Contribution of bovine papillomavirus type 1 E1 protein residue 48 to replication function

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The E1 protein of bovine papillomavirus type 1 (BPV-1) is the origin recognition protein and is essential for the initiation of viral DNA replication. We reported previously that there is a conserved motif between residues 25 and 60 of all papillomavirus E1 proteins that resembles a casein kinase II (CKII) phosphorylation site. The corresponding serine in BPV-1, serine-48, is an efficient substrate for CKII in vitro. To examine the functional role of this potential phosphorylation site, three amino acid substitutions were constructed at serine-48. Conversion of serine-48 to a glycine (S48G) resulted in a BPV-1 genome that was unable to replicate and had reduced transformation capacity. The S48G E1 protein also failed to support replication of a BPV-1 origin-containing plasmid when expressed from a heterologous vector rather than the viral genome, indicating a direct replication defect. In contrast, conversion of serine-48 to acidic residues (S48D or S48E), which mimic the charge and structure of phosphoserine, maintained the wild-type replication phenotype. These mutational results are consistent with a replication requirement for a negative charge at serine-48, presumably supplied by in vivo phosphorylation. The mechanistic basis for the negative charge requirement was examined by testing several activities of the S48G mutant E1 protein in vivo using yeast one- and two-hybrid systems. No gross defect was observed for stability, origin binding or interaction with E2 or for E1–E1 interaction, although subtle defects in these activities would not likely be detected. Overall, the results suggest that important phospho-regulatory control of E1 replication function is mediated through the N-terminal region of this protein.

Introduction

Papillomaviruses are the causative agent of warts in humans, other mammals and some avian species. Most human papillomaviruses (HPVs) cause benign cutaneous warts, although some ‘high-risk’ HPV variants are linked to the development of anogenital cancers (Syrjanen, 1989). Papillomaviruses display both species-specificity and tissue-tropism and infect and proliferate specifically in squamous epithelial cells of the host species. Following infection of basal epithelium, the DNA from the infecting virus undergoes a transient period of amplification during which the copy number typically is increased to several hundred copies per cell, after which the viral episomes are maintained at a relatively constant copy number (Belyavskyi et al., 1996; Berg et al., 1986; Bergman et al., 1988; Ravnan et al., 1992). Completion of the virus life-cycle depends on host cell differentiation, with vegetative virus DNA replication and virus particle production occurring only in terminally differentiated epithelial cells.

Bovine papillomavirus type 1 (BPV-1) serves as a prototype for the study of papillomavirus mechanisms of transcriptional regulation, transformation and DNA replication. BPV-1 is a member of a class of papillomaviruses that are able to induce proliferation in dermal fibroblasts as well as latent infection of basal epithelium and productive infection of differentiated epithelium. Wild-type BPV-1 replicates as an episome and is capable of inducing transformation when transfected into certain rodent cell types (Law et al., 1981). These characteristics of BPV-1 have been exploited for the identification of the viral...
gene products and mechanisms involved in the processes of BPV-1 DNA replication and transformation. While much information exists on the structure and function of the viral gene products, much less is known about host–virus interactions, how viral proteins are modified by host systems and how modification may regulate the activities of viral products.

The major viral DNA replication protein of BPV-1 is the 68 kDa E1 protein, an ATP-dependent helicase that specifically binds and unwinds the virus replication origin (See et al., 1993a; Wilson & Ludes-Meyers, 1991; Yang et al., 1993). The binding site for the E1 protein lies within the replication origin in the BPV-1 long control region and consists of an 18 bp imperfect inverted repeat centred around the HpaI site at nt 1 (Holt & Wilson, 1995; Holt et al., 1994; Ustav et al., 1991). Mapped functional domains of the E1 protein include a DNA-binding domain, an ATP-binding domain, a nuclear localization sequence and regions for interaction with the viral transcriptional transactivator protein E2 (Leng & Wilson, 1994; Lentz et al., 1993; MacPherson et al., 1994; Sarafi & McBride, 1995; Thorner et al., 1993). Both the E1 and full-length E2 proteins are required in vivo for replication of BPV-1 origin-containing DNA (Spalholz et al., 1993; Ustav & Stenlund, 1991). Complex formation of the E1 protein with the 48 kDa transcriptional transactivator product of the E2 ORF enhances E1 binding to the origin in the presence of binding sites for the E1 and E2 proteins (Blitz & Laimins, 1991; Mohr et al., 1990; Sedman & Stenlund, 1995; See et al., 1993b). Apart from the E1 and E2 proteins, the DNA replication machinery is supplied by the host cell. E1 interacts with cellular polymerase pol-α (Park et al., 1994) and replication factor A (Han et al., 1999) and may serve to recruit these and other cellular replication factors to the virus replication origin.

