

Proteasome-Mediated Degradation of the Papillomavirus E2-TA Protein Is Regulated by Phosphorylation and Can Modulate Viral Genome Copy Number

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The bovine papillomavirus E2 proteins regulate viral transcription, replication, and episomal genome maintenance. We have previously mapped the major phosphorylation sites of the E2 proteins to serine residues 298 and 301 and shown that mutation of serine residue 301 to alanine leads to a dramatic (10- to 20-fold) increase in viral DNA copy number. In this study we analyzed how phosphorylation regulates E2 protein function. S301 is located in a PEST sequence; these sequences are often found in proteins with a short half-life and can be regulated by phosphorylation. We show here that the E2 protein is ubiquitinated and degraded by the proteasome. Mutation of serine 301 to alanine increases the half-life of E2 from approximately 50 min to 160 min. Furthermore, the A301 E2 protein shows greatly reduced ubiquitination and degradation by the proteasome. These results suggest that the E2 protein level is regulated by phosphorylation, which in turn determines viral episomal copy number.

Papillomaviruses are small DNA viruses that induce persistent epithelial lesions, known as warts or papillomas. The viral genomes are maintained as episomes in the lower layers of these lesions; vegetative viral DNA replication and capsid protein synthesis occur only in the more differentiated layers of the epithelium. The papillomavirus E2 gene encodes several proteins that are involved in viral DNA replication, transcriptional regulation, and viral genome segregation and maintenance. The E2 protein contains two major phosphorylation sites at serine residues 298 and 301 (19) and a minor site at serine residue 235 (14). Mutation of serine 301 leads to a dramatic (10- to 20-fold) increase in viral DNA copy number (20). This suggests that the E2 proteins regulate viral genome copy number.

There are a number of steps at which the E2 proteins could regulate genome copy number. The bovine papillomavirus (BPV) type 1 E2 open reading frame encodes both a transcriptional transactivator and two shorter transcriptional repressors (reviewed in reference 21). The transactivator contains a 200-amino-acid transactivation domain and a 100-amino-acid DNA binding and dimerization domain separated by a flexible hinge region. The repressors do not contain the transactivation domain. The E2-TA transactivator activates transcription from several viral promoters by binding to specific E2 binding sites within the viral enhancers. The shorter repressor proteins can antagonize the function of E2-TA by competing for binding to the enhancer elements and by forming inactive heterodimers with the transactivator. Phosphorylation of the E2 proteins could modulate DNA replication indirectly by altering the transcriptional regulatory properties of the E2 proteins and changing the levels of other viral gene products. However, we have shown using mutational analyses that expression of viral open reading frames, other than E1 and E2, is not required for the high-copy phenotype and that this phenotype is observed

even when E1 is expressed from a heterologous promoter (A. A. McBride, unpublished observations).

E2-TA plays an auxiliary role in initiation of viral DNA replication; E2-TA cooperatively binds to the replication origin with the viral E1 protein (3, 24). E2-TA also alleviates nucleosomal repression of the replication origin and interacts with cellular replication proteins (15, 16). Phosphorylation could directly modulate one or more of the activities of E2 that are required for initiation of viral DNA synthesis, and this could result in higher levels of DNA replication. However, in transient replication experiments in which E1 and E2 were synthesized from expression vectors, no differences in levels of replication between wild-type E2-TA and E2-TA with mutation of serine residue 301 could be observed (A. A. McBride, unpublished observations). On the contrary, in transient replication experiments with the entire viral genome, the high-copy phenotype could be observed within 2 to 3 days of transfection (19).

E2-TA is also required for episomal maintenance of the viral genome (25). Our research group has previously demonstrated that both E2-TA and the viral genomes are linked to mitotic chromosomes during cell division and has proposed that this is the mechanism by which E2-TA segregates and maintains the viral genomes (31). Ilves et al. have extended these observations and demonstrated that E2 is required to link plasmids containing E2 DNA binding sites to mitotic chromosomes (11). Lehman et al. have demonstrated that, in cells transformed with BPV-1 containing mutations in several E2 phosphorylation sites, the E2-TA protein is unable to interact with mitotic chromosomes (13). However, in the absence of other viral gene products, E2-TA proteins with mutations in the phosphorylation sites can interact with chromosomes as efficiently as wild-type E2 (2). We propose that E2-TA phosphorylation can regulate copy number by modulating genome segregation but that this is indirect. In this study we show that phosphorylation regulates the stability of the E2 protein and that mutation of S301 results in higher steady-state levels of E2, which can explain the higher copy number.

Many regulatory proteins contain identifiable signals that

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target them for degradation by the ATP-dependent proteasome or calcium-dependent calpain proteases. The region surrounding the major E2 phosphorylation sites (S298 and S301) constitutes a good PEST sequence. PEST sequences are often found in proteins with short half-lives and are proposed to play a role in protein turnover (27). These sequences are rich in proline, glutamic acid, aspartic acid, serine, and threonine and are often flanked with basic residues. There are examples in which PEST sequences are conditional and phosphorylation serves as a mechanism to expose them. Phosphorylation of PEST sequences has been implicated in targeting proteins for degradation by the calcium-dependent calpain proteases (30) and by the ubiquitin-dependent proteasome pathway (9, 26). Phosphorylation is known to be vital for the degradation of numerous proteins, such as replication initiation protein Cdc6p (7), cyclin D1 (6), and cyclin E (34). Multiple phosphorylation events are also required for efficient degradation of the NF- κ B inhibitor, I κ B α (5, 29, 32), and the transcription factor, E2F-1 (33). In this study, we show that the wild-type E2-TA protein is degraded by the ubiquitin-mediated proteasome pathway. Furthermore, mutation of E2 serine 301 results in a protein with increased half-life and greatly reduced susceptibility to ubiquitination and proteasomal degradation. This increase in protein stability is likely to be responsible for the increase in episomal maintenance and genome copy number.

MATERIALS AND METHODS

Cell culture. CV-1-derived lines were cultured in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. CV-1 cells expressing the E2 proteins under the control of a metallothionein promoter were generated by transfection of pMEP-4 plasmids (Invitrogen) expressing either the wild-type E2 proteins or E2_{A301} proteins. Hygromycin B-resistant colonies were pooled and used for all experiments.

Plasmids. To generate pMEP-E2 and pMEP-E2_{A301}, the *Bst*EII-*Bst*XI fragment from pPAVA E2_{kz}A301 (19), containing an E2 gene with mutation of serine to alanine at position 301, was cloned into the corresponding fragment of pTZE2_{kz} (19), resulting in pTZE2_{kz}A301. *Bam*HI-*Hind*III fragments taken from pTZE2_{kz} and pTZE2_{kz}A301 were subcloned into the episomal vector, pMEP-4 (Invitrogen).

