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The E1 Replication Protein of Bovine Papillomavirus Type 1 Contains an Extended Nuclear Localization Signal That Includes a p34^{cdc2} Phosphorylation Site

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Bovine papillomavirus (BPV) DNA replication occurs in the nucleus of infected cells. Most enzymatic activities are carried out by host cell proteins, with the viral E1 and E2 proteins required for the assembly of an initiation complex at the replication origin. In latently infected cells, viral DNA replication occurs in synchrony with the host cell chromosomes, maintaining a constant average copy number of BPV genomes per infected cell. By analyzing a series of mutants of the amino-terminal region of the E1 protein, we have identified the signal for transport of this protein to the cell nucleus. The E1 nuclear transport motif is highly conserved in the animal and human papillomaviruses and is encoded in a similar region in the related E1 genes. The signal is extended relative to the simple nuclear localization signals and contains two short amino acid sequences which contribute to nuclear transport, located between amino acids 85 and 108 of the BPV-1 E1 protein. Mutations in either basic region reduce nuclear transport of E1 protein and interfere with viral DNA replication. Mutations in both sequences simultaneously prevent any observable accumulation of the protein and reduce replication in transient assays to barely detectable levels. Surprisingly, these mutations had no effect on the ability of viral genomes to morphologically transform cells, although the plasmid DNA in the transformed cells was maintained at a very low copy number. Between these two basic amino acid blocks in the nuclear transport signal, at threonine 102, is a putative site for phosphorylation by the cell cycle regulated kinase p34^{cdc2}. Utilizing an E1 protein purified from either a baculovirus vector system or *Escherichia coli*, we have shown that the E1 protein is a substrate for this kinase. An E1 gene mutant at threonine 102 encodes for a protein which is no longer a substrate for the p34^{cdc2} kinase. Mutation of this threonine to isoleucine had no observable effect on either nuclear localization of E1 or DNA replication of the intact viral genome.

Bovine papillomavirus type 1 (BPV-1) has become a useful model for the study of eukaryotic DNA replication. The virus has the unusual property that in stably transformed cells, the genome maintains itself as a stable nuclear plasmid. The viral DNA replicates in the S phase of the cell cycle, and when cells are forced to exit the cell cycle by serum deprivation, the viral DNA stops replicating. Thus, transformed cells provide a model replicon for the study of regulated DNA synthesis.

Recently, progress in this field has begun to unravel in detail the role of viral proteins in the replication process. Genetic studies have shown that the E1 and E2 open reading frames encode for proteins having critical roles in viral replication. Although the role of the E2 protein in viral transcription has previously been described (21), in vitro and in vivo evidence points to a direct role for E2 in viral replication (24, 46, 48, 49). E1, a 68-kDa phosphoprotein found in the nucleus of infected cells (33, 43, 46), recognizes and binds to the viral origin of replication (24, 46, 48, 49). With significant functional homologies to the simian virus (SV40) large T antigen (TAg), E1 is believed to function by coordinating the assembly of an initiation complex at the replication origin. Given observations which have shown that the E1 protein has helicase and DNA unwinding activities (23, 38) and that the protein can interact with the cellular DNA polymerase alpha (26), this analogy to the large T antigens as a central factor in initiating viral replication is clear. E2 is known to help E1 associate with DNA through both protein-protein and protein-DNA interactions (24, 41, 48, 49). However, it is likely that E2 plays additional roles in replication, as its synergy in stimulating DNA replication in vitro goes beyond its cooperative interaction with E1 in DNA binding (48). The overall evolutionary conservation of replication strategies for the animal and human papillomaviruses is suggested by the findings that only the E1 and E2 gene products are required in vivo for transient replication of a large number of viral DNAs (3, 5, 45) and that the expression cassettes for E1 and E2 can be interchanged between species, with little indication of incompatibility (3).

E1 also has several regions of sequence similarity to SV40 large TAg (4). These patches of sequence similarity between E1 and the well-characterized TAg have been helpful in identifying homologous activities in the E1 protein. For example, E1 contains sequences similar to the nucleotide binding and ATPase regions of SV40 large TAg, and these sequences appear to have functional homology as well (43). Mutations in the E1 gene which disrupt the conserved motif (GPXGXGK[T/S]) associated with an ATP binding domain destroy E1 helicase and ATPase activities (23).

Perhaps the region with the greatest similarity exists between amino acids 94 and 108 of E1 and the nuclear localization region of SV40 large TAg (Fig. 1). The nuclear localization signal of SV40 large TAg was the first well-defined sequence to be identified as a signal for protein transport to the nucleus (13, 17). The most striking feature of this signal is a stretch of five consecutive basic amino acids

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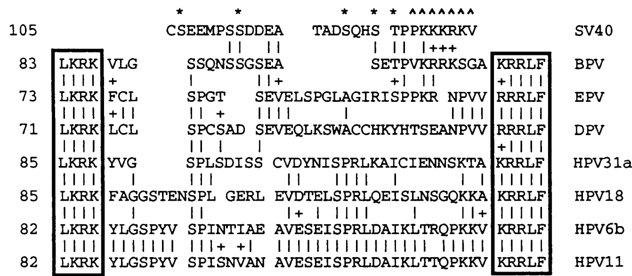

FIG. 1. Sequence homology between the region of SV40 large TAg containing the nuclear localization signal and the corresponding region of the E1 protein of several papillomaviruses. |, sequence identity between proteins; +, sequence similarity; *, amino acids phosphorylated in SV40 large TAg (34). The three basic peptide clusters in BPV E1 are underlined. The nuclear localization signal sequence for SV40 TAg is highlighted by carets. Two highly conserved clusters in the papillomavirus sequences are enclosed by boxes. EPV, elk papillomavirus; DPV, deer papillomavirus; HPV, human papillomavirus.

(PKKKRKV). The nuclear localization signals for a number of other nuclear proteins have since been identified, and most show similarity to the SV40 large TAg signal (8, 39), retaining the positively charged character of the signal.

Nuclear localization is a two-step process, consisting of (i) transport and binding to the nuclear pore complex and (ii) translocation across the membrane. A current model for nuclear transport predicts that the nuclear localization signal is recognized by a transport protein or receptor located in the cytoplasm, which ferries the nuclear protein to the nuclear pore, where binding occurs (1). After association with the nuclear pore, two pathways for entry have been described. Either the transported protein is released from the receptor and moved across the nuclear membrane independently or the complex of receptor and nuclear protein is transported into the nucleus, after which the receptor is released and returns to the cytoplasm to repeat the cycle. The translocation step is dependent on ATP, whereas the initial binding to the nuclear pore complex is not (reference 39 and references therein).