Recently, E1 has been shown to interact with and be phosphorylated by cyclin E/cyclin-dependent kinase (CDK) complexes, and this phosphorylation appears to be required for replication function (Cueille et al., 1998; Ma et al., 1999). Other phosphorylation sites have also been identified, although their functional significance remains less clear (Lentz et al., 1993; Santucci et al., 1990; Zanardi et al., 1997).

From the above reports, it is apparent that the E1 protein is multifunctional and has complex regulation mediated through both post-translational modification and protein–protein interactions. Mutational studies map most E1 functions to the central and C-terminal portions of the protein and it has been reported that deletion of the first 131 amino acids yields an E1 protein that retains replication capacity, though at a reduced level (Ferran & McBride, 1998). In order to evaluate possible functional contributions of the N-terminal region, we previously compared the available E1 sequences for conserved features within the first 100 amino acids (McShan & Wilson, 1997a). Our examination revealed an N-terminal motif consisting of a serine followed by a stretch of acidic residues that was consistently present between amino acids 25 and 60 in human and animal papillomavirus E1 proteins. This conserved motif resembled the recognition sequence for casein kinase II (CKII) and we showed that the corresponding serine in BPV-1 E1, serine-48, was an efficient substrate for CKII in vitro. In the current study, we performed a mutational analysis of this serine to evaluate its potential functional contribution to viral DNA replication. The mutational results are consistent with a requirement for a negative charge at this residue, such as would be supplied by phosphorylation, and support a positive phosphoregulatory role for this residue in activating E1 replication function.

Methods

- **Plasmid construction.** Plasmids were constructed according to standard cloning procedures and the identity of clones and mutants was confirmed by sequencing with the dsDNA cycle sequencing kit (Gibco-BRL). Site-directed mutagenesis was performed with either the USB T7 in vitro mutagenesis kit or the Stratagene QuickChange kit. Plasmid pdBPV.1 (ATCC #37134) was used as the parent for construction of E1 mutations in the context of BPV-1 genomic DNA. For heterologous expression of wild-type and mutant E1 proteins, the appropriate E1 fragments were cloned into the pCH110 vector (Pharmacia; SV40 early promoter) and the resulting constructs were designated pCH-E1.

- **Cell culture.** C127 cells used in focus formation and transient DNA replication assays were grown in an atmosphere of 5% CO₂ in Dulbecco’s modified Eagle’s medium (IRH BioSciences) supplemented with 10% foetal bovine serum (HyClone), 0.02% antibiotics and 25% fungizone (both from JRH BioSciences). Chinese hamster ovary (CHO) cells used in transient DNA replication assays were grown in HAM’S F12 medium supplemented as above and were also maintained in 5% CO₂.

- **Focus formation assay.** C127 cells were 80% confluent when harvested for electroporation. Electroporations for transformation assays were done with 50 ng or 1 µg supercoiled pdBPV.1 and pdBPV.1(S48G) DNA, harvested for electroporation. Following electroporation, the contents of each cuvette were resuspended in 10 ml DMEM growth medium with 5 mM BES and 1 ml of the final cell suspension was applied to each of three 100 mm dishes. Transfected cells were maintained for 21 days (50 ng electroporations) or 14 days (1 µg electroporations), at which time they were fixed with 70% isopropanol and stained with 1% methylene blue in 70% isopropanol. Foci were then counted.

- **Transient DNA replication assays.** Transient in vivo replication assays were performed as described previously (McShan & Wilson, 1997b). Wild-type and mutant pdBPV.1 DNAs were assayed in C127 cells and the BPV-1 sequences were separated from the vector by BamHI digestion prior to electroporation. Five µg wild-type or mutant BPV DNA was used per electroporation. The wild-type or mutant pCH-E1 expression vectors were tested for ability to support replication of an origin-positive plasmid, pBOR, in CHO cells. Electroporations were done with 5 µg supercoiled pBOR (Holt & Wilson, 1992). 5 µg wild-type or
mutant pCH-E1 and 3 μg of the E2 protein-expression vector pCEAG-E2 (provided by Arne Stenlund, Cold Spring Harbor Laboratory), with carrier DNA to a total DNA content of 50 μg.