Transient expression and immunofluorescence. To determine appropriate induction methods, cells were plated onto glass slides 16 h before induction of the metallothionein promoter by the addition of CdSO₄ at concentrations ranging from 0.5 to 10 μ M, for time periods of up to 24 h. Induction times of 3 to 5 h and concentrations of 1 to 1.5 μ M CdSO₄ were used for all subsequent experiments. For immunofluorescence, cells were fixed for 20 min in 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.1% Triton X-100 in PBS for 10 min. E2 was detected with a 1:10 dilution of monoclonal antibody B201 (provided by Elliot Androphy) and goat anti-mouse immunoglobulin G (IgG) conjugated to fluorescein isothiocyanate (1:50 dilution; Jackson Immunochemicals). Slides were mounted in Fluoromount G (Southern Biotechnology Associates, Inc.) containing 25 μ g of propidium iodide per ml. Immunofluorescence was detected and photographed with a Bio-Rad MRC1024 confocal laser scanning imaging system.

Protease inhibition. For inhibition experiments, cells were induced as described previously, washed twice with PBS, and treated with either 50 μ M lactacystin, 5 μ M clasto-lactacystin β -lactone, 20 μ M MG132, 25 mM NH₄Cl, a caspase inhibitor mix of 100 μ M caspase 1 inhibitor V and 100 μ M caspase 3 inhibitor II, 20 μ M calpain inhibitor 2 (*N*-acetyl-Leu-Leu-methionine [ALLM]), or 50 μ M calpain inhibitor 1 (*N*-acetyl-Leu-Leu-norleucine [ALLN]), for 5 to 6 h. All inhibitors were purchased from Calbiochem, except NH₄Cl and ALLM, which were purchased from Sigma Aldrich. Cellular proteins were extracted in modified radioimmunoprecipitation assay (RIPA) buffer (20 mM HEPES [pH 7.0], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing protease inhibitor cocktail Complete (Roche) and *Staphylococcus aureus* protein A (Pansorbin; Calbiochem) for preclearing. The pellet was resuspended in 1 $\times$ SDS sample buffer containing 2% SDS, 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 0.1% bromophenol blue, and 10% glycerol, heated for 10 min at 100°C, and sonicated to shear cellular DNA. Total protein concentrations were determined using a bicinchoninic acid assay kit (Pierce).

Western blotting. Equivalent amounts of protein were heated in an equal volume of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer at 100°C for 4 min. Samples were separated on an SDS-10% polyacrylamide gel and transferred onto Immobilon P membranes (Millipore). Western blotting was performed following standard protocols with anti-E2 antibody B201 (Elliot An-

drophy) followed by peroxidase-conjugated anti-mouse IgG (Pierce). E2 proteins were detected using chemiluminescence reagent Super Signal Dura (Pierce) or ECL Plus (Amersham Pharmacia Biotech).

In vitro ³⁵S-labeling and immunoprecipitation. Cells were incubated with medium deficient in methionine and cysteine and supplemented with 5% dialyzed calf serum (Gibco/BRL) for 2 h, followed by a 2-h induction with 1.5 μ M CdSO₄ and concomitant radiolabeling with Promix L-[³⁵S] in vitro cell labeling mix (0.2 mCi/ml) (Amersham Pharmacia Biotech). Cells were washed twice with PBS and chased with medium containing excess amounts of unlabeled methionine and cysteine. Treatment with protease pathway inhibitors was initiated immediately after washing, where indicated. Cell lysates were prepared in modified RIPA buffer (20 mM HEPES [pH 7.0], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% deoxycholate, 0.1% SDS) containing protease inhibitor cocktail Complete (Roche). Equivalent trichloroacetic acid (TCA)-precipitable counts were immunoprecipitated using the B201 antibody (Elliot Androphy). Immune complexes were collected on protein A-Sepharose, washed five times with alternative washes of RIPA buffer and RIPA buffer containing 1 M NaCl. Proteins were eluted in SDS-PAGE sample buffer, boiled, and separated by 10% SDS-PAGE. Gels were fixed, treated with Enlightening (Dupont NEN Research Products), and autoradiographed. Quantitation was performed using a PhosphorImager and ImageQuant software (Molecular Dynamics).

Immunofluorescence and in situ hybridization on tissue sections. Portions of frozen bovine wart tissue were cut into 10- μ m-thick sections and placed on silanated slides. For immunofluorescence studies, slides were fixed for 20 min in 3.7% formaldehyde-300 mM sucrose solution in PBS, permeabilized with 0.1% Triton X-100 in PBS, and blocked in 0.25% gelatin-0.25% bovine serum albumin in PBS. E2 proteins were detected with B201 as described above. For in situ hybridization, 4 μ m-thick, frozen sections were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and digested with 0.1 mg of RNase A per ml and 200 U of *Aspergillus oryzae* RNase per ml. The DNA in the tissue was denatured for 15 min at 75°C in 50% formamide-5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and the slides were dehydrated in a graded series of chilled ethanol. A BPV-1 DNA probe was prepared by labeling p142-6 (28) with fluor-12-dUTP using a Prime-It Fluor fluorescence labeling kit (Stratagene). Sections were hybridized in a solution containing 50% formamide, 10% dextran sulfate, 4 $\times$ SSC, 50 ng of probe, and 6 μ g of sheared competitor DNA at 37°C overnight. Slides were washed three times in 50% formamide-2 $\times$ SSC at 50°C and three times in 0.1 $\times$ SSC at 65°C. Slides were mounted in Vectashield mounting fluid (Vector Laboratories) containing 0.2 μ g of propidium iodide per ml. Fluorescence was detected and photographed with a Bio-Rad MRC1024 confocal laser scanning imaging system.