The putative nuclear localization signal for BPV E1 had previously been mapped to the amino-terminal 223 amino acids, since a construct expressing a fragment of E1 from which the first 223 amino acids were deleted accumulated exclusively in the cytoplasm (43). There are three sequences of three or four consecutive basic amino acids in the amino-terminal region of E1, all between amino acids 84 and 114. The middle sequence, (102)TPVKRRK(108), is most similar to the nuclear localization signal of SV40 large TAg.

Another feature of this conserved region between SV40 large TAg and E1 is a predicted phosphorylation site for the $p34^{cdc2}$ protein serine/threonine kinase. This kinase is an important regulator of the cell cycle, active at both the G_1/S and G_2M boundaries. There appear to be different substrates for this kinase during the cell cycle. There is no defined consensus recognition site for this kinase at the G_1/S boundary, although most substrates so far identified are phosphorylated at threonine or serine followed by proline (K/R)(T/S)PX(R/K) appears to be the consensus sequence recognized at the G_2/M boundary (27). This sequence, minus the first basic residue, overlaps the amino-terminal end of the SV40 nuclear localization signal and is also found in a region of chemical similarity in the E1 protein. Phosphorylation of threonine 124 in SV40 large TAg by $p34^{cdc2}$ has been shown to be important for the replication activities of this protein (22, 25, 37). In the absence of phosphorylation of threonine 124, TAg binds without cooperation to the origin palindrome (site 2), and as measured by the McKay assay, with a much weaker affinity to this site. Phosphorylation of threonine 124 shifts the binding preference of TAg from site 1, a major *cis* determinant for transcriptional autoregulation, to site 2, thus stimulating DNA replication. The region just amino terminal to the $p34^{cdc2}$ site and nuclear localization signal in SV40 TAg contains several serine phosphorylation sites. A casein kinase II site in this region has been shown to influence the rate of nuclear accumulation of TAg in the nucleus (29, 30). E1 is heavily phosphorylated on serine, and there are numerous potential serine phosphorylation sites in the corresponding region of E1. Threonine 102 of BPV E1 is in a domain of the protein thus potentially similar in function to the domain surrounding threonine 124 of SV40 large TAg.

In this report, we investigate whether the sequence conservation between amino acids 80 and 120 of BPV E1 and the corresponding region of SV40 large TAg correlate to similar functional and regulatory properties. A series of E1 constructs containing mutations in this region was generated, and the effects of these mutations on intracellular distribution, protein phosphorylation, and viral DNA replication were determined. These studies show that despite the high degree of sequence homology, there are significant differences between the nuclear localization signals of E1 and SV40 large TAg as well as the requirement for phosphorylation at the conserved $p34^{cdc2}$ site.

MATERIALS AND METHODS

Construction of recombinant plasmids and mutagenesis. Expression plasmids for making E1 proteins in COS-7 cells were constructed with the vector pMT21 as previously described (44). To efficiently shuttle a fragment of E1 for mutagenesis into and out of various vectors, pMTE1 was partially digested with *Eco*RI, filled in with the Klenow fragment of DNA polymerase I, and religated. A clone in which the *Eco*RI site in the vector had been destroyed was used for all further experiments. Likewise, replication experiments were done with plasmid pMLBPV R118, in which the *Eco*RI site of the pML plasmid had been removed.

DNA sequences coding for the wild-type E1 protein and a mutant containing a threonine-to-isoleucine mutation at amino acid 102 were recombined into the *Autographa californica* nuclear polyhedrosis virus genome, using the transfer vector pAcc13E1. Recombinant baculovirus was prepared as described previously (42).

Site-directed mutants were prepared by two protocols. The 1,168-bp *Eco*RI-*Sma*I fragment of E1 (nucleotides 945 to 2113) was cloned into M13mp18 and used as a source of single-stranded DNA for mutagenesis according to established protocols (32). Alternatively, the same fragment was cloned into the phagemid vector pBluescript (Stratagene) and a single-stranded templates were generated by using helper phage. Mutations were transferred between all plasmids on the *Sma*I-to-*Eco*RI fragment. These sites were unique in all E1-containing plasmids used.

Cell culture, electroporation, and replication assays. Monkey COS-7 cells, mouse C127 cells, and cell lines carrying wild-type or mutant BPV genomes were grown in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% fetal bovine serum (J. R. Scientific), penicillin, and streptomycin. Culture of *Spodoptera fru-*

giperda Sf9 cells and infection of these cells by recombinant baculoviruses were done as described elsewhere (24).

Electroporations and transient replication assays were performed as described previously (45) except that C127 cells were subject to a 250-V discharge and COS-7 cells were subjected to a 230-V discharge. C127 cells used in transient assays were plated onto eight 100-mm-diameter dishes, and time points were taken at 24-h intervals on days 2 to 6. A modified Hirt extraction protocol was used to isolate replicated BPV DNA (11). Briefly, each plate of cells was lysed by addition of 800 μl of Hirt buffer (10 mM Tris-HCl [pH 7.8], 10 mM EDTA, 0.6% sodium dodecyl sulfate [SDS]). Plates were rocked gently for several minutes and then tilted to drain lysate to one side of dish. Lysates were pipetted into a microcentrifuge tube, and 200 μl of 5 M NaCl was added. Tubes were mixed and incubated in ice for 30 to 60 min and then centrifuged for 30 min at 4°C. Supernatants were transferred to a 2-ml microcentrifuge tube and extracted with 800 μl of 50% phenol–50% chloroform. An 850-μl volume of the aqueous phase was transferred to a clean tube and extracted again with 800 μl of chloroform; 700 μl of aqueous layer was removed to a clean 1.5-ml tube, 40 μl of 5 M NaCl was added, and the sample was precipitated with 670 μl of isopropanol. Samples were stored at −20°C until all time points were collected. DNA was pelleted, washed with 70% ethanol, air dried, and reprecipitated from 300 μl of 0.3 M sodium acetate with 750 μl of ethanol. After pelleting and air drying, samples were treated and digested as described prior to separation on agarose gels (45). Southern blot analysis was performed according to established protocols (2, 32).

To determine relative copy number for the various mutants in transformed cells, pools of colonies were used. BPV-transformed foci were picked at 2 to 3 weeks after electroporation, and 10 to 12 foci from one plate were pooled. Pooled foci were expanded, and the population was used to isolate high-molecular-weight cellular DNA. Equal cellular DNA from the pools was analyzed by Southern blot analysis and probed with nick-translated BPV DNA.

