaio was shown to express the E2 transactivation function at high levels (38), but for these studies we replaced the original E2 ORF with one which contained a Kozak consensus initiation codon and in which 200 base pairs of upstream untranslated sequences had been removed. This recombinant virus-vector is shown in Fig. 1B. By immunoprecipitation with E2-specific antisera, we were able to show that the latter virus, designated $vSBE2_{kz}$, expressed the E2 protein at about 10-fold higher levels than that of the original pPAVA-1 virus (data not shown). In addition to the full-length E2 protein, this virus expressed a second E2-derived polypeptide with an apparent molecular mass of 31 kilodaltons. We believe this protein to be the E2-TR repressor, expressed from its own promoter located at nucleotide 3080 within the E2 ORF.

SF9 insect cells and COS-1 monkey cells were each infected with the E2-expressing recombinant viruses at a high multiplicity of infection, and at between 40 and 48 h postinfection, the cells were metabolically labeled with either [^{35}S]methionine and [^{35}S]cysteine or with $^{32}P_i$. E2 proteins were immunoprecipitated from each cell type by using the E2-specific anti-peptide antisera, SRQE (27). Both the full-length E2 and shorter E2-TR polypeptides are labeled with ^{32}P in both cell lines, indicating that they are potentially phosphoproteins (Fig. 1C and D).

The E2 proteins are phosphorylated at similar sites in both SF9 and COS-1 cells. For many of the following studies described, we used E2 polypeptides derived from SF9 cells. It has been reported that mammalian polypeptides expressed from recombinant baculoviruses are authentically posttranslationally modified in insect cells (23, 30, 39, 40). To ensure that the E2 phosphorylation sites were similar in insect cells and in mammalian cells, partial proteolytic cleavage was carried out on the E2 proteins derived from each cell type. Full-length E2 proteins, metabolically labeled with either $^{32}P_i$ or ^{35}S-labeled amino acids, were immunoprecipitated from both cell types and separated on a SDS-polyacrylamide gel. Gel slices containing the proteins were incubated with increasing amounts of *S. aureus* V8 protease, and the resulting peptides were analyzed on an SDS-polyacrylamide gel. An identical pattern of phosphopeptides was obtained with the E2 proteins derived from either mammalian COS-1 cells or insect SF9 cells, indicating that they were modified at similar sites (Fig. 2A).

The E2 polypeptides are phosphorylated on serine and threonine residues. To identify which type of amino acid was phosphorylated, ^{32}P-labeled E2 and E2-TR polypeptides were purified from infected SF9 cells by immunoprecipitation and gel purification. The purified proteins were hydro-

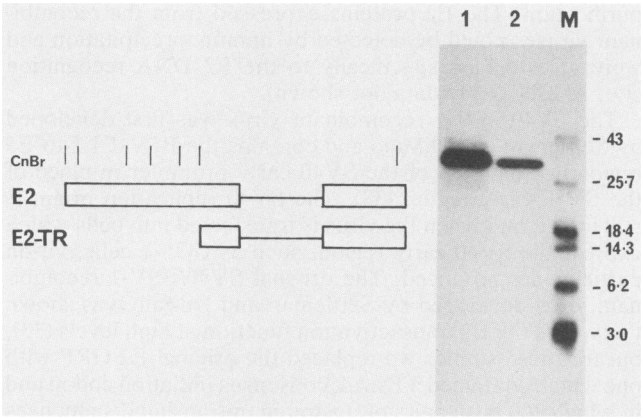

FIG. 3. Cyanogen bromide cleavage of ^{32}P-labeled E2 polypeptides derived from SF9 cells. The diagram indicates the position of methionine residues in the E2 and E2-TR polypeptides. The boxes represent the functional domains of the proteins. The E2 polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis after cleavage with cyanogen bromide. Lanes: 1, E2; 2, E2-TR; M, molecular mass standards given in kilodaltons.

lyzed, and the resulting amino acids were separated by two-dimensional thin-layer electrophoresis. The position of phosphoserine, phosphothreonine, and phosphotyrosine were identified by using unlabeled standards which could be detected by staining with ninhydrin. For the full-length E2 protein derived from insect cells, the majority of the ^{32}P label was present as phosphoserine, with a small amount of phosphothreonine and no phosphotyrosine (Fig. 2B).