Western blotting of pCH-E1 and pCH-E1(S48G) expressed proteins. Ten μg pCH-E1, pCH-E1(S48G) and control pCH110 was transfected individually into COS-1 cells by electroporation as described previously (Leng & Wilson, 1994). At 72 h post-transfection, cells were lysed on ice in culture dishes by using SDS gel sample buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 2% mercaptoethanol, 0.01% bromophenol blue). Fifteen μl of each sample was loaded on 10% SDS–polyacrylamide gels and electrophoresed (Laemmli, 1970). Proteins were transferred electrophoretically from the SDS gels to Hybond-ECL Western nitrocellulose membrane (Amersham) at 2.5 mA/cm² of gel in a Polyblot unit (American Bioinetics). Immunological detection of the expressed wild-type and S48G E1 proteins was performed by using E1-specific polyclonal antisera (5990 and 5997; Sandler et al., 1993), according to the manufacturer’s recommendations for the enhanced chemiluminescence detection procedure (Amersham). Western blots of bacterially expressed E1 protein were performed as described previously (Wilson & Ludes-Meyers, 1991).

Yeast one-hybrid assay. The one-hybrid system is as described previously and consisted of two reporter strains, p53BS-LACZ and E1BST-LACZ, containing minimal promoters with triplet copies of either the p53-binding site or E1-binding site, respectively (Gonzalez et al., 2000). Introduction of pGAD-E1(F), which expresses a GAL4 activation domain (AD)–E1 fusion protein, induces β-galactosidase in the E1BST-LACZ strain with minimal induction in the p53BS-LACZ strain. For the current study, the S48G and S48D mutations were introduced into pGAD-E1(F) by using the QuikChange kit (Stratagene) as above. Each mutant plasmid DNA was transformed into both the E1BST-LACZ and p53BS-LACZ strains and five independent clones were tested for β-galactosidase activity by a colony filter assay, as described previously (Gonzalez et al., 2000). All five clones for each mutant exhibited similar phenotypes and a representative clone for each was chosen for quantitative β-galactosidase measurement by liquid culture assay with CRPG (chlorophenol red β-d-galactopyranoside) as the substrate (Gonzalez et al., 2000).

Yeast two-hybrid system. The ability of wild-type and mutant E1 proteins to interact with themselves and with E2 protein was tested in vivo in yeast SFY526 cells. For the E1–E2 studies, the wild-type and mutant (S48G or S48D) E1 genes were cloned into pGBT9 to produce vectors (pGBT9E1) that expressed GAL4 DNA-binding domain (DBD)–E1 fusion proteins. Likewise, the full-length E2 gene was cloned into pGAD424 to generate a vector (pGAD2E1) that expressed a GAL4 AD–E2 fusion protein. Each purified pGBT9E1 DNA was co-transfected into SFY526 cells along with pGAD2E1 DNA and double transformants were selected on minimal medium lacking leucine and tryptophan (SD/–L/–T). Corresponding control clones were generated by co-transforming the wild-type and mutant pGBT9E1 DNA's with the parental pGAD424 vector that expresses only the unfused AD. Five independent clones were chosen from each transformation and fusion protein expression was verified by Western blotting. For each set of five clones, the β-galactosidase expression phenotypes were similar by the colony filter assay, so a representative clone was chosen for quantitative liquid culture β-galactosidase determination as above.

The E1–E1 interaction was examined in a similar way to the E1–E2 interaction. To complement the pGBT9E1 vectors (DBD–E1 fusions), wild-type and mutant E1 DNAs were cloned into pGAD424 to generate the corresponding AD–E1 fusion proteins. Homologous pairs of constructs (DBD–E1 plus AD–E1) were co-transfected into SFY526 cells and selected on SD/–L/–T medium and five clones for each pair were characterized as for the E1–E2 pairs.