Fluorescent staining of E1 protein. COS-7 cells transfected with pMTE1 DNA by the method of electroporation were grown on coverslips. At 48 h after electroporation, cells were washed with phosphate-buffered saline (PBS), fixed with 50% methanol–50% acetone (vol/vol) for 5 min at room temperature and washed again with PBS. Fixed cells were blocked by the addition of 3% (wt/vol) bovine serum albumin (BSA) in PBS for 30 min at room temperature. Anti-M polyclonal antiserum (directed against the amino-terminal portion of E1 [44]) was diluted 1:500 in PBS containing 0.1% BSA and 0.1% Triton X-100 (PBST/BSA), added to the plates and incubated at room temperature for 45 to 60 min in a humidified chamber. The cells were then washed four times for 5 min with PBST/BSA. Goat anti-rabbit polyclonal antiserum conjugated to fluorescein isothiocyanate was diluted 1:50 in PBST/BSA and added to cells for 30 min at room temperature in a humidifying chamber, and then the cells were washed as described above. Coverslips were rinsed briefly in water and mounted on microscope slides with glycerol containing 50 mM Tris (pH 7.8) and 2.5% DABCO (10).

In vivo labeling of E1 and phosphoamino acid analysis. Recombinant baculoviruses expressing wild-type or mutant E1 protein were used to infect Sf9 cells on 100-mm-diameter dishes. At 48 h postinfection, cells were washed with phosphate-free Grace's medium containing 10% dialyzed fetal bovine serum. Cells were starved for 2 h in the same medium; 2 ml of this medium supplemented with 2 mCi of $^{32}PO_4$ was added to each plate, and the plates were incubated for 3 h. Extracts of cells were prepared following lysis in radioimmunoprecipitation assay buffer as described previously (44).

E1 proteins were isolated from extracts by immunoprecipitation with anti-M antisera as previously described (44). Immunoprecipitates were electrophoresed on SDS–10% preparative polyacrylamide gels, and resolved proteins were transferred in a semidry Western immunoblot apparatus (Bio-Rad) to polyvinylidene difluoride membranes according to protocols supplied with the apparatus. E1 protein was visualized by autoradiography, and the bands containing E1 proteins were excised from the membrane. Hydrolysis of labeled protein to amino acids was done directly on the membrane, using 6 N HCl at 100°C for 2 h. Labeled amino acids were dried and separated as described previously (44).

In vitro labeling of E1 protein and synthetic peptides by $p34^{cdc2}$ protein. Synthetic peptides corresponding to E1 amino acids 90 to 112 were synthesized on an Applied Biosystems 431A synthesizer. Peptides corresponded to the wild-type sequence or contained a mutation of serine 100 to alanine or threonine 102 to alanine or both. Peptides were synthesized by using solid-phase methodology (benzotriazolyltetramethyluronium hexafluorophosphate-activated 9-fluorenylmethoxycarbonyl-amino acids), employing user-devised cycles. Cleavage and protection were accomplished with reagent K (14), and the crude peptides were purified to >95% by reverse-phase high-pressure liquid chromatography with a gradient of acetonitrile in water (both containing 0.1% trifluoroacetic acid). Structure and purity were confirmed in all cases by electrospray ionization-mass spectrometry (VG Bio Q); 30 μg of peptide was used in a single in vitro labeling reaction.

$p34^{cdc2}$ protein was prepared from M-phase HeLa cells and supplied by D. Beach (Cold Spring Harbor Laboratory). The $p34^{cdc2}$ kinase from *Xenopus* eggs was also used and was obtained from J. Newport (University of California, San Diego). The E1 protein from baculovirus-infected Sf9 cells was purified as described by Yang et al. (48). The same epitope-tagged E1 open reading frame was also used to purify E1 from *Escherichia coli* cells harboring a plasmid with an inducible T7 phage expression vector. Kinase reactions were carried out in buffer containing 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 83 mM NaCl, 1 mM rATP, and 50 mCi of [γ-^{32}P]ATP for 30 min at 30°C. Reaction mixtures were loaded directly onto SDS-polyacrylamide gels (20% for peptides and 10% for E1); 20% gels were exposed directly to X-ray film, and others were transferred to polyvinylidene difluoride or nitrocellulose membranes in a semidry Western blot apparatus prior to exposure.

RESULTS

Within the E1 coding sequence, there are only three regions bearing three or more consecutive basic amino acids. All are located within the first 114 amino acids (Fig. 2, regions A, B, and C). The N-terminal-most sequence (A) is highly conserved among all known papillomavirus E1 sequences, while the second sequence (B) is the most conserved between BPV E1 and the SV40 large TAg nuclear localization signal. Region B is not as well conserved among the papillomaviruses. Adjacent to this basic amino acid group is a putative $p34^{cdc2}$ phosphorylation site, also conserved with SV40 large TAg. A third region (C) lies just downstream of region B and is also highly conserved among the known papillomavirus sequences.

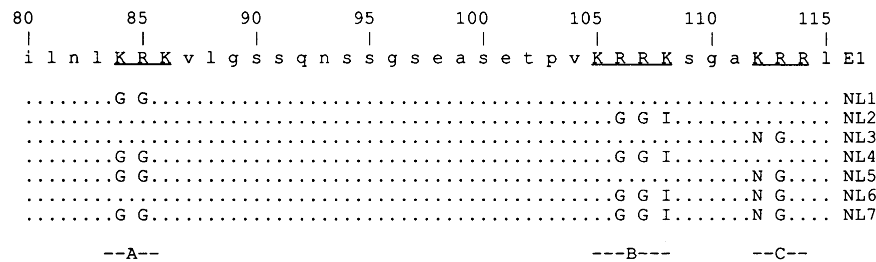

FIG. 2. Amino acid sequences of the BPV E1 protein from residues 80 to 115 (top line; see Fig. 1) and of constructs containing mutations in the region of the putative BPV-1 E1 nuclear localization signal. Regions A, B, and C are indicated and underlined in the wild-type E1 sequence. Only amino acid substitutions are shown for the mutants.

The amino acid sequence in this region of BPV E1 is compared with other papillomavirus E1 sequences and the sequence of SV40 large TAg in Fig. 1. Functionally important domains in the SV40 large TAg sequence are identified, including the nuclear localization signal and several phosphorylation sites, among them the site for $p34^{cdc2}$ phosphorylation, which is required for activation of DNA replication. A set of constructs was prepared in which all three of the basic regions in E1 (A, B, and C) were mutated, leaving a single charged amino acid at each site (Fig. 2). Mutations were made in single sites, in all combinations of pairs of sites, and in all three sites simultaneously. All mutants were cloned back into the BPV genome for analysis of replication activity or into a heterologous E1 expression system by using the adenovirus major late promoter. This latter vector was necessary for visualization of the cellular location of the wild-type and mutant proteins, since they are not synthesized in sufficient quantities in the context of the BPV genome to be detected by immunofluorescence.