RESULTS

Expression system to study E2 protein turnover and stability. The location of the serine 301 phosphorylation site in the PEST region of E2 suggested to us that the stability of the E2 protein might be responsible for the increase in viral copy number in cells. Therefore, an E2 expression system was established to investigate this further. In initial experiments in which E2 was transiently expressed from transfected plasmids, problems with protein function, nuclear localization, and solubility that were probably due to overexpression and misfolding were encountered. Overexpression of E2 under these circumstances would not allow us to study normal protein turnover, and a system that attained functional but detectable levels of E2 was required. To achieve this, a stable expression system was established using Epstein-Barr virus derived extrachromosomal vectors that expressed E2 from an inducible metallothionein promoter (Invitrogen). Using this system, we were able to establish stable CV-1 cell lines that could be induced to express E2 at a wide range of concentrations. E2 levels could be regulated both by titration of CdSO₄ levels in the medium (Fig. 1A) and by varying the length of induction time (Fig. 1B). Cells induced with low levels of CdSO₄ or induced for shorter induction times exhibited appropriate nuclear localization of E2. As the levels of E2 increased, cytoplasmic accumulation began to occur, as shown in Fig. 1A (2.5 and 5 μ M CdSO₄) and Fig. 1B (6 and 21 h). Cytoplasmic accumulation of E2 also correlates with protein insolubility. Titratable amounts of E2 protein could be observed by Western blot analysis at induction times of up to 6 h with 1 μ M CdSO₄ (see Fig. 1C). However, by 21 h large quantities of E2 could be observed by immunofluorescence but could not be

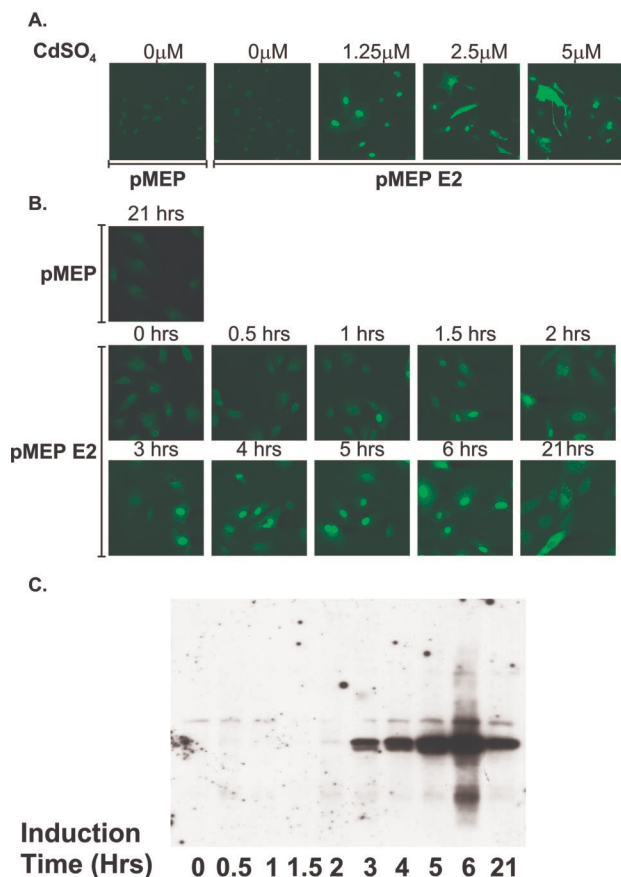


FIG. 1. (A) Induction of E2 from the metallothionein promoter in the pMEP and pMEP-E2 CV-1 cell lines. The metallothionein promoter was induced for 16 h prior to immunofluorescence using various concentrations of CdSO₄ in the medium, as indicated. E2 proteins were detected by indirect immunofluorescence using the E2-specific antibody, B201. (B) Effect of induction times on E2 expression. E2 expression was induced in pMEP and pMEP-E2 CV-1 cell lines with 1 μM CdSO₄ for variable lengths of time, as shown above the photomicrographs. E2 proteins were detected by indirect immunofluorescence using the E2-specific antibody B201. The negative control pMEP CV-1 line was fixed 21 h after induction. (C) Extraction of E2 at different times of induction. E2 expression was induced in the pMEP-E2 CV-1 cell line with 1 μM CdSO₄ for varying lengths of time. The E2 proteins were extracted in RIPA buffer and detected by Western blot analysis using the E2-specific antibody, B201.

efficiently extracted by RIPA buffer, suggesting that the protein had become insoluble. Observations obtained with these lines indicated that, when overexpressed, E2 is susceptible to aggregation in the cytoplasm. We determined that 1 to 1.5 μM CdSO₄ induction for time periods of from 3 to 5 h is sufficient for optimal nuclear localization and solubilization.

E2_{A301} displays an extended half-life compared to wild type. To investigate the role of phosphorylation in E2 protein turnover, the half-life of E2 and E2_{A301} proteins was measured by pulse-chase analysis in the CV-1 pMEP-E2 cell line (Fig. 2A and B). The CV-1 pMEP cell lines were induced with 1.5 μM CdSO₄ and labeled with [³⁵S]methionine and [³⁵S]cysteine simultaneously for 2 h. The medium containing the heavy metal and the ³⁵S-amino acids was removed, and samples were extracted in RIPA buffer at hourly time points up to 5 h. The E2 proteins were immunoprecipitated and analyzed by SDS-PAGE (Fig. 2A). A graphical representation of the experiment, shown in Fig. 2B, illustrates that E2_{A301} displays an extension in half-life that is greater than three times that of wild-type E2. The estimation of the wild-type E2 half-life of 50

min is similar to that previously described for E2 in BPV-1-transformed cells (10). The E2_{A301} half-life is extended to approximately 160 min. Therefore, an E2 protein that is unable to be phosphorylated at serine residue 301 is much more stable, implying that phosphorylation regulates the stability of E2.

E2 is degraded by the proteasome pathway. Proteins utilize a variety of protease pathways for their destruction. These include nonspecific lysosomal proteases; proteases involved in apoptosis such as caspases; calcium-activated proteases or calpains; and the ubiquitin-dependent proteasome. To identify which protease pathway degrades the E2 protein, pulse-chase experiments were conducted in the presence of various protease pathway inhibitors. The CV-1 pMEP cell lines were induced with 1.5 μM CdSO₄ and labeled with ³⁵S-amino acids simultaneously for 2 h. Medium containing heavy metals and ³⁵S-amino acids was removed and replaced with medium containing the various protease inhibitors for 5 h. Dimethyl sulfoxide (DMSO) (the solvent for all inhibitors except NH₄Cl) was used as a control. The inhibitors used were 5 μM clasto-lactacystin β-lactone, 20 μM MG132, 25 mM NH₄Cl, a caspase inhibitor mix, 20 μM ALLM, or 50 μM ALLN. Proteins were extracted in RIPA buffer and analyzed by immunoprecipitation with the E2-specific antibody, B201. Figure 3 illustrates the effect of these inhibitors on E2 protein stability. In untreated cells, E2 is degraded to 5% (12% with DMSO) of the initial protein amounts within 5 h. Addition of clasto-lactacystin β-lactone or MG132, inhibitors of the proteasome, inhibits this degradation, and 74 or 69% of initial E2 proteins, respectively, remain after 5 h. All other treatments display minimal effects