Results

Conservation of a serine followed by acidic residues within the N terminus of E1

Several discrete functional domains of the BPV-1 E1 protein have been characterized and include a nuclear localization sequence at amino acids 84–112 (Leng & Wilson, 1994; Lentz et al., 1993), an ATP-binding site (MacPherson et al., 1994) and a DNA-binding domain located between amino acids 140 and 300 (Chen & Stenlund, 1996; Leng et al., 1997; Sarafi & McBride, 1995; Thorner et al., 1993). The DNA-binding domain region interacts with the viral E2 protein (Chen & Stenlund, 1996; Leng et al., 1997), as do sequences in the C-terminal region of E1 (X. Leng, K. Woytek and V. G. Wilson, unpublished results; Lusky & Fontane, 1991; Moscufo et al., 1999; Thorner et al., 1993). In addition, the central and C-terminal regions of E1 contain the ATnPase and helicase domains, although the boundaries are not well defined (MacPherson et al., 1994; Mansky et al., 1997; Sarafi & McBride, 1995; Yang et al., 1993). Interestingly, none of these mapped activities requires E1 sequences in the N-terminal 80 amino acids, and if this region is deleted entirely the protein still has some replication function (Ferran & McBride, 1998). In order to address possible roles for the N-terminal region, all available papillomavirus E1 protein predicted amino acid sequences were examined for conserved sequences. A motif found to be conserved between amino acids 25 and 60 of all E1 sequences was that of a serine followed by a stretch of acidic residues (Fig. 1) and we recently showed that this motif in BPV-1 E1 was an efficient CKII substrate (McShan & Wilson, 1997a). The high degree of conservation of this motif and its potential modification by a host kinase suggest a possible functional role for the N terminus that has not been defined previously. To test the functions of this conserved motif, serine-48 in BPV-1 E1 was mutated to glycine in the context of the viral genome and tested for its effect on the properties of BPV-1 in vivo. A serine-to-glycine conversion (S48G) rather than serine-to-alanine was chosen for this site because both serine and glycine are considered poor helix formers, while alanine is a good helix former. Secondary structure predictions of this region suggested that the CKII motif is in a random coil order to address possible roles for the N-terminal region, all available papillomavirus E1 protein predicted amino acid sequences were examined for conserved sequences. A motif found to be conserved between amino acids 25 and 60 of all E1 sequences was that of a serine followed by a stretch of acidic residues (Fig. 1) and we recently showed that this motif in BPV-1 E1 was an efficient CKII substrate (McShan & Wilson, 1997a). The high degree of conservation of this motif and its potential modification by a host kinase suggest a possible functional role for the N terminus that has not been defined previously. To test the functions of this conserved motif, serine-48 in BPV-1 E1 was mutated to glycine in the context of the viral genome and tested for its effect on the properties of BPV-1 in vivo. A serine-to-glycine conversion (S48G) rather than serine-to-alanine was chosen for this site because both serine and glycine are considered poor helix formers, while alanine is a good helix former. Secondary structure predictions of this region suggested that the CKII motif is in a random coil with an adjacent, downstream helical segment and that substitution with alanine might potentially extend the helical region causing a greater structural perturbation than that caused by substitution with glycine (not shown).

Focus-formation capacity of BPV-1 genomic DNA containing the S48G mutation in the E1 ORF

The ability to induce focus formation in cultured mouse cells is a well-characterized property of the wild-type BPV-1
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Fig. 1. Conservation of a motif consisting of a serine and an acidic residue-rich region in the N-terminal region of papillomavirus E1 proteins. Shown are the predicted amino acid sequences (one-letter code) for residues 1–60 of ten representative papillomaviruses. The group shown includes two members each of the papillomaviruses that cause fibropapillomas [BPV-1, deer papillomavirus (DPV)], cutaneous lesions (HPV-1a, HPV-7), genital lesions (HPV-16, HPV-18), oral lesions (HPV-13, HPV-32) and those associated with epidermodysplasia verruciformis (HPV-5, HPV-15). The motif is enclosed by rectangles. For a more extensive depiction of this motif among a larger collection of E1 sequences, see McShan & Wilson (1997).

Table 1. Focus formation in C127 cells by wild-type and mutant genomic BPV-1 DNAs

Each experiment consisted of three independent electroporations with 50 ng of each purified DNA. Each batch of electroporated cells was split into three plates for incubation. The numbers shown for each experiment are the mean numbers of foci on all nine plates. Experiments 1–3 were each performed with independent preparations of the two DNAs. The final column is the overall mean for experiments 1–3.