Cellular compartmentalization. The subcellular location of wild-type and mutant E1 proteins was determined by indirect immunofluorescence analysis of cells expressing these proteins under the control of the heterologous promoter. Representative pictures of the fluorescent patterns detected with the various mutant and wild-type genes are shown in Fig. 3, and a statistical analysis of the cellular location of each mutant protein is presented in Table 1. In all cases, equivalent amounts of E1 protein were synthesized, as measured by immunofluorescence (data not shown), although cellular compartmentalization was clearly affected by the mutations. In all cells expressing wild-type E1 protein, the protein was located exclusively in the cell nucleus. The same result was true for cells expressing the NL3 mutant (region C). In contrast, all other E1 mutants were defective to some extent in the ability to express protein which localized to the nucleus. Mutant NL1, which is in region A, was the most severely affected of the single mutants, with the majority of the E1 expressing cells staining only in the cytoplasm. NL2, which is in region B, was also defective but not as severely as NL1. Fifty-seven percent of the cells had strong cytoplasmic stain, but many (56%) also had noticeable nuclear stain as well. As expected, all mutants which combine the defects of NL1 and NL2 were at least defective as the NL1 mutant itself. Interestingly, mutant NL6, which combines the amino acid substitutions of mutants NL2 and NL3 (regions B and C), was noticeably more defective than mutant NL2 by itself, although the NL3 mutation alone appeared to have no effect.

The results from the expression of mutant E1 proteins in COS-7 cells demonstrate that basic region A is required for nuclear localization. Any mutant in which the basic character is removed at this position is severely defective for nuclear transport. Basic region B plays an important role as well, however, since the region A basic peptide cluster cannot completely compensate for mutations in region B. Furthermore, when the mutations in regions A and B were combined in a double mutant, they were additive in their defects. Therefore, it appears that the nuclear localization signal for BPV E1 is bipartite, encompassing two nearby regions of basic amino acids. How these regions are related to each other in the three-dimensional structure of the protein would be of interest. It is conceivable that in the properly folded protein, these regions form a single, larger area of charged character. Bipartite nuclear localization signals have been identified for several proteins, including nucleoplasmin and the large TAg of polyomavirus (8, 28, 31).

The nuclear localization mutants affect BPV replication in vivo. As BPV DNA replication occurs exclusively in the nucleus of infected cells, any mutation affecting the ability of the E1 protein to be transported to the nucleus would be expected to disturb its replication functions as well. A transient replication assay was used to determine the effects of the E1 mutations on the replication capacity of these proteins. In these experiments, the various mutations NL1 through NL7 were transferred into the intact BPV-1 DNA, and the mutant or wild-type DNA was transfected into the recipient C127 cells. This assay measures the accumulation of BPV DNA during the initial amplification phase of the viral infection, when the copy number per cell is increasing from the input amount to the stable, latently transformed level. In the assays, the circular plasmid DNAs are linearized to facilitate quantitation. Replicating DNA is observed as an increase in the intensity of *Dpn*I-resistant linear DNA over a time course extended for 6 days.

The results of transient replication assays with the nuclear localization mutants are shown in Fig. 4. The replication data correlate well with the ability of the mutant E1 protein to be transported into the cell nucleus. The level of replication varies up to 10-fold, depending on the severity of the nuclear accumulation defect. The most defective replication mutants are those in which region A has been disturbed, namely, NL1, NL4, NL5, and NL7 (mutations in regions A, A and B, A and C, and A, B, and C, respectively). Region B mutants are also significantly reduced in replication efficiency. These mutants include NL2 (region B alone) and NL6 (region B and C) in addition to the combinations including mutations in region A mentioned above. NL3, which has a mutation confined to region C, is the least defective for replication and nuclear accumulation. In several independent experiments, this mutant replicates consistently at a level near 80% of the wild-type level, as judged