Mapping the region of phosphorylation in the E2 proteins. To map the region of phosphorylation of the E2 proteins, ^{32}P-labeled E2 and E2-TR were purified from insect cells, as described above, and cleaved with cyanogen bromide. This reagent cleaves polypeptides after methionine residues, which are located at residues 1, 16, 75, 103, 139, 162, and 399 in the 410-amino-acid full-length E2 protein, as shown in the diagram in Fig. 3. Cyanogen bromide cleavage demonstrated that the phosphorylation site mapped to a fragment with an apparent molecular mass of 29 kilodaltons which corresponds to a region between amino acids 163 and 399, common to both the E2 and E2-TR polypeptides. Furthermore, this experiment demonstrated that there were no additional major phosphorylation sites in the amino-terminal region unique to the full-length E2 protein.

The phosphorylation site was further mapped by cleaving the E2 polypeptides with endoproteinase LysC. This enzyme cleaves proteins at the C-terminal side of lysine residues, which are present at amino acid positions 21, 25, 48, 70, 84, 107, 111, 149, 226, 322, 339, 346, and 391 in the full-length E2 protein, as indicated in the diagram in Fig. 4. ^{32}P- and ^{35}S-labeled E2 and E2-TR proteins were purified from infected SF9 cells and digested with endoproteinase LysC. Several ^{35}S-labeled peptides were observed from both E2 and E2-TR polypeptides which corresponded in size to those predicted from the amino acid sequence (Fig. 4). However, only one peptide fragment from each protein was labeled with ^{32}P, and a comparison of the ^{32}P- and ^{35}S-labeled peptides clearly demonstrates that it is the largest fragment which is phosphorylated. This maps the phosphorylation site between amino acids 227 and 322 of the E2 polypeptides. We had suspected that the phosphorylation site might have been located in the peptide extending from amino acid residue 150 to residue 226, because this region

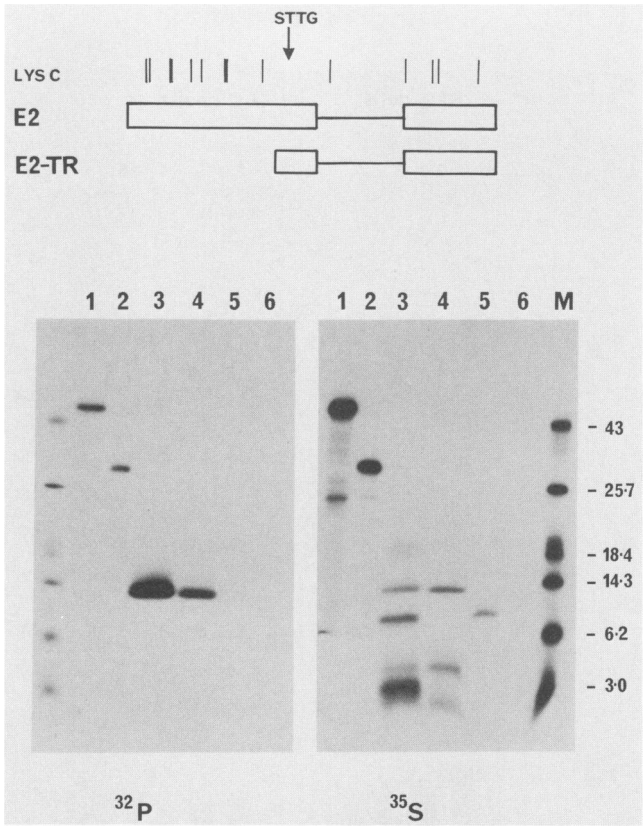

FIG. 4. Digestion of E2 polypeptides with endoproteinase LysC. The diagram indicates the position of lysine residues in E2 and E2-TR polypeptides. The arrow indicates the region of the polypeptide recognized by the STTG antisera. ^{35}S-labeled (left gel) and ^{32}P-labeled (right gel) polypeptides were purified from infected SF9 cells and digested with endoproteinase LysC. Lanes: 1, undigested E2; 2, undigested E2-TR; 3, E2 digested with LysC; 4, E2-TR digested with LysC; 5, E2 digested with LysC and immunoprecipitated with STTG antisera; 6, E2-TR digested with LysC and immunoprecipitated with STTG antisera; M, protein molecular mass markers given in kilodaltons.