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>Genotype</th>
<th>Number of foci</th>
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<tr>
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<td>Expt 1</td>
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<tr>
<td>BPV.1</td>
<td>Wild-type E1 ORF</td>
<td>86</td>
</tr>
<tr>
<td>BPV.1(S48G)</td>
<td>S48G mutant E1 ORF</td>
<td>39</td>
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genome (Law et al., 1981). Previous studies have shown that many mutations in the E1 ORF can decrease the transformation efficiency of the BPV-1 genome as assayed by focus formation (Chiang et al., 1992a). The transformation capacity of wild-type and S48G mutant BPV-1 DNAs was tested in C127 cells by using three independent preparations of each DNA. The BPV-1 genome containing the S48G mutation in the E1 ORF was reduced by an average of 70% in its ability to induce focus formation in C127 cells in comparison with the wild-type (Table 1). The same reduction was also observed at high input DNA content (1 µg; data not shown). In addition, foci formed by cells transfected with the S48G BPV-1 genome were consistently smaller and slower to form than those formed by wild-type BPV-1. Since the transformation results reflect multiple electroporations using independent DNA preparations, the reduction in focus formation was not an artifact of DNA preparation or individual electroporation conditions.

One potential explanation for this transformation defect in BPV-1 S48G is an inability to replicate the viral genome, in which case the resultant transformants should contain only integrated viral genomes. Unfortunately, foci induced by the S48G mutant genome were resistant to passage and consistently died several days after the transfer of the disrupted focus to a slide well, in contrast with foci induced by the wild-type BPV-1 genome, which grew well and were readily passaged after disruption from the plate surface. This characteristic of the mutant genome prevented both establishment of an S48G BPV-1-transformed cell line and subsequent characterization of the integration state of the BPV-1 genome in these cells.

E1 protein with the S48G mutation fails to support transient DNA replication in vivo

Since the stable replication ability of S48G BPV-1 could not be assessed, the replication function was examined in transient assays. The ability to replicate transiently as an episome when transfected into C127 cells is a well-established property of the wild-type BPV-1 genome and requires a functional E1 protein (Spalholz et al., 1993; Ustav & Stenlund, 1991; Ustav et al., 1991). BPV-1 genomic DNA containing the S48G mutation was compared with the wild-type genome in a standard transient replication assay. The wild-type genomic BPV-1 DNA replicated as expected, producing a DpnI-resistant
Mutational analysis of BPV-1 E1 serine-48

Fig. 2. Typical transient replication assay with wild-type and S48G mutant BPV-1 genomic DNAs in C127 cells. Transfections and Southern analysis were done as described in Methods. Lanes 1 and 2 contain 20 and 200 pg HindIII-linearized input BPV-1 DNA. Lanes 3–7 show DpnI- and HindIII-digested Hirt lysis products for wild-type BPV-1 (lane WT) and BPV-1 (S48G) (Mutant lanes). Hirt lysates were prepared at 96 h post-electroporation. Replication assays with BPV-1 (S48G) (lanes 4–7) were performed using four independent preparations of S48G mutant BPV-1 input DNA (a–d). DpnI-sensitive digestion products of non-replicated input DNA are visible at bottom of autoradiograph.

Fig. 3. Stability and transient replication capacity of heterologously expressed wild-type and S48G E1 proteins. (A) Transient replication assays were performed as described in Methods using the BPV-1 origin-containing plasmid, pBOR (lanes 4–6), or control pUC18 DNA (lane 3). Replication samples (lanes 3–6) all received an E2-expression vector along with the vectors expressing either wild-type E1 (WT lanes) or the S48G mutant E1 (M lanes). The two lanes shown for the S48G mutant (lanes 5 and 6) represent separate electroporations. Samples were Hirt-extracted at 96 h post-electroporation and then all samples were digested with DpnI and HindIII prior to Southern blot analysis. Lanes 1 and 2 show 1 ng and 200 pg HindIII-linearized input pBOR DNA. (B) Comparison of wild-type and S48G E1 protein expression by Western blot. Plasmids pCH110, pCH-E1 and pCH-E1(S48G) were transfected into COS-1 cells. Cell lysates were harvested, electrophoresed and immunoblotted using N terminus-specific anti-E1 serum 5997 as described in Methods. Lanes 1–3 show the immunoblot of the pCH110 extract (C), the wild-type pCH-E1 extract (WT) and the mutant pCH-E1(S48G) extract. The position of immunoreactive E1 is indicated. Identical results were obtained with a C terminus-specific anti-E1 serum (not shown).

Acidic amino acid substitutions at serine-48 retain replication function

Our previous observation that serine-48 is phosphorylated by CKII in vitro (McShan & Wilson, 1997a) raises the possibility that this residue could be involved in the regulation of E1 replication function. Furthermore, the observation that serine-48 is not directly involved in the replication process suggests that its role may be indirect. To test this hypothesis, we subjected the S48G E1 protein to several functional assays and found that it retains replication function. This finding is consistent with the idea that serine-48 may be involved in the regulation of E1 replication function by indirect mechanisms. 