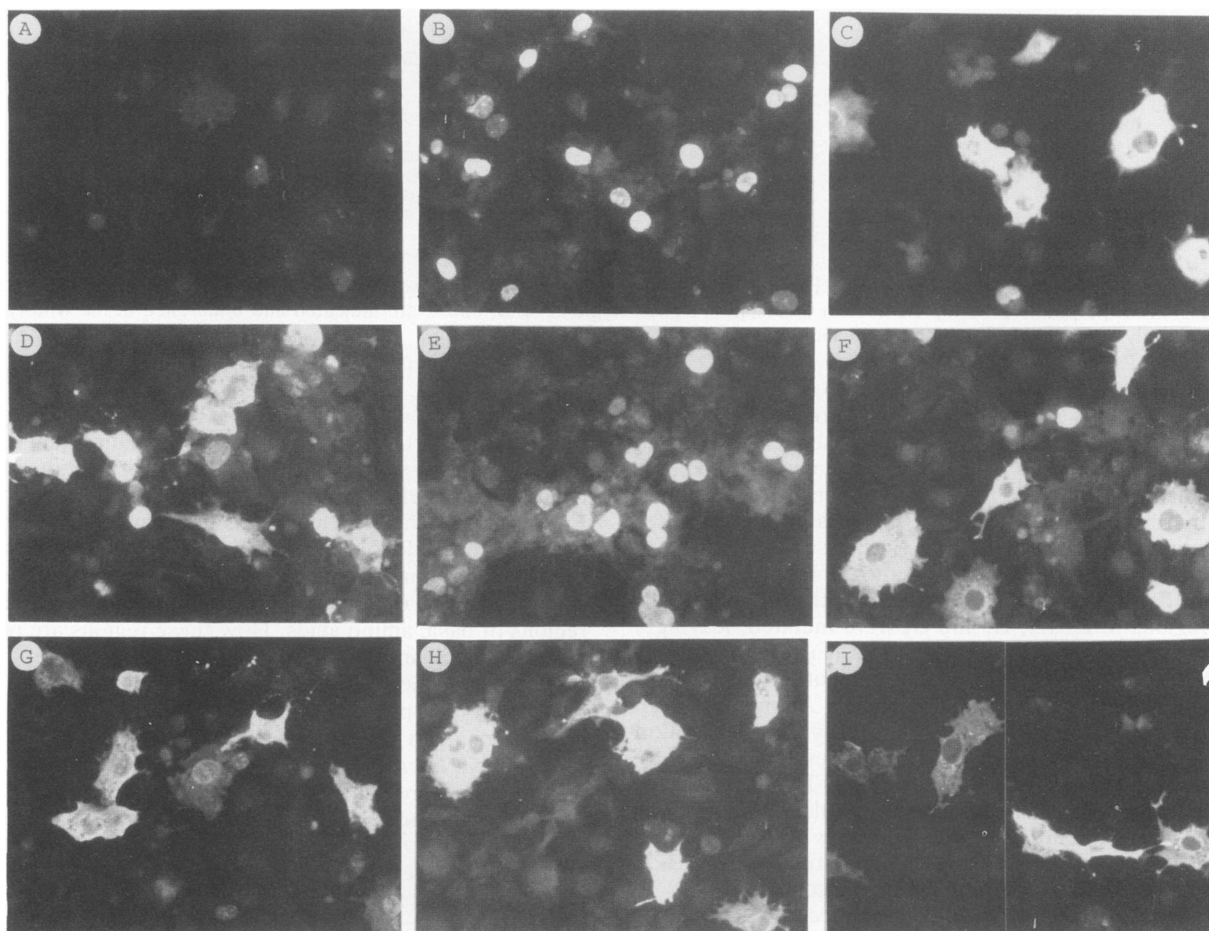

FIG. 3. Immunofluorescent staining of cells expressing wild-type and mutant E1 proteins. E1 proteins were expressed from an adenovirus late promoter vector in COS-7 cells, and the protein distribution was measured as detailed in Materials and Methods. (A) Mock-treated cells; (B to I) cells expressing wild-type (B), NL1 (C), NL2 (D), NL3 (E), NL4 (F), NL5 (G), NL6 (H), and NL7 (I) E1 proteins.

from the relative signal in the BPV band on day 6 of a transient replication assay.

The mutant BPV genomes were assayed for the ability to transform mouse C127 cells. In several independent experiments, we found no difference in transformation efficiencies between any of the mutants and the wild type. Stable plasmid copy number was measured in pools of colonies to determine the relative copy number of BPV genomes harbored by the wild-type- and E1 mutant-containing cells (Fig. 5). Interestingly, despite equivalent transformation efficiencies, marked differences in plasmid copy number were observed. Mutations that affect either region A or region B or both result in a reduced copy number of viral genomes. This result further supports the identification of regions A and B as critical for E1 function and is consistent with the notion that E1 concentration in the nucleus regulates copy number.

TABLE 1. Cellular distribution of E1 proteins[a]

Protein	% Nuclear	% Cytoplasmic	% Nuclear and cytoplasmic	n
Wild type	100.0	0.0	0.0	325
NL1	4.1	71.1	24.8	121
NL2	23.0	24.0	53.0	100
NL3	99.1	0.0	0.9	348
NL4	1.7	96.1	2.2	181
NL5	14.5	49.3	36.2	138
NL6	3.6	87.9	8.5	165
NL7	1.3	93.7	5.1	158

[a] COS-7 cells expressing wild-type or mutant E1 protein from the adenovirus late promoter were stained with anti-E1 antibodies and fluorescently labeled. Fields of cells were photographed, and all cells expressing E1 were counted and placed into categories based on intracellular location of E1 protein.

In vivo phosphorylation of E1 at threonine 102. Threonine 102 was mutated to isoleucine as described in Materials and Methods, and this mutation was transferred to an infectious baculovirus E1 expression vector, an *E. coli* E1 expression cassette, and the intact BPV-1 genome. Phosphoamino acid analysis was performed on wild-type and mutant E1 proteins labeled in baculovirus-infected cells (Fig. 6). The wild-type E1 protein has no detectable phosphotyrosine and only barely detectable phosphothreonine. The remaining phosphate is on serine and probably represents phosphorylation of several serine residues on the E1 protein. The threonine 102-to-isoleucine mutant, however, had no detectable phosphothreonine. This finding indicates that the weak phosphothreonine signal observed in the wild-type protein is due to phosphorylation at threonine 102 within infected insect cells.

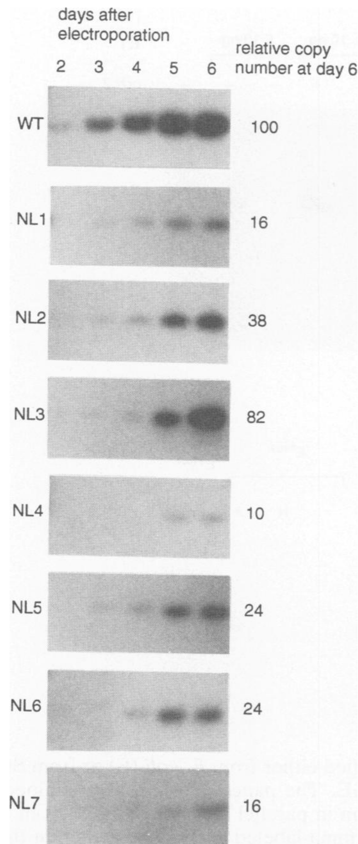

FIG. 4. Transient replication of wild-type BPV and BPV genomes containing mutations in the putative nuclear localization signal. Time points were taken at days 2 through 6 as indicated. Samples were treated with *Dpn*I and *Xba*I, separated on agarose gels, and blotted to nitrocellulose membranes. Blots were probed with nick-translated BPV plasmid DNA. Relative copy number was determined by quantitation of the day 6 band from each sample on a Betagen phosphorimager. The wild type (WT) was assigned a value of 100.

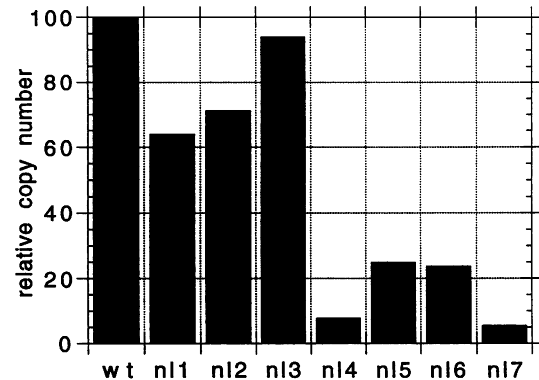

FIG. 5. Stable replication assay. Total cellular DNA was isolated from pools of colonies transformed by wild-type (wt) or E1 mutant BPV DNA. Relative copy number was determined by quantitating a Southern blot of total cell DNA probed with a BPV-specific probe on a Betagen phosphorimager.