contains a cluster of serine and threonine residues which are relatively well conserved among the various papillomavirus E2 proteins. Therefore, the peptides resulting from digestion with LysC were immunoprecipitated with an antipeptide antiserum, STTG, which recognizes this region of the E2 polypeptide. This antiserum precipitated a fragment of approximately 8 kilodaltons from the full-length E2 polypeptide, which was labeled with ^{35}S but not with ^{32}P. The corresponding fragment from E2-TR could not be detected by ^{35}S labeling, because the N-terminal methionine residue, which is the only sulfur-containing amino acid in this peptide fragment, is cleaved from the E2-TR protein (unpublished observations). This confirmed that the major phosphorylation site was located between amino acids 227 and 322 of the E2 polypeptides.

Mapping the region of phosphorylation by deletion analysis. To further establish the region of phosphorylation of the E2 polypeptides, we made use of two plasmids that express hybrid E2 proteins. In these constructs, amino acid sequences corresponding to the nonconserved hinge region between residues 220 and 309 of the E2 ORF had been replaced with a short amino acid sequence, RNGSEVRD PLVTYEGSLPPA, to which we have a high-affinity antipep-

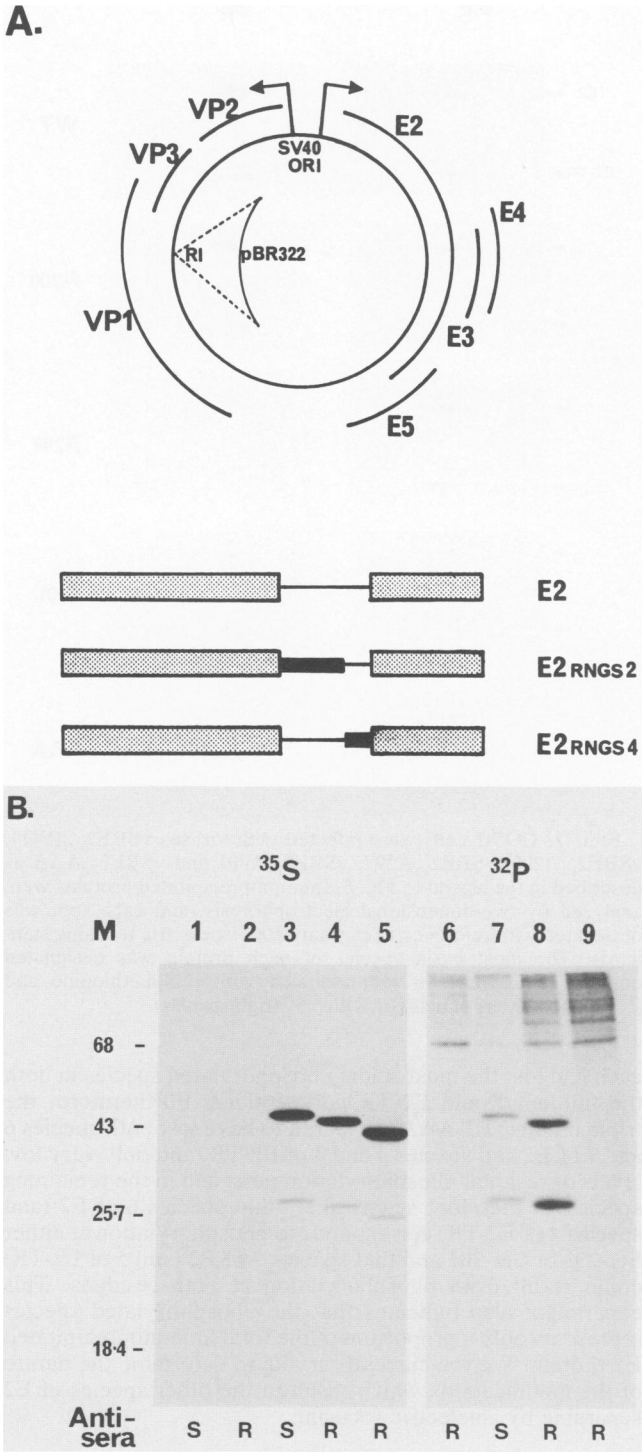

FIG. 5. (A) Structure of pSBE2$_{kz}$E2RNGS contructs. The wild-type E2 ORF in pSBE2$_{kz}$ was replaced with E2 ORFs E2RNGS2 and E2RNGS4. The E2 polypeptides expressed from these constructs contain a synthetic epitope, RNGS, in place of part of the hinge region (■). (B) Immunoprecipitation of E2 polypeptides from COS-1 cells infected with the vSBE2$_{kz}$RNGS recombinant viruses. COS-1 cells were either mock infected (lanes 1, 2, and 6) or infected with vSBE2$_{kz}$ (lanes 3 and 7), vSBE2$_{kz}$RNGS2 (lanes 4 and 8), or vSBE2$_{kz}$RNGS4 (lanes 5 and 9). Cells were labeled 42 h postinfection with either [^{35}S]methionine and [^{35}S]cysteine (left panel) or $^{32}P_i$ (right panel). E2 polypeptides were immunoprecipitated with SRQE (S) (lanes 1, 3, and 7) or RNGS (R) (lanes 2, 4, 5, 6, 8, and 9) antisera. The apparent molecular mass of the E2 proteins is several kilodaltons larger than that predicted from their amino acid sequences. This is due to the region between amino acids 286 and 309 (unpublished observations) and explains why E2RNGS2 has a lower mobility than that of E2RNGS4, despite a larger deletion.