The ability of S48G BPV-1 to replicate in the transient assay confirmed that serine-48 is critical for E1 function; however, it did not distinguish between direct and indirect effects on replication. In addition to its role in replication initiation, the wild-type BPV-1 E1 protein has also been shown to influence transcription from E2-responsive promoters when expressed in the context of the viral genome (Le Moal et al., 1994; Sandler et al., 1993). Consequently, the observed replication defect of S48G BPV-1 could result from disruption of the normal transcriptional pattern of the genome rather than a specific loss of E1 replication activities. To eliminate transcriptional effects, the S48G E1 protein was expressed from a heterologous vector and was tested for the ability to support replication in a triple plasmid assay utilizing a BPV-1 origin-containing plasmid, pBOR, and an E2-expression vector (Holt & Wilson, 1995). The pBOR plasmid was replicated in the presence of the wild-type pCH-E1 expression vector (Fig. 3A, lane 4), but not in two independent experiments shown for the pCH-E1(S48G) expression vector (Fig. 3A, lanes 5 and 6). The parental vector for the origin construct, pUC18, did not replicate in the presence of wild-type E1 and the E2-expression vector (Fig. 3A, lane 3), demonstrating the origin specificity of this system. Western blotting confirmed that the S48G E1 protein was not inherently unstable (Fig. 3B) and suggested that there should be equivalent amounts of the wild-type and S48G proteins under replication conditions. Therefore, the observed replication defect in S48G E1 protein is likely due to disruption of some critical functional activity.

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possibility that the basis for the S48G replication defect is the inability of the glycine residue to be phosphorylated. To examine this possibility, pCH-E1 was mutated to convert the E1 serine-48 residue to either aspartic acid (S48D) or glutamic acid (S48E), since the structure and charge of these acidic residues resembles a constitutive phosphoserine. The pCH-E1(S48D) and (S48E) mutants were tested in the triple plasmid transient replication assay shown in Fig. 4. As before, the S48G mutant was completely defective and no replicated pBOR DNA was detected throughout the 6-day time-course of this experiment. In contrast, both the S48D and S48E mutants supported robust pBOR replication, with the kinetics and amount of replicated material at 144 h comparable to wild-type E1. These results indicate that a serine is not actually necessary at residue 48 and that a negative charge is likely to be the functionally significant requirement.

The S48G E1 protein is functional for several known E1 activities

In order to investigate the mechanistic basis for the defectiveness of the S48G mutant E1 protein, several known properties of E1 were examined. Origin-specific DNA binding is necessary for replication (Holt & Wilson, 1995; Sedman et al., 1997; Seo et al., 1993,a; Ustav et al., 1991) and, while the actual DNA-binding domain for E1 is located downstream from S48G (Chen & Stenlund, 1998; Leng et al., 1997; Sarafi & McBride, 1995; Thorner et al., 1993), it is possible that mutation of S48G adversely affects this critical function. To determine whether the functional and structural integrity of the mutant protein were intact for origin binding, McKay immunoprecipitation assays (Holt & Wilson, 1995) were performed using E. coli extracts expressing no E1 protein, wild-type E1 or the S48G mutant E1 protein. In repeated experiments, there was no quantitative difference in the binding abilities of the wild-type and S48G E1 proteins in vitro (data not shown).

Recently, we developed a yeast one-hybrid system for assaying binding of E1 to its recognition sequence in vivo (Gonzalez et al., 2000). In this system, wild-type E1 is expressed as a fusion with the GAL4 AD and is tested in reporter strains that have an integrated lacZ gene downstream of a minimal promoter containing triple copies of either the E1-binding site (E1BS) or the control p53-binding site (p53BS). The wild-type AD–E1 fusion protein produced substantially higher β-galactosidase expression in the E1BS reporter strain compared with the p53BS strain, while the AD protein alone could not activate either promoter (Fig. 5A; data not shown). These results confirmed that E1 could bind its recognition sequence specifically in vivo in the context of the yeast genome. To examine the binding properties of the serine-48 mutants in vivo, the S48G and S48D mutations were each engineered into the AD–E1-expression vector (pGBT9E1) and were tested for promoter activation (Fig. 5A). Both mutants activated the E1BS promoter specifically compared with the control p53BS promoter and the levels of β-galactosidase expression were comparable to those observed with the wild-type AD–E1 fusion. These combined in vitro and in vivo results demonstrate that the structural integrity of the DNA-binding domain was not disrupted significantly by the S48G substitution and, consequently, lack of origin recognition cannot account for the observed total replication defect. Furthermore, introduction of the constitutive negative charge at residue 48 did not increase the origin-binding capacity of E1, so it is unlikely that the phosphorylation of this residue is necessary to activate DNA binding by E1.