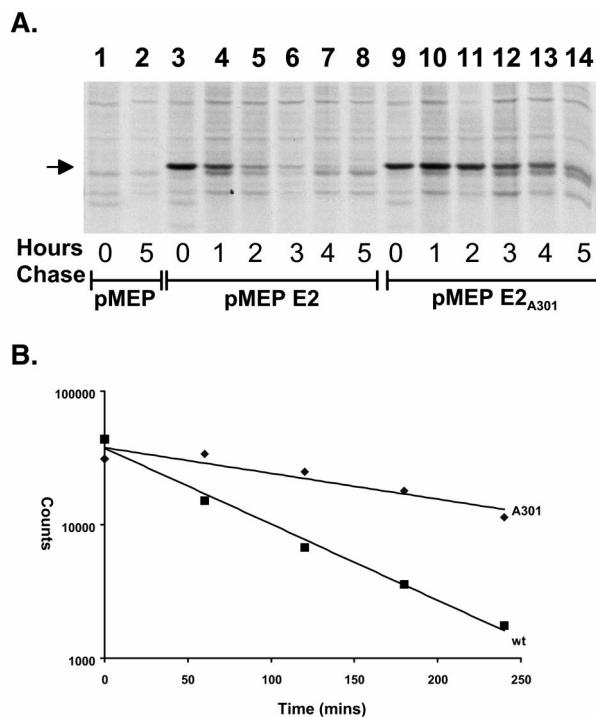
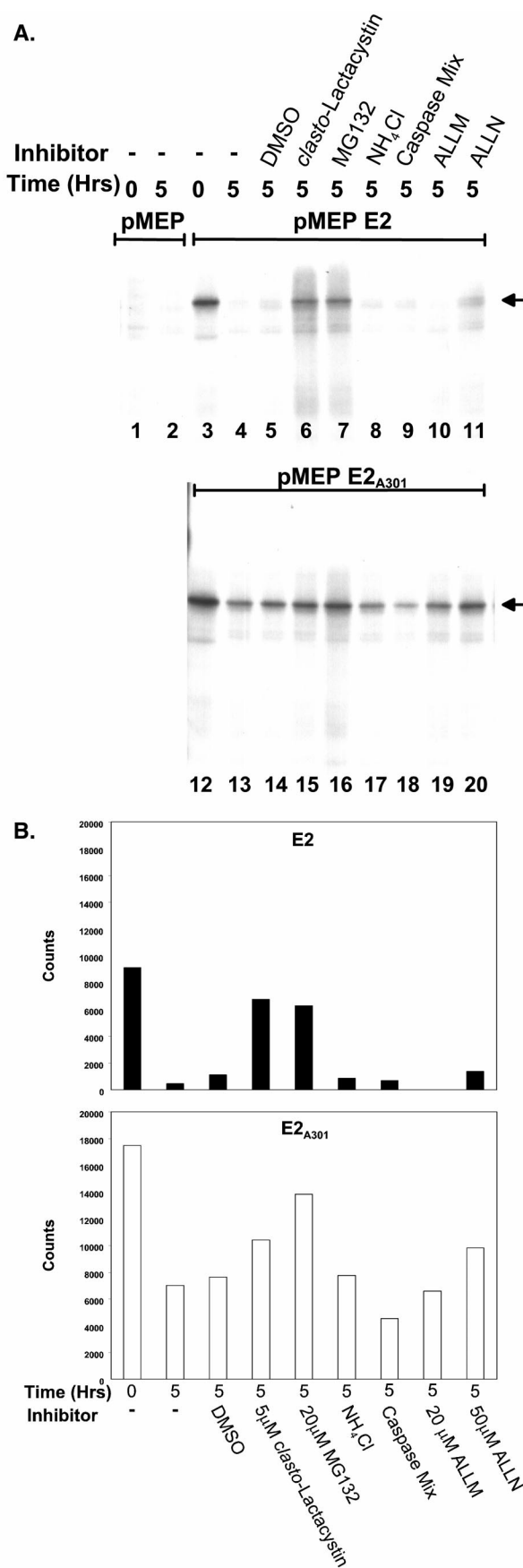


FIG. 2. Half-life determination of the E2 proteins. (A) pMEP (lanes 1 and 2), pMEP-E2 (lanes 3 to 8), and pMEP-E2_{A301} (lanes 9 to 14) CV-1 cell lines were induced with 1.5 μM CdSO₄, pulsed with ³⁵S-amino acids simultaneously for 2 h, and chased for up to 5 h. Proteins were extracted every hour and normalized by TCA-precipitable counts. E2 proteins were isolated by immunoprecipitation with B201 and analyzed by SDS-PAGE. (B) Graphical representation of data collected for panel A. Proteins were quantitated using a PhosphorImager, and counts were normalized using several stable cellular bands as internal controls.



on E2 turnover. NH_4Cl is an inhibitor of lysosomal proteolysis. The caspase inhibitor mix of caspase 1 inhibitor V and caspase 3 inhibitor II inhibits members of the ICE family of cysteine proteases that are involved in apoptosis. ALLM and ALLN are inhibitors of the calcium-regulated proteases calpain I and II and cathepsin L and B.

In this experiment, E2_{A301} also displays an extension in half-life compared to wild-type E2 and only decreases to 40% (44% with DMSO) of the initial protein amount in the 5-h time period. *clasto*-Lactacystin β -lactone and MG132 inhibit the degradation of E2_{A301} slightly by increasing the percentage of protein remaining after 5 h from 44 to 60 and 79%, respectively, of the initial proteins. A slight inhibition of degradation is also observed with ALLN, which can be a less-specific inhibitor of the proteasome as well as of calcium-regulated proteases. All other treatments have minimal effects on E2_{A301} stability. This experiment confirms our observation that E2_{A301} has increased stability over wild-type E2. There is a 20-fold decrease in wild-type E2 levels within 5 h, while E2_{A301} only decreases to about half of the initial amount. This screen of protease pathway inhibitors demonstrates that wild-type E2 is degraded by the proteasome pathway. E2_{A301} also appears to be degraded by the proteasome pathway, though much less efficiently than wild-type E2.