tide antiserum, RNGS (46). This sequence is derived from p56^{lck} and does not contain any phosphorylation sites. In addition, p56^{lck} is normally expressed exclusively in cells of lymphoid origin and is not expressed in SF-9 cells or in COS-1 cells (25, 45). To generate pTZE2$_{kz}$RNGS2, synthetic double-stranded oligonucleotides encoding this sequence were cloned between the *Dra*II (nucleotide 3259) and *Kpn*I (nucleotide 3460) sites of the E2 ORF, and to generate pTZE2$_{kz}$RNGS4, the oligonucleotide sequence replaced the region between the *Kpn*I (nucleotide 3460) and *Sty*I (nucleotide 3535) sites. The hybrid polypeptides are more useful than those which simply contain internal deletions because the E2-specific antiserum used in these studies is directed against amino acid sequences between residues 290 and 310. Thus, we were able to use antiserum directed against the synthetic epitope, RNGS, to immunoprecipitate the hybrid E2 proteins. The hybrid E2 ORFs were cloned into the SV40/BPV-1 recombinant virus and virions produced. The resulting virus preparations, vSBE2$_{kz}$RNGS2 and vSBE2$_{kz}$ RNGS4, were used together with vSBE2$_{kz}$ (wild-type E2) to infect COS-1 cells. The cells were metabolically labeled with $^{32}P_i$ or [^{35}S]methionine and [^{35}S]cysteine at 42 h postinfection, and the E2 polypeptides were analyzed by immunoprecipitation with either the SRQE or RNGS antisera. All three ^{35}S-labeled E2 proteins could be detected, but only the E2 polypeptides expressed from vSBE2$_{kz}$RNGS2 and vSBE2$_{kz}$ were strongly labeled with ^{32}P (Fig. 5). Only a minimal level of phosphate labeling was observed in both the E2 and E2-TR polypeptides expressed from vSBE2$_{kz}$RNGS4, suggesting that the sequence replaced by the synthetic epitope in E2$_{kz}$RNGS4, from amino acid 286 to 309, contains the major phosphorylation site within the E2 proteins.

Site-specific mutagenesis of potential phosphorylation sites in the E2 polypeptide. The region of the E2 polypeptide which has been replaced by the synthetic epitope in E2$_{kz}$RNGS4 has the sequence VDLASRQEEEEQSPD STEEEPVTL and contains three serine residues, located at amino acids 290, 298, and 301. This sequence also contains two threonine residues, but for this study we have focused on the serine residues since they are the major phosphorylated species. Each of the serine residues was specifically and individually mutated to an alanine residue, and the resulting mutated E2 ORFs were cloned into the SV40/BPV-1 recombinant virus. These viruses were designated vSBE2$_{kz}$A290, vSBE2$_{kz}$A298, and vSBE2$_{kz}$A301. In addition, the virus vSBE2$_{kz}$AAA was generated, in which all three serines have been changed to alanine residues.