In vitro phosphorylation of E1 by p34^{cdc2}. The in vivo labeling results implicate threonine 102 as a substrate for phosphorylation in the E1 protein. It is likely, given the sequence conservation of this site in E1 and known substrates for the p34^{cdc2} kinase, including SV40 large TAg, that the p34^{cdc2} kinase is one kinase responsible for phosphorylating E1 at threonine 102. To test this hypothesis directly, wild-type and mutant E1 proteins were labeled in vitro, using p34^{cdc2} kinase prepared from M-phase HeLa cells or *Xenopus* oocytes (Fig. 7). A picture of the silver-stained gel (Fig. 7A) indicated the mass of E1 prepared either from bacterial cells or from infected insect cells. These proteins were the substrates for the kinase labeling reactions shown in Fig. 7B. After a 30-min incubation period, plus or minus the cdc2 kinase, the total reaction was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The results show that the baculovirus-produced E1 protein is associated with a kinase, as ^{32}P incorporation is detected at a low level even without incubation with the p34^{cdc2} protein. We believe that this activity is not intrinsic to E1, as it does not track with the bulk of the protein in a sedimentation glycerol gradient profile, and its activity varies from preparation to preparation (data not shown). Furthermore, the *E. coli*-produced E1 protein does not show this activity (Fig. 7B and D). Nevertheless, this activity is tightly associated with E1, and it can be assayed in extensively washed immunoprecipitates of the E1 protein. It is not likely to be the cdc2 kinase, as the endogenous activity phosphorylates E1 predominantly on serine residues (data not shown), while the cdc2 kinase phosphorylates E1 on threonine residues. As is shown in Fig. 7B, the cdc2 kinase phosphorylates both the *E. coli* E1 and the baculovirus E1. Although in this latter case it cannot be firmly ruled out that the E1-associated kinase is activated by the cdc2 enzyme, we feel that this is unlikely, in that the phosphoamino acid composition of the cdc2-treated baculovirus E1 is heavily skewed toward the threonine residue and the *E. coli* E1 treated with the cdc2 kinase shows only threonine as a radioactive residue after analysis (Fig. 7C). Figure 7D shows that both the human and *Xenopus* p34^{cdc2} kinases can phosphorylate *E. coli*-produced E1 and that the mutant E1 with threonine 102 converted to isoleucine dramatically loses its ability to serve as a substrate. In these reactions, 30 ng of E1 was used; when mutant and wild-type proteins were mixed in equal proportions, no inhibition of the kinase, acting presumably on the wild-type protein, was detected. It is interesting to note that the E2 protein in the isolated E1-E2 complex is also phosphorylated in the pres-

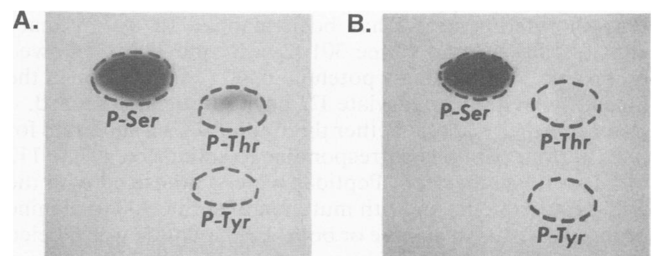

FIG. 6. Phosphoamino acid analysis of E1 proteins. E1 proteins were expressed from recombinant baculovirus expression vector in infected insect cells. Proteins were labeled in vivo, and E1 was purified by immunoprecipitation. E1 was hydrolyzed to amino acids that were then separated by two-dimensional electrophoresis on thin-layer plates. The positions of phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) standards are indicated by the dashed ovals. (A) Wild-type E1; (B) El with a threonine 102-to-isoleucine mutation.

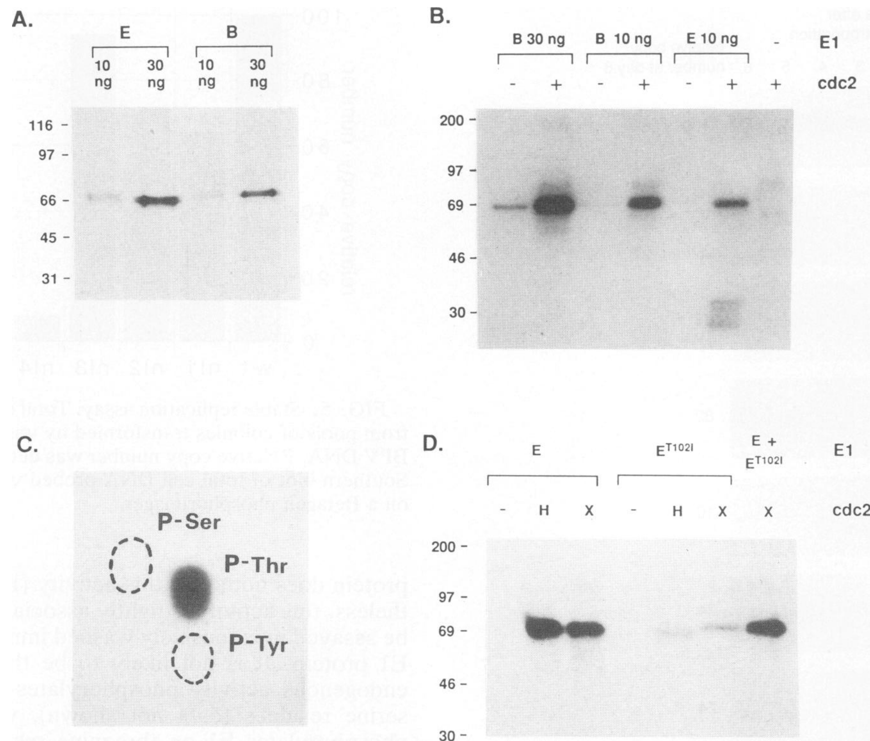

FIG. 7. Phosphorylation of E1 in vitro by the p34^{cdc2} kinase. (A) E1 protein was purified either from *E. coli* (E) or from Sf9 cells infected with a baculovirus vector (B); 10 or 30 ng of each protein was analyzed by SDS-PAGE. The panel shows a silver-stained picture of the detected protein. In panels A, B, and D, positions of the molecular weight standards run in parallel are listed at the left in kilodaltons. (B) The purified E1 proteins from either source were treated with the p34^{cdc2} kinase and gamma-labeled ATP as described in the text and then analyzed by electrophoresis. The autoradiogram shows the action of the kinase. (C) The E1 protein purified from *E. coli* was treated with the p34^{cdc2} kinase, and the labeled material was excised from a gel similar to the one shown in panel B. After elution, the phosphoamino acid composition of the material was determined. P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine. (D) Purified mutant (E^{T102I}) and wild-type (E) E1 proteins (30 ng), both from *E. coli*, were treated with p34^{cdc2} kinase. The kinase from either human cells (H) or *Xenopus laevis* (X) was used. In the far right lane, mutant and wild-type E1 (15 ng of each) were mixed, treated with kinase, and analyzed.

ence of the p34^{cdc2} kinase. E2 has three serine-proline dipeptides and one threonine-proline peptide. In contrast, purified E2 labels poorly (if at all) with the p34^{cdc2} kinase (data not shown). These results are complicated at present by the possibility that the E1-associated kinase may have altered activity in the presence of p34^{cdc2} kinase, for which E2 is now a substrate. Another, more intriguing possibility is that the E1-E2 complex alters the conformation of E2, exposing these appropriate residues for phosphorylation. Phosphorylation of E2 has been mapped to two primary sites, serine 298 and serine 301 (20). Serine 298 is followed by proline, making this a potential p34^{cdc2} site, although the kinases which phosphorylate E2 have not been analyzed.