Wild-type E2 shows modification characteristic of ubiquitination that is reduced by mutation of serine 301. Most substrates of the proteasome pathway utilize covalent linkage of ubiquitin for targeting proteins for degradation. It has been shown, in some cases, that phosphorylation can promote ubiquitination and further proteasomal degradation. We sometimes observed higher-molecular-weight E2 bands in experiments with proteasome inhibitors, so to determine if these were due to ubiquitination, we enhanced the levels of expression of E2 to visualize these products more clearly while still maintaining correct E2 function and cellular localization. The pMEP CV-1 cell lines were induced with 1 μM CdSO_4 for 5 h, followed by immediate treatment for 5 h with proteasome inhibitors, 50 μM lactacystin, and 20 μM MG132. We have also observed that as E2 protein expression increases, the protein becomes insoluble (Fig. 1C). This is consistent with the accumulation of insoluble, ubiquitinated proteins described by Johnston et al. and Anton et al. for other proteins that are degraded by the proteasome (1, 12). Therefore, in this experiment, RIPA-insoluble proteins were further extracted in 2% SDS and analyzed by Western blot analysis using the E2-specific antibody, B201. In cells treated with the proteasome inhibitors, lactacystin, and MG132, wild-type E2 levels were greatly increased and displayed a high-molecular-weight ladder pattern that is characteristic of highly ubiquitinated proteins (Fig. 4, lanes 5 and 6) (23). The ladder pattern observed correlates with that expected by addition of multiple 8-kDa ubiquitin moieties. Mutation of serine residue 301 to alanine dramatically reduces the amounts of higher-molecular-weight products (Fig. 4, lanes 11 and 12), indicating that phosphorylation of this

FIG. 3. E2 is degraded by the proteasome. (A) E2 expression was induced in the CV-1 pMEP (lanes 1 and 2), pMEP-E2 (lanes 3 to 11), and pMEP-E2_{A301} (lanes 12 to 20) cell lines with 1.5 μM CdSO_4 , and cells were simultaneously labeled with ^{35}S -amino acids for 2 h. The medium containing CdSO_4 and ^{35}S -amino acids was removed, and samples either were not treated or were immediately treated with medium containing either DMSO, 5 μM *clasto*-lactacystin, 20 μM MG132, NH_4Cl , a caspase inhibitor mix, 20 μM ALLM, or 50 μM ALLN, as indicated, for 5 h. Samples were extracted in RIPA buffer, immunoprecipitated with B201, and analyzed by SDS-PAGE. (B) Graphical representation of data collected for panel A.

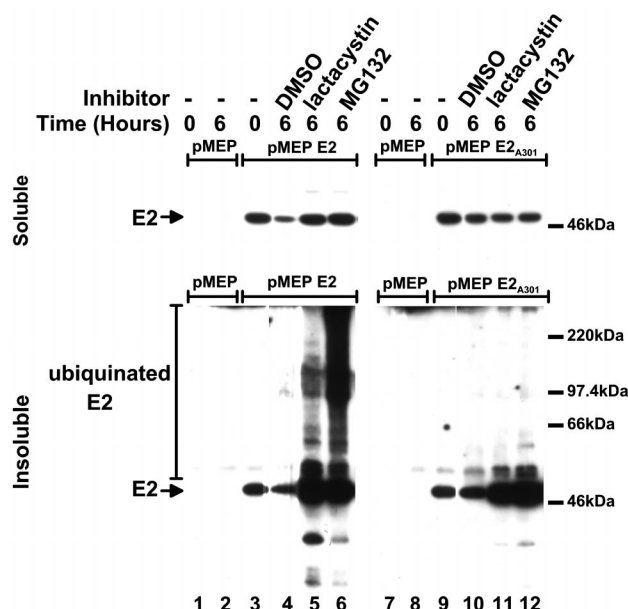


FIG. 4. E2 displays modification characteristic of ubiquitination. CV-1 pMEP (lanes 1, 2, 7, and 8), pMEP-E2 (lanes 3 to 6), and pMEP-E2_{A301} (lanes 9 to 12) cell lines were induced with 1 μ M CdSO₄ for 5 h. CdSO₄ was removed and samples were immediately treated with DMSO, 20 μ M lactacystin, or 20 μ M MG132, as indicated, for 6 h. Soluble portions were extracted in RIPA buffer, and RIPA-insoluble portions were resuspended in SDS sample buffer. Samples were separated by SDS-PAGE and analyzed by Western blot using the E2-specific antibody, B201.

residue is required for efficient ubiquitination and subsequent degradation of E2 by the ubiquitin-proteasome pathway.

Colocalization of cells expressing high levels of E2 and vegetatively amplifying DNA in bovine wart tissue. Our studies indicate that increased levels of E2 can result in an increased number of episomal viral genomes in cell lines containing BPV-1. We predict that phosphorylation regulates episomal maintenance, which is crucial for persistence of viral genomes in the proliferating basal cells of a papilloma. We have observed another situation in which the level of E2 correlates with the viral DNA copy number. In a papilloma, vegetative viral DNA amplification is restricted to cells in the stratum spinosum and above. We and others have previously noted that E2 is also expressed at very high levels in a subset of cells in the stratum spinosum (4, 22). Figure 5A shows the pattern of E2 expression in a bovine papilloma, as detected by immunofluorescence with the B201 monoclonal antibody. This antibody recognizes both transactivator and repressor species of E2. Low levels of E2 proteins are observed in all basal cells, and very high levels of E2 are found in a portion of the cells in the stratum spinosum. To determine whether the same subset of cells in the stratum spinosum contained high levels of E2 and viral DNA, immunofluorescence for E2 and in situ hybridization for BPV-1 DNA were carried out on serial sections of wart tissue. As shown in Fig. 5B2 to B9, most cells containing high levels of E2 (Fig. 5B2, B4, B5, B6) also contained large amounts of amplified viral DNA (Fig. 5B3, B7, B8, and B9). Viral DNA is present in all layers above the stratum spinosum (Fig. 5B3), but E2 is not present in cells above this layer. Replication takes place only in the stratum spinosum, but viral DNA persists as cells differentiate into the upper layers and is encapsidated. This correlation suggests that the increase in E2 levels may be responsible for activation of vegetative DNA replication.