As described above, COS-1 cells were infected with the viruses and the E2 polypeptides were labeled with $^{32}P_i$ and [^{35}S]methionine and [^{35}S]cysteine. All E2 polypeptides could be detected by ^{35}S labeling, showing that the SRQE antisera was able to recognize the mutant polypeptides, even though the mutations are in the vicinity of the antibody epitope (Fig. 6). All three E2 proteins mutated in a single amino acid were labeled with ^{32}P; however, polypeptides E2-A298 (Fig. 6, lane 10) and E2-A301 (Fig. 6, lane 11) showed a reduction in phosphate labeling and the triple mutant E2-AAA (Fig. 6, lane 12) demonstrated very little labeling. This suggested

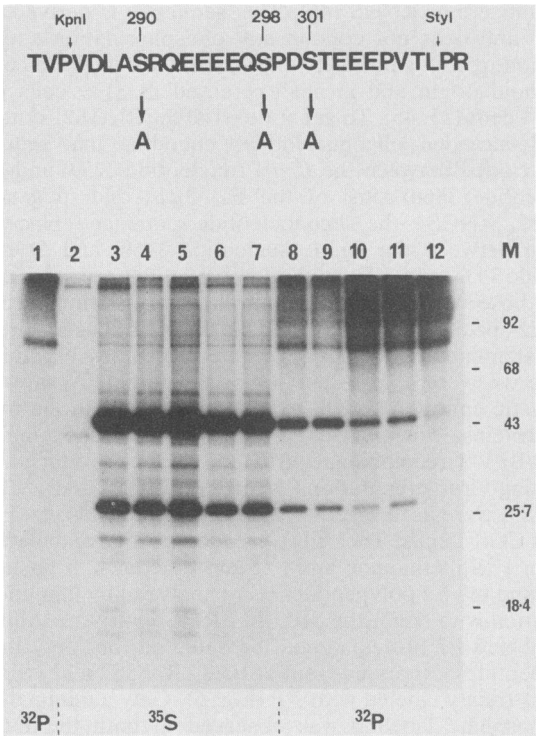

FIG. 6. The amino acid sequences surrounding the major phosphorylation sites of the E2 proteins are shown at the top. The region indicated between the *Kpn*I and *Sty*I restriction sites is eliminated in the polypeptide $E2_{kz}$RNGS4. This region contains serine residues at positions 290, 298, and 301, each of which has been mutated to an alanine residue. Immunoprecipitation from COS-1 cells of E2 polypeptides mutated at serine in positions 290, 298 and 301 is shown at the bottom. COS-1 cells were infected with SV40 (lanes 1 and 2), $vSBE2_{kz}$ (lanes 3 and 8), $vSBE2_{kz}A290$ (lanes 4 and 9), $vSBE2_{kz}A298$ (lanes 5 and 10), $vSBE2_{kz}A301$ (lanes 6 and 11), and $vSBE2_{kz}AAA$ (lanes 7 and 12). Cells were labeled 42 h postinfection with either [^{35}S]methionine and [^{35}S]cysteine (lanes 2 through 7) or $^{32}P_i$ (lanes 1 and 8 through 12). E2 polypeptides were immunoprecipitated with SRQE antisera. M, Molecular mass standards (given in kilodaltons).