As a further test of whether threonine 102 is a substrate for p34^{cdc2}, four peptides corresponding to amino acids 90 to 112 of E1 were synthesized. Peptides were synthesized with the wild-type sequence or with mutation of serine 100 to alanine or threonine 102 to alanine or both. Each peptide was labeled in vitro with the p34^{cdc2} kinase under the same conditions used for phosphorylation of the purified E1 protein and then run on a 20% acrylamide gel (Fig. 8). Only the peptides with threonine at amino acid 102 can be labeled with this kinase. This finding substantiates the in vivo and in vitro result obtained for the full-length protein, that threonine 102 is a substrate for the p34^{cdc2} kinase enzyme. The in vitro data are quite clear and show that E1 can be a substrate for p34^{cdc2}. We have not, however, proven that the in vivo kinase in Sf9 cells is truly p34^{cdc2}. Even though threonine 102 lies within a consensus site for this kinase and its mutation results in a loss of phosphothreonine in the protein, it is possible that mutation at this site results in a loss of a phosphorylation elsewhere. Alternatively, some other kinase may be phosphorylating threonine 102 in vivo. The rate or extent of phosphorylation of threonine 102 in vertebrate cells will need to be directly studied to fully assess the potential role of this modification on papillomavirus DNA replication.

The threonine 102-to-isoleucine mutation was subcloned into the full-length viral genome to determine the effect of the mutation on viral replication. Several transient replication assays using this DNA were performed. The results consistently showed that the mutant DNA could replicate as well as the wild type could (Fig. 9). In several stable replication assays, mutant viral DNA copy number was equivalent to wild-type DNA copy number in the transformed cells (data not shown). These results indicate that phosphorylation of E1 on this residue is not critical for plasmid replication.

DISCUSSION

Nuclear localization. We have identified the signal for transport of the BPV E1 protein to the nucleus of infected cells. The signal is more complex than expected from the sequence conservation to SV40 large TAg and consists of

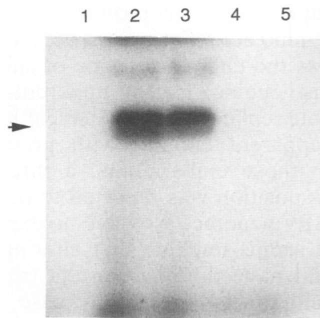

FIG. 8. Phosphorylation of synthetic E1 peptides by $p34^{cdc2}$ kinase. In vitro-labeled peptides were separated on a 20% polyacrylamide gel and autoradiographed. Lanes: 1, no peptide; 2, peptide SSQNSSGSEASETPVKRRKSGAK (wild-type E1 sequence from amino acids 90 to 112); 3, peptide SSQNSSGSE AAETPVKRRKSGAK; 4, peptide SSQNSSGSEASEAPVKRRK SGAK; 5, peptide SSQNSSGSEAAEAPVKRRKSGAK. Alanine substitutions are underlined. The arrow indicates the position of the labeled peptides.

two regions of basic charge in the amino-terminal portion of the protein. Region A is the dominant signal. Mutations in this region reduce nuclear accumulation of E1 protein dramatically, and transient replication is reduced to less than one-sixth of the wild-type level. Region B also has an effect on nuclear localization of E1, as the majority of the protein synthesized from a heterologous vector is localized in the cytoplasm of the cell. This mutation has less of an effect on BPV DNA replication, however, suggesting that enough E1 protein is accumulating in the nucleus to allow significant replication to occur. Mutations in region C had no observable effect on nuclear accumulation and minimal effect on DNA replication. This is an interesting result, as this short region is extremely well conserved among all known papillomavirus E1 protein sequences. It is possible that this region has some important role in another function of E1 for the virus. Double mutations in regions A and B were additive, with nuclear localization detected in only a rare nucleus and transient replication reduced by 10-fold. From our mutational analysis, we cannot conclude that sequences between or outside of regions A and B are irrelevant to the nuclear localization function. Four other E1 mutations were created in flanking regions and expressed transiently from the adenovirus vector. These mutants were S100A (E1 amino acid 100 serine codon converted to an alanine), E101A, T102I, and S109L. All of these mutants behaved like wild-type E1 with respect to nuclear localization and were thus not characterized further (data not shown). Given what is known for other nuclear localization signals, we would speculate that regions A and B define a bipartite nuclear transport signal.

In general, bipartite nuclear localization signals contain two short stretches of basic residues (2–4) separated by a space sequence of variable length (6). Very little is conserved with respect to structure or distance between the two basic clusters, and indeed insertions and deletions can be tolerated. Dingwall and Laskey (7) have recently proposed that the two domains make contact sequentially with HSC-70 and then the nuclear pore receptor at the positive domain. According to this model, the space between the basic residues is denatured for transport, and the distinction between single domain nuclear localization signals (e.g., SV40) and extended (or bipartite) signals would be the number of basic residues at the protein-protein contact points. For simple signals, one large patch of basic residues is sufficient for contact with HSC-70 and the nuclear pore receptor; for bipartite signals, both domains are required for full function.

The majority of nuclear proteins whose signals have been analyzed contain only a single target sequence. A number of other proteins with multiple signals have been identified, however, with polyomavirus large TAg and nucleoplasmin being the best-characterized examples (28, 31). It is generally believed that the activity of dual signals is additive; that is, the activity of both signals together is better than that of either one alone. This appears to be the case for the bipartite E1 signal, although the A-region signal is clearly stronger than the B-region signal. Several explanations for the existence of more than one signal which diverge from the one summarized above have been proposed. Different signals may be involved in different steps in the nuclear transport process, as has been proposed for the dual signals of the yeast α2 protein, for which mutations in each signal appear to have different effects (9). For most bipartite signal proteins such as E1, mutation of one signal impairs nuclear transport but does not eliminate it. If each signal were separately involved in a distinct step of transport, one would predict that a defect in one signal would likely render the protein completely defective for nuclear accumulation. This does not generally appear to be the case for dual-signal proteins. Alternatively, nuclear localization signal constraints may have evolved to be more relaxed, since the signal must function in the context of the active protein. This is in contrast to signals which mediate transport into the endoplasmic reticulum, for example, which are cleaved from the mature protein following transport (47). This requirement for some flexibility in sequence would be compatible with the model proposed by Dingwall and Laskey (7).