DISCUSSION

It was previously shown that mutation of one of the major phosphorylation sites of the E2 protein results in a virus that has a greatly increased extrachromosomal copy number in BPV-transformed cells (20). In this study we show that this mutation substantially reduces apparent E2 ubiquitination and subsequent degradation by the proteasome. This results in a protein with a half-life much longer than that of wild-type E2. It is likely that the increased amount of E2 protein is directly responsible for the higher genome copy number in cells containing the mutated E2 protein. No difference could be detected in the ability of wild-type E2 and E2_{A301} to support DNA replication of a minimal origin in a transient assay. However, E2 is also essential for long-term maintenance of viral genomes (25) due to the ability of E2 to link viral genomes to mitotic chromosomes in dividing cells (11, 13, 31). If the amount of E2 protein is normally limiting, then an increase in the level of E2 could result in increased copy number by in-

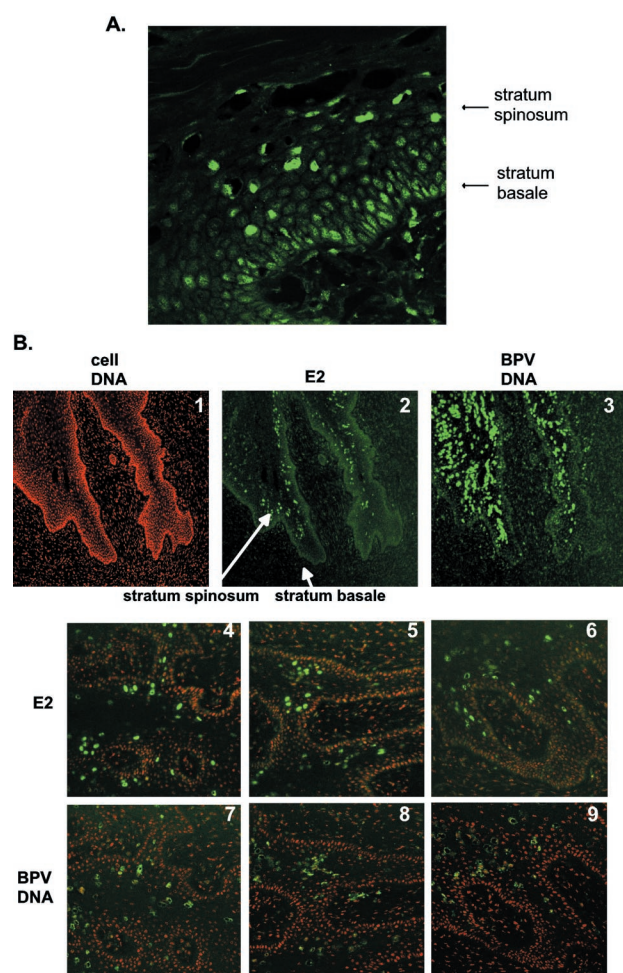


FIG. 5. Expression of E2 protein in a papilloma. E2-specific immunofluorescence and BPV-specific fluorescent in situ hybridization (FISH) were performed on bovine wart tissue. (A) E2 protein expression detected by immunofluorescence with B201. (B) Colocalization of E2 protein and BPV-1 DNA in serial bovine wart sections by immunofluorescence and FISH. Panel 1, cellular DNA staining by propidium iodide (red); panels 2, 4, 5 and 6, E2 protein as detected by immunofluorescence (green); and panels 3, 7, 8, and 9, BPV-1 DNA as detected by FISH (green). Propidium iodide staining (red) is also shown in panels 2 to 9. Panels 1, 2, and 3; panels 4 and 7; panels 5 and 8, and panels 6 and 9 show images from a serial section of the papilloma.

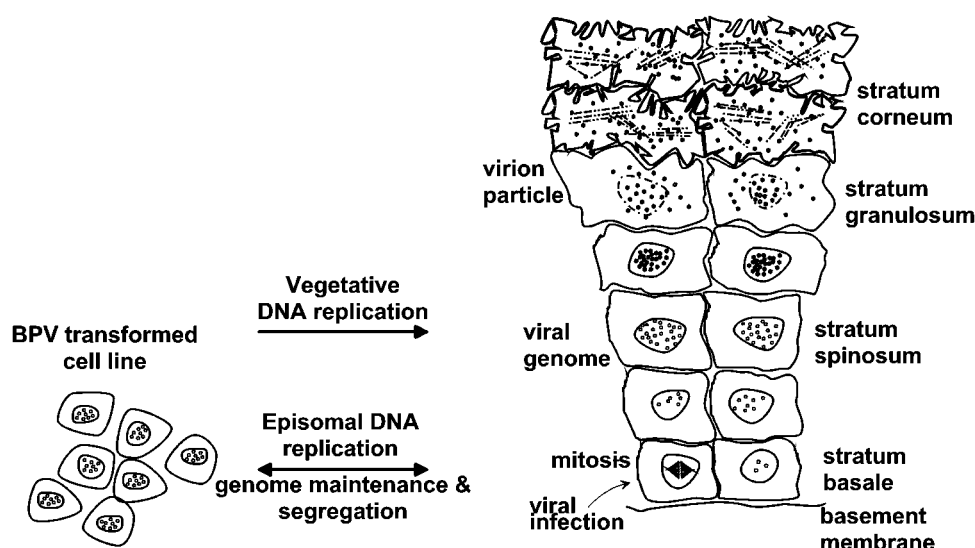


FIG. 6. Two phases of the viral life cycle where E2 protein levels could regulate viral DNA copy number.

creasing the percentage of genomes attached to chromosomes at each cell division.

It has been reported that E2 phosphorylation regulates the interaction of E2 with mitotic chromosomes in BPV-transformed cells; however, S235, S298, and S301 must be mutated to abrogate this interaction (13). When E2 is expressed in the absence of the viral genome and other viral genes, mutation of the phosphorylation sites has no effect on the ability of E2 to directly interact with chromosomes (2). We have not analyzed the half-life of E2 proteins with mutations in other or all phosphorylation sites (S235, S298, and S301) in this study. We propose that phosphorylation indirectly determines the number of viral genomes bound to chromosomes by regulating the half-life of the E2 protein. E2 proteins that are phosphorylated would be degraded rapidly, and unphosphorylated E2 would have an extended half-life. The extended half-life could be important to ensure that E2-genome complexes are stable throughout the length of mitosis. In this scenario, E2 might be phosphorylated by cell cycle-specific kinases, which could be regulated by interaction with the viral DNA and the E1 protein. Yang et al. have examined the levels of E2 proteins throughout the cell cycle in BPV-1-transformed cells and find that E2-TA is highest in the S and G₂ phases and decreases in mitosis and G₁ phases (35). Although this could be due, at least in part, to transcriptional or translational regulation, it would also be consistent with our hypothesis that E2 phosphorylation and turnover are regulated at specific stages of the cell cycle. When the replication and transactivation functions of the E2 and E2_{A301} proteins are compared in transient assays as well as in the absence of the viral genome, no obvious differences can be detected (A. A. McBride, unpublished data). If prevention of E2 phosphorylation merely increases the levels of E2, then E2_{A301} would be expected to function more efficiently in these assays. However, if phosphorylation and degradation are cell cycle regulated, then the effect may be cumulative over several cell cycles.