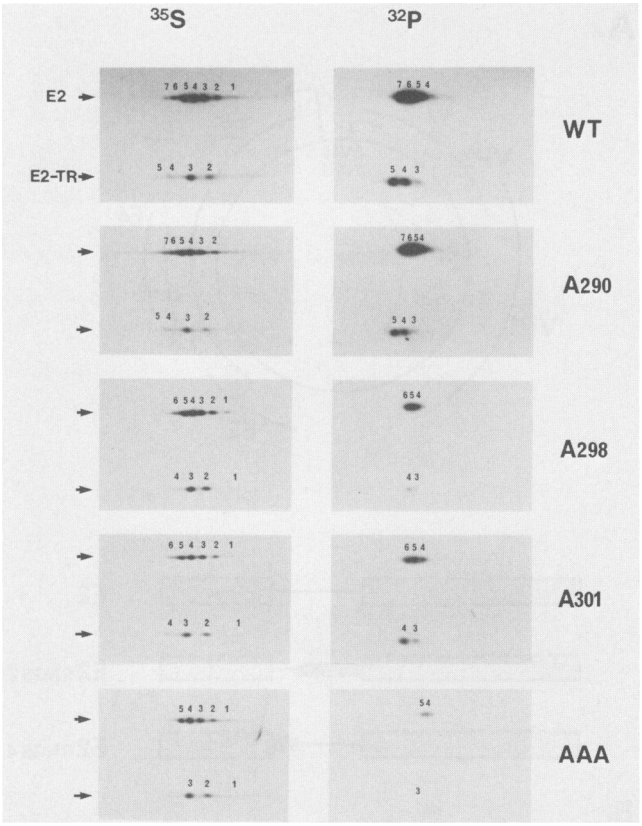

FIG. 7. COS-1 cells were infected with viruses $vSBE2_{kz}$ (WT), $vSBE2_{kz}A290$, $vSBE2_{kz}A298$, $vSBE2_{kz}A301$ and $vSBE2_{kz}AAA$ as described in the legend to Fig. 6. Immunoprecipitated proteins were analyzed by two-dimensional electrophoresis, and each spot was designated with reference to carbamylated isoelectric focusing standards. The most basic species of each protein was designated number 1. E2 proteins were labeled with [^{35}S]methionine and [^{35}S]cysteine (left panels) or with $^{32}P_i$ (right panels).

that the serine residues at amino acid positions 298 and 301 were the major phosphorylation sites in the E2 proteins.

Analysis of the E2 proteins by two-dimensional electrophoresis. To further confirm that serine residues 298 and 301 are the major phosphorylation sites of the E2 polypeptides, we analyzed polypeptides E2, E2-A290, E2-298, E2-301, and E2-AAA by two-dimensional electrophoresis. Polypeptides immunoprecipitated from COS-1 cells were first resolved by isoelectric focusing and then by SDS-polyacrylamide gel electrophoresis. This revealed that the full-length E2 polypeptide was quite heterogeneous and consisted of at least seven different isoelectric species, while the E2-TR protein exhibited five species (Fig. 7). Characterization of each species is not simple, since each species probably does not represent a single posttranslational modification but rather a combination of several modifications. However, only the most acidic species, those designated 4 to 7 of E2 and 4 and 5 of E2-TR, showed high levels of phosphate labeling, although low amounts could be detected in other species. The pattern observed with the E2-A290 polypeptide was indistinguishable from wild-type, in agreement with the finding in Fig. 6 that serine residue 290 is not a major phosphorylation site. In contrast, E2-A298 and E2-A301 each had lost the most acidic phosphorylated species in both the full-length and E2-TR polypeptides. Furthermore, the triple mutant, E2-AAA, appeared to have lost both species 6 and 7 of E2 and species 4 and 5 of E2-TR, and only very low levels of residual phosphorylation persisted in the remaining species. It therefore seems likely that species 6 of E2 (and species 4 of E2-TR) corresponds to phosphorylation at either Ser-298 or Ser-301 and that species 7 of E2 (and 5 of E2-TR) could result from phosphorylation at both residues. This experiment also indicates that the phosphorylated species represents only a proportion of the total immunoprecipitated E2 protein. We are currently trying to determine the nature of the modifications which result in the other species of E2 separable by isoelectric focusing.

DISCUSSION

The E2 proteins play a pivotal role in the control of BPV-1 gene expression. BPV-1 is able to transform certain rodent cell lines in which the viral genome is maintained extrachromosomally and only early viral genes are expressed at very low levels (15, 22). This latent nonproductive infection is thought to be analogous to that of the basal epithelial cells and dermal fibroblasts of a bovine fibropapilloma. Several viral promoters are dependent on E2 activation (13, 16, 42), and it has been postulated that the low level of virus

transcription results from the relative levels of transactivator and repressor proteins. Posttranslational modifications, such as phosphorylation, of the E2 proteins could provide an additional level of regulation of viral gene expression.

A number of cellular transcription factors have been shown to be regulated by phosphorylation. The yeast transcriptional activator, ADR1, is inactivated by phosphorylation by cAMP-dependent protein kinase (5), but phosphorylation increases DNA binding of the yeast heat shock factor (41), the adenovirus-induced E4F transcription factor (36), and the serum response factor which binds to the serum response element in the c-*fos* enhancer (35). In addition, dimerization and transcriptional efficacy of the nuclear factor CREB are induced by phosphorylation (47).

In this study, we have demonstrated that the BPV-1 E2 transactivator and repressor (E2-TR) proteins are phosphorylated predominantly on serine residues. By a variety of techniques, we have mapped the major sites of phosphorylation to serine residues at positions 298 and 301 in the E2 protein. These sites are present in all three E2 transcriptional regulatory proteins.