In addition to origin binding, E1 replicative function requires interaction with E2 protein (Chiang et al., 1992b; Sedman & Stenlund, 1995; Seo et al., 1993,b; Ustav & Stenlund, 1991) and self-oligomerization (Fouts et al., 1999; Sedman & Stenlund, 1996, 1998). The ability of wild-type and serine-48 mutants of E1 to bind E2 protein or themselves was examined in vitro by using a yeast two-hybrid system (Fig. 5B, C). In the E1–E2 interaction assay (Fig. 5B), wild-type E1 produced detectable β-galactosidase activity only when the E2 partner was present. A similar specific response to the E2 partner was observed for both the S48D and S48G mutants, and their β-galactosidase levels were actually twice that of the wild-type E1 protein. From these results, it is clear that the S48G mutant has no gross defect in E2 interaction that would account for its complete inability to support replication. We do not believe that the increased β-galactosidase activity of S48G compared with wild-type E1 is significant, since both the S48D and S48G mutants gave nearly identical responses and the S48D mutant was fully functional for replication.

In order to evaluate E1–E1 interaction, wild-type and mutant E1 proteins were tested as homologous pairs in the two-hybrid system (Fig. 5C). When wild-type E1 was expressed as a GAL4 DBD–E1 fusion in conjunction with the unfused AD, there was no detectable β-galactosidase expression, while the converse pair (AD–E1 plus unfused DBD)
gave a low but consistent background of β-galactosidase activity. Co-expressing the AD–E1 plus DBD–E1 fusions resulted in a 10-fold stimulation of β-galactosidase activity, consistent with an E1–E1 interaction in vivo. Both of the AD–E1 and DBD–E1 fusions have also been tested against other partners not known to bind E1, and no stimulation of β-galactosidase activity above background was observed (data not shown), confirming that E1 is not highly promiscuous for protein binding. We conclude from these studies that E1 exhibits intrinsic self-association in vivo that is consistent with the observed capacity of E1 to oligomerize in vitro (Fouts et al., 1999; Sedman & Stenlund, 1996, 1998). Like wild-type E1, both the S48D and S48G mutants exhibited in vivo self-association, although their fold stimulations over background were less than for wild-type (approximately 3- and 4-fold, respectively, compared with 10-fold for wild-type). Nonetheless, both mutants clearly retained self-interaction capacity and, again, the similarity between the S48D and S48G mutants suggests that any absolute difference from wild-type is not sufficient to explain replication differences.

Discussion

We noted previously that all available papillomavirus predicted E1 amino acid sequences contained a conserved motif located between amino acids 25 and 60 that consisted of a serine followed by a stretch of acidic residues, which resembles a CKII phosphorylation site (McShan & Wilson, 1997a). In BPV-1, the serine in this motif is residue 48 and this residue can be phosphorylated efficiently by CKII in vitro (McShan & Wilson, 1997a). This high degree of conservation of this motif and its potential phosphorylation by a host kinase led us to investigate the functional significance of the serine residue by mutational analysis. Conversion of BPV-1 E1 serine-48 to glycine resulted in a mutant E1 protein the stability of which in vivo was similar to the wild-type E1 protein, as shown by Western blotting. However, introduction of the S48G mutation to the E1 ORF in genomic BPV-1 rendered the genome both deficient for episomal DNA replication in C127 cells and reduced in transformation capacity. Furthermore, unlike wild-type E1 protein, the S48G E1 mutant produced from a heterologous expression vector was unable to support replication of a BPV-1 origin plasmid in the presence of E2 protein. This latter experiment excluded an E1-dependent transcriptional effect on genomic DNA replication and confirmed that the S48G E1 protein was directly defective for some requisite replication function. In contrast, conversion of serine-48 to aspartic or glutamic acid resulted in a mutant E1 protein that exhibited intrinsic self-association, although their fold stimulations over background were less than for wild-type (approximately 3- and 4-fold, respectively, compared with 10-fold for wild-type). Nonetheless, both mutants clearly retained self-interaction capacity and, again, the similarity between the S48D and S48G mutants suggests that any absolute difference from wild-type is not sufficient to explain replication differences.