We have identified an additional stage of the viral life cycle that might be regulated by levels of E2 protein. Staining for the E2 protein in bovine wart tissue demonstrated that there are two distinct areas in which the E2 protein can be detected. Low levels of E2 protein are found in all basal cells of the papilloma, where E2 is probably required for transcriptional regu-

lation and episomal genome replication. In addition, E2 is expressed at very high levels in a subset of cells in the stratum spinosum. Burnett et al. have also described the E2-expressing cells in the stratum spinosum and have noted that this is the layer in which vegetative viral DNA replication initiates (4). In this study we have colocalized the cells expressing high levels of E2 protein and vegetatively replicating viral DNA and have established that this is the same population. E2 is expressed at high levels in the stratum spinosum but is undetectable in the upper, more differentiated layers, indicating that its expression is tightly regulated (Fig. 5B2). However, viral DNA is present in all layers above the stratum spinosum (Fig. 5B3) suggesting that replication occurs in the stratum spinosum and viral DNA persists as cells differentiate into the upper layers and it is encapsidated. This could indicate that E2 is involved in the switch to vegetative DNA replication. Very little is known about the requirements for this stage of replication, so it is difficult to speculate about the exact role of E2. Upregulation of E2 protein in certain cells of the stratum spinosum could be dependent on differentiation-dependent transcriptional or translational regulation but could also be due to differentiation-dependent stabilization and subsequent degradation of E2. Further experiments are necessary to distinguish between these possibilities. The diagram in Fig. 6 shows the two phases where modulation of E2 protein turnover could regulate viral DNA copy number. In support of our hypothesis that the levels of E2 modulate viral genome copy number, Frattini et al. have demonstrated that infection of cells containing episomal human papillomavirus type 31 with recombinant adenoviruses expressing E2 results in a fivefold increase in viral DNA (8). Furthermore, these cells are blocked in S phase and continue to synthesize DNA without undergoing mitosis, similar to the cells in the stratum spinosum of a papilloma. If E2 degradation is cell cycle regulated and occurs between S phase and mitosis, then E2 protein levels would be expected to accumulate in these cells.

The wild-type E2 transactivator protein has a half-life of 40 min; it is ubiquitinated and degraded by the proteasome. Mutation of the serine residue 301 phosphoacceptor site in the E2 protein substantially increases the half-life of E2, suggesting that E2 degradation is regulated by phosphorylation. Proteins targeted for degradation by the proteasome pathway are usu-

ally marked by a covalent linkage of the C-terminal glycine of the 76-amino-acid ubiquitin protein and the ϵ -amino group of specific lysine residues on the target protein. One class of amino acid sequences thought to be important for targeting proteins for degradation is PEST sequences (27). PEST sequences are rich in proline, glutamic and aspartic acid, serine, and threonine, and have been identified in numerous proteins that are rapidly turned over. These sequences often contain consensus sites for protein kinases, and phosphorylation of PEST sequences has been shown to trigger ubiquitination and degradation (reviewed in reference 9). Ubiquitin ligases can recognize different phosphorylation patterns in combination with other motifs in the protein substrates. In some cases PEST sequences are transplantable and, when fused to a stable protein, render it unstable (17, 27). The E2_{A301} protein has a significantly longer half-life than wild-type E2 (160 min versus 50 min), but it is still turned over at a moderate rate and a low level of ubiquitination can be detected. Therefore, phosphorylation of serine 301 has a major role in modulating protein stability, but other features must also determine half-life. In some proteins, such as yeast uracil permease, mutation of multiple phosphorylation sites is required to completely inhibit degradation (18). Further studies are required to determine whether the other mapped E2 phosphorylation sites, serines 235 and 298, are also involved in determining protein stability. Serine 301 has a consensus site for casein kinase II (CKII) and can be phosphorylated by this kinase *in vitro* (A. A. McBride, unpublished observations). Serine 298 has a minimal consensus site (S/TP) for phosphorylation by cyclin-dependent kinases and could also be phosphorylated by CKII if serine 301 is phosphorylated first, providing a negative charge for CKII recognition. It is likely that phosphorylation of each site affects phosphorylation of the other, as they are only separated by two amino acids.

The E2 protein accumulates in the cytoplasm when it is substantially overexpressed in mammalian cell lines, and this accumulation correlates with protein insolubility (Fig. 1 to 3). These findings support the recently described model that proposes that proteins normally degraded by the proteasome form aggregates when misfolded or overexpressed or when the capacity of the proteasome is exceeded (1, 12). This model proposes that proteins that are targeted for proteasomal degradation undergo ubiquitination, followed by either degradation or deubiquitination. In cases where the proteasome capacity is exceeded or blocked, ubiquitinated proteins form aggregates in the cytoplasm. Over time, microtubule retrograde transport mediates transport of these aggregated proteins to the microtubule organizing center. Anton et al. (1) further demonstrate that these ubiquitinated proteins also accumulate in nuclear promyelocytic leukemia oncogenic domains (PODs). In cells overexpressing E2 we have also observed aggregates of E2 located in a spot close to the nucleus that would be consistent with the microtubule organizing center. Johnston et al. (12) studied the turnover of the cystic fibrosis transmembrane conductance regulator (CFTR), and Anton et al. (1) studied a mutated form of influenza virus nucleoprotein. Our studies show that the same pathway can be observed when E2, a nonmutated viral nuclear protein normally degraded by the proteasome, is overexpressed. Therefore, to study the regulated turnover of E2, we have developed an inducible system so that physiological levels of functional E2 can be analyzed.

The E2 proteins function at several different stages of the viral life cycle. Most regulatory proteins have a short half-life so that they may carry out their function and quickly be inactivated. There are several phases in the papillomavirus life cycle where modulation of E2 turnover might be important,

and we have described two of these above. In both cases, there is evidence that increased levels of E2 protein result in increased genome copy number.