It has been suggested that phosphorylation may play a role in the transport of SV40 TAg into the nucleus (16, 12, 35). Rihs and Peters (30) have reported that while the TAg signal at residues 126 or 127 to 132 was sufficient for transport of β-galactosidase to the nucleus, the kinetics of transport were greatly enhanced by inclusion of residues 111 to 125. They

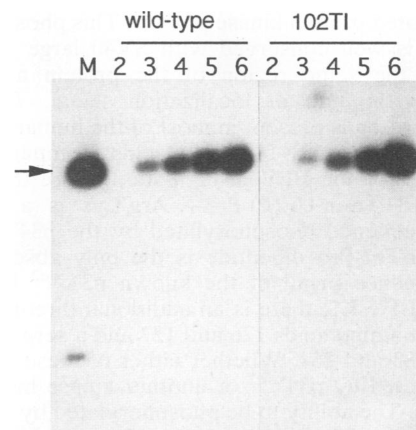

FIG. 9. Transient replication of wild-type E1 and E1 with a threonine-to-isoleucine mutation at amino acid 102. Time points were taken on days 2 through 6 following electroporation of DNA into C127 cells. Samples were digested with *Dpn*I and *Xba*I, separated on agarose gels, and transferred to nitrocellulose. Blots were probed with nick-translated BPV plasmid DNA.

have demonstrated that the enhanced kinetics of transport is a direct result of phosphorylation by casein kinase II at serine 111 or 112 (29). In E1, a number of potentially phosphorylated serines, including a casein kinase II consensus site, reside in a position analogous to this transport-enhancing region of SV40 TAg. However, there was no detectable effect on nuclear localization when the $p34^{cdc2}$ site was mutated in the E1 protein (data not shown).

Perhaps the most curious result reported here that requires further study concerns the phenotype of the nuclear localization mutants in transformation. The nuclear localization mutants in both domains A and B are reduced in transient replication assays by at least 10-fold, and we would have expected that these effects would be amplified in stable assays wherein the reduction in replication would be cumulative as the cells continued to be passasged. This did not occur; instead, the populations seemed to carry the genomes at a stable yet reduced copy number. Perhaps this finding implies that a constant amount of E1 protein enters the nucleus at each division for each mutant and that this amount is sufficient for that copy number. Indeed, the nuclear localization assays, performed in COS-7 cells, show that nuclear compartmentalization is very dramatically reduced for these proteins. Conceivably, some of the protein joins up with the viral DNA upon nuclear membrane breakdown in the stable assays; alternatively, very little E1 may be required for some replication. Equally puzzling is the finding that these mutants, which establish at a very low copy number (10% of the wild-type level), oncogenically transform as does the wild type. One would have expected that at such a low copy number, not enough transcription of the viral oncogenes would be possible. A simple prediction would be that the transforming DNA integrates where competition between replication and transcription complexes are not a significant factor. Perhaps, with less E1 in the nucleus, transcription per genome is higher, thus compensating for the low copy number. Given previous reports that E1 may have such a repressive effect upon transcription (15, 36) and recent direct demonstration of such effects (18, 40), this seems a strong possibility. It will then be interesting to measure the transcriptional effects of such mutants in transformed cells.

Phosphorylation of E1. We have determined that threonine 102 of the E1 protein can be phosphorylated by the cell cycle-regulated protein kinase $p34^{cdc2}$. This phosphorylation site in E1 is well conserved with SV40 large TAg, being located in the same region of the protein and directly adjacent to the nuclear localization signal. The peptide Ser-Pro-Arg-Leu is present in most of the human papillomaviruses and is located 16 amino acids, compared with 10 amino acids for the BPV $p34^{cdc2}$ site, before the region C basic peptide. Ser/Thr(P)-Pro-X-Arg/Lys is a commonly observed sequence phosphorylated by the $p34^{cdc2}$ kinase, but the Ser/Thr-Pro dipeptide is the only absolutely conserved sequence in all of the known $p34^{cdc2}$ kinase substrates. In BPV E1, there is an additional threonine-proline dipeptide at amino acids 126 and 127 and a serine-proline at dipeptide 283 and 284. Whether either of these two sites is phosphorylated by $p34^{cdc2}$ or another kinase has yet to be determined. The ability to be phosphorylated by $p34^{cdc2}$ will likely have to be determined for the E1 protein of different papillomaviruses empirically.

The role, if any, of the $p34^{cdc2}$ phosphorylation in the function of the BPV E1 protein remains to be determined. Moreover, it has not been shown to be phosphorylated in stably infected mammalian cells. This analysis is particularly difficult, as little E1 protein is produced. Mutation of threonine 102 to an amino acid that cannot be phosphorylated and does not possess the charge or shape of such a phosphorylated residue (isoleucine) had no apparent effect on either transient or stable replication or on nuclear localization. This finding is in apparent conflict with previously reported results (19). In those experiments, a threonine-to-alanine mutation at this position was reported to result in a replication-deficient BPV genome. We have no explanation for this discrepancy. It seems unlikely that altering a threonine to isoleucine would have different results from changing the same amino acid to alanine; in any case, neither mutant would be phosphorylated.

Recent studies on the phosphorylation pattern of SV40 TAg point to interaction between phosphorylation sites distantly removed from one another in the primary sequence (34). Phosphorylation of SV40 TAg is clustered in two sites in the amino-terminal and carboxy-terminal regions of the protein. These recent results suggest that the two sites may be in close proximity in the three-dimensional structure of the protein. E1 protein phosphorylation may also occur in clusters at the amino- and carboxy-terminal regions of the protein. A predicted site for phosphorylation by casein kinase II (Ser flanked by several acidic residues) exists at amino acid 584 as well as Ser-48 of BPV E1, and similar sites are conserved in the same region of other papillomaviruses. We are presently mapping the serine phosphate sites of the E1 protein in order to determine a more complete role for phosphorylation in the activity of the E1 protein in BPV replication. It is possible that some of the potential phosphorylation sites on E1, such as the $p34^{cdc2}$ site discussed in this work, play a more significant role in productive vegetative viral replication than in plasmid replication. To test such ideas, a new assay system will be required.

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