Two different systems were used to overexpress the E2 polypeptides in insect cells and in SV40-transformed monkey cells. The polypeptides appeared to be modified at similar sites in both cell types, and it seems unlikely that the phosphorylation sites would differ in either bovine cells or in mouse C127 cells (in which BPV-1 transformation and replication studies have traditionally been carried out). In addition, the majority of studies on E2-dependent transactivation have been carried out in monkey CV-1 cells. When the E2 polypeptides were separated by two-dimensional electrophoresis, it was observed that only a proportion of the total E2 protein appeared to be phosphorylated in COS-1 cells. However, because the E2-specific antibody used in these studies was generated against a peptide which spans the phosphorylation site, the possibility remains that the ratios of the different species results from different affinities of the antibody for the phosphorylated and nonphosphorylated polypeptides. It will be interesting to analyze the proportion of phosphorylated and nonphosphorylated E2 proteins in BPV-1-transformed cells and to determine whether this ratio changes under a variety of conditions.

The region of the E2 polypeptide containing the phosphorylation sites has previously been designated the hinge region, as it can be deleted from the protein without notable effect on the transactivation function (26). Therefore, not surprisingly, preliminary results indicate that E2 proteins with mutations in serine residues 298 and 301 are not defective in transactivation. However, this region is immediately adjacent to the DNA binding domain and, while it is not required for DNA binding in vitro, this short region is required in addition to the DNA binding domain for repression in vivo (A. McBride, unpublished observations). This sequence could therefore play some role in cellular localization or stabilization of the E2 proteins. Mutations in the E2 ORF are pleiotropic, affecting both transformation and replication functions, and while it is assumed that this is due to the transcriptional regulatory functions encoded by the E2 ORF, it cannot be excluded that the E2 proteins may also play a more direct role in these functions. We are currently assaying the serine mutants in the background of the entire BPV-1 virus, as this may enable us to detect a more subtle phenotype for these mutations. The region of the E2 polypeptide containing the major phosphorylation sites, immediately adjacent to the DNA binding domain, is not conserved among the other papillomavirus proteins, perhaps arguing against an important regulatory role for these modifications. It has previously been described that the cottontail rabbit papillomavirus is a phosphoprotein (3), although there is no homology between the region shown to contain the phosphoserine residues in the BPV-1 E2 polypeptide and the corresponding region in cottontail rabbit papillomavirus E2. However, the serine residue at amino acid 298 and the following two residues, SPD, are conserved among the viruses BPV-1, BPV-2, deer papillomavirus, and European elk papillomavirus, all of which cause fibropapillomas in their natural hosts.

The region surrounding the major phosphorylation sites, TVPVDLASRQEEEEQSPDSTEEEPVTLPR, has an unusual amino acid composition and constitutes a good PEST sequence. PEST sequences are polypeptide regions rich in proline, glutamic acid, aspartic acid, serine, and threonine residues and are found mainly in proteins with short half-lives. These sequences have been proposed to play a role in protein turnover (37). The half-life of the E2 proteins has been determined to be 40 min for the full-length polypeptide and 10 min for the E2-TR repressor (17). The serine residues at positions 290 and 301 represent excellent potential casein kinase II sites. Casein kinase II sites are generally followed by stretches of acidic residues, and the most critical position is at the third position after the serine residue (18). Theoretically, Ser-298 could also form a casein kinase II site when Ser-301 is phosphorylated, therefore providing the necessary negative charge at position 3 (28, 29). A similar situation occurs in glycogen synthetase, in which prior phosphorylation by casein kinase II is required for substrate recognition by glycogen synthetase kinase 3 (11). However, this does not seem to be the case in the E2 polypeptides, since serine residues 298 and 301 appear to be phosphorylated independently from each other. Intriguingly, many PEST sequences contain casein kinase II consensus sites (37), and an interesting possibility is that phosphorylation at these sites plays some role in the regulation of protein turnover. Notably, there are comparable regions in proteins such as c-*myc*, c-*fos*, and Epstein-Barr virus BZLF1 which are located in a position upstream from the DNA binding domain similar to that of the E2 proteins (10, 24, 37).

The identification of the major phosphorylation sites of the BPV-1 E2 proteins will enable us to determine the functional significance of these modifications, and identification of the protein kinases responsible may contribute to our understanding of how these factors regulate viral gene expression.

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