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REFERENCES

- Anton, L. C., U. Schubert, I. Bacik, M. F. Princiotta, P. A. Wearsch, J. Gibbs, P. M. Day, C. Realini, M. C. Rechsteiner, J. R. Bennink, and J. W. Yewdell. 1999. Intracellular localization of proteasomal degradation of a viral antigen. *J. Cell Biol.* **146**:113–124.
- Bastien, N., and A. A. McBride. Interaction of the papillomavirus E2 with mitotic chromosomes. *Virology*, in press.
- Blitz, I. L., and L. A. Laimins. 1991. The 68-kilodalton E1 protein of bovine papillomavirus is a DNA binding phosphoprotein which associates with the E2 transcriptional activator *in vitro*. *J. Virol.* **65**:649–656.
- Burnett, S., A.-C. Ström, N. Jareborg, A. Alderborn, J. Dillner, J. Moreno-Lopez, U. Pettersson, and U. Kiessling. 1990. Induction of bovine papillomavirus E2 gene expression and early region transcription by cell growth arrest: correlation with viral DNA amplification and evidence for differential promoter induction. *J. Virol.* **64**:5529–5541.
- DiDonato, J., F. Mercurio, C. Rosette, J. Wu-Li, H. Suyang, S. Ghosh, and M. Karin. 1996. Mapping of the inducible I κ B phosphorylation sites that signal its ubiquitination and degradation. *Mol. Cell Biol.* **16**:1295–1304.
- Diehl, J. A., F. Zindy, and C. J. Sherr. 1997. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev.* **11**:957–972.
- Elsasser, S., Y. Chi, P. Yang, and J. L. Campbell. 1999. Phosphorylation controls timing of cdc6p destruction: a biochemical analysis. *Mol. Biol. Cell* **10**:3263–3277.
- Frattini, M. G., S. D. Hurst, H. B. Lim, S. Swaminathan, and L. A. Laimins. 1997. Abrogation of a mitotic checkpoint by E2 proteins from oncogenic human papillomaviruses correlates with increased turnover of the p53 tumor suppressor protein. *EMBO J.* **16**:318–331.
- Hershko, A., and A. Ciechanover. 1998. The ubiquitin system. *Annu. Rev. Biochem.* **67**:425–479.
- 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.
- Ilves, I., S. Kivi, and M. Ustav. 1999. Long-term episomal maintenance of bovine papillomavirus type 1 plasmids is determined by attachment to host chromosomes, which is mediated by the viral E2 protein and its binding sites. *J. Virol.* **73**:4404–4412.
- Johnston, J. A., C. L. Ward, and R. R. Kopito. 1998. Aggresomes: a cellular response to misfolded proteins. *J. Cell Biol.* **143**:1883–1898.
- Lehman, C. W., and M. R. Botchan. 1998. Segregation of viral plasmids depends on tethering to chromosomes and is regulated by phosphorylation. *Proc. Natl. Acad. Sci. USA* **95**:4338–4343.
- Lehman, C. W., D. S. King, and M. R. Botchan. 1997. A papillomavirus E2 phosphorylation mutant exhibits normal transient replication and transcription but is defective in transformation and plasmid retention. *J. Virol.* **71**:3652–3665.
- Li, R., and M. R. Botchan. 1993. The acidic transcriptional activation domains of VP16 and p53 bind the cellular replication protein A and stimulate *in vitro* BPV-1 DNA replication. *Cell* **73**:1207–1221.
- Li, R., and M. R. Botchan. 1994. Acidic transcription factors alleviate nucleosome-mediated repression of DNA replication of bovine papillomavirus type 1. *Proc. Natl. Acad. Sci. USA* **91**:7051–7055.
- Loetscher, P., G. Pratt, and M. Rechsteiner. 1991. The C terminus of mouse ornithine decarboxylase confers rapid degradation on dihydrofolate reductase. Support for the pest hypothesis. *J. Biol. Chem.* **266**:11213–11220.
- Marchal, C., R. Haguenaer-Tsapis, and D. Urban-Grimal. 1998. A PEST-like sequence mediates phosphorylation and efficient ubiquitination of yeast uracil permease. *Mol. Cell Biol.* **18**:314–321.
- 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.
- 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.
- McBride, A. A., and G. Myers. 1997. The E2 proteins: an update, p. III 54–III 99. In G. Myers, C. Baker, K. Munger, F. Sverdrup, A. McBride, and H.-U. Bernard (ed.), *Human papillomaviruses 1997*. Los Alamos National Laboratory, Los Alamos, N.Mex.
- McBride, M., E. J. Androphy, and K. Munger. 1999. Regulation of the

- papillomavirus E6 and E7 oncoproteins by the viral E1 and E2 proteins, p. 35–52. In G. Myers (ed.), *Viral regulatory structures and their degeneracy*. Addison-Wesley, Reading, Mass.
23. **Mimnaugh, E. G., P. Bonvini, and L. Neckers.** 1999. The measurement of ubiquitin and ubiquitinated proteins. *Electrophoresis* **20**:418–428.
 24. **Mohr, I. J., R. Clark, S. Sun, E. J. Androphy, P. MacPherson, and M. R. Botchan.** 1990. Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. *Science* **250**:1694–1699.
 25. **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.
 26. **Rechsteiner, M., and S. W. Rogers.** 1996. PEST sequences and regulation by proteolysis. *Trends Biochem. Sci.* **21**:267–271.
 27. **Rogers, S., R. Wells, and M. Rechsteiner.** 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234**:364–368.
 28. **Sarver, N., J. C. Byrne, and P. M. Howley.** 1982. Transformation and replication in mouse cells of a bovine papillomavirus—pML2 plasmid vector that can be rescued in bacteria. *Proc. Natl. Acad. Sci. USA* **79**:7147–7151.
 29. **Scherer, D. C., J. A. Brockman, Z. Chen, T. Maniatis, and D. W. Ballard.** 1995. Signal-induced degradation of I κ B α requires site-specific ubiquitination. *Proc. Natl. Acad. Sci. USA* **92**:11259–11263.
 30. **Shumway, S. D., M. Maki, and S. Miyamoto.** 1999. The PEST domain of I κ B α is necessary and sufficient for in vitro degradation by μ -calpain. *J. Biol. Chem.* **274**:30874–30881.
 31. **Skiaopoulos, M. H., and A. A. McBride.** 1998. BPV1 viral genomes and the E2 transactivator protein are associated with cellular metaphase chromosomes. *J. Virol.* **72**:2079–2088.
 32. **Van Antwerp, D. J., and I. M. Verma.** 1996. Signal-induced degradation of I κ B α : association with NF- κ B and the PEST sequence in I κ B α are not required. *Mol. Cell. Biol.* **16**:6037–6045.
 33. **Vandel, L., and T. Kouzarides.** 1999. Residues phosphorylated by TFIIF are required for E2F-1 degradation during S-phase. *EMBO J.* **18**:4280–4291.
 34. **Won, K. A., and S. I. Reed.** 1996. Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. *EMBO J.* **15**:4182–4193.
 35. **Yang, L., I. Mohr, R. Li, T. Nottoli, S. Sun, and M. Botchan.** 1991. Transcription factor E2 regulates BPV-1 DNA replication in vitro by direct protein-protein interaction. *Cold Spring Harbor Symp. Quant. Biol.* **56**:335–346.