While a cellular kinase that modifies serine-48 in vivo has
not been defined, the most likely candidate is CKII. CKII is a ubiquitous enzyme that is an essential serine/threonine kinase in *Saccharomyces* and *Dictyostelium* (Litchfield et al., 1994). CKII phosphorylates numerous substrates, including many transcription factors (Allende & Allende, 1995; Issinger, 1993) and the simian virus 40 origin recognition protein, large T antigen (Rihs & Peters, 1989; Rihs et al., 1991). Phosphorylation by CKII has been shown to modulate both DNA binding and protein–protein interactions and these activities were examined as possible explanations for the replication defect of the S48G mutant E1 protein. However, the S48G mutant showed no gross defect in origin binding, E2 interaction or E1 interaction in *vivo* compared to the wild-type E1 or the pseudophosphorylated S48D E1 mutant. It should be noted, however, that subtle defects in any of these interactions might be sufficient to prevent formation of a functional replication complex, yet not be detected in the yeast one- and two-hybrid systems. In addition, there are several known interactions between E1 and host cell proteins (Bonne-Andrea et al., 1995; Han et al., 1999; Liu et al., 1998; Park et al., 1994; Swindle & Engler, 1998; Yasugi et al., 1997), any one of which could be affected by phosphorylation of serine-48. Consequently, the mechanistic basis for the severe replication defect of the S48G mutant remains to be determined.

The global phosphoregulation of papillomavirus E1 proteins is still largely unexplored. The full-length BPV-1 E1 protein exhibits multiple phosphorylations on serine residues and at least one threonine phosphorylation at residue 102, although mutation of threonine-102 had no effect on viral DNA replication (Lentz et al., 1993). More recently, both serine-90 (Lambert et al., 1990; Zanardi, 1997) and serine-109 (Zanardi et al., 1997) of BPV-1 E1 were shown to be phosphorylated in *vivo*. Mutation of either serine-90 or -109 to an alanine resulted in increased transient DNA replication in *vivo*. Consistent with the increase in replication, mutant E1 protein expressed in and purified from Sf9 cells exhibited increased origin binding (S90A) and increased helicase activity (S109A) in *vitro*, suggesting that phosphorylation at either site had a negative regulatory effect. Conversely, positive phosphoregulation of E1 replication activity has been demonstrated for HPV-11 E1 via CDKs (Ma et al., 1999), although the mechanistic basis for the enhanced replication was not explored. As both the cyclin/CDK-binding motif (RXL motif) and candidate phosphorylation sites for CDKs are present in other E1 proteins, it is likely that E1 activation by CDK phosphorylation will be a general regulatory mechanism. Our results with the serine-48 mutations are consistent with phosphorylation of this residue also being required for activation of some E1 replication function. Interestingly, while our S48G mutant was totally unable to support detectable replication, Ferran & McBride (1998) demonstrated that an E1 protein deleted for the first 131 amino acids retained at least minimal replication function. A possible explanation consistent with the available phosphorylation data is that the N terminus of E1 protein contains phosphorylation sites that regulate E1 activity positively (serine-48 and possibly other residues) and negatively (serine-90, serine-109 and possibly other residues) in a combinatorial fashion. Mutation of serine-48 to a non-phosphorylatable residue would eliminate an activation event without affecting phosphorylation of the negative regulatory sites, the net result being a constitutively inactive E1 protein. In contrast, removal of the entire N-terminal region might eliminate both the positive and negative regulatory control, resulting in an E1 protein with the observed basal activity. The existence of multiple positive and negative modification sites suggests a complex regulatory network that could modulate E1 activity incrementally as a function of both cell cycle and cell differentiation state. This combinatorial system may provide the precise control of virus replication required for the long-term persistence of papillomavirus infections in the epidermis.

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originate and stimulate cell-free DNA replication. Proteins facilitate human papillomavirus-11 E1 protein binding to the signal sequence of bovine papillomavirus E1 protein is necessary and sufficient for the nuclear localization of E1–β-galactosidase fusion protein. 

The E1 replication protein of bovine papillomavirus type 1 contains an extended nuclear localization signal that includes a p34cdc2 phosphorylation site. 

The cellular DNA polymerase alpha-primase is required for papillomavirus DNA replication and efficient transformation in cells. 

The rate of nuclear transport kinetics depend on phosphorylation-site-containing sequences flanking the karyophilic signal of the simian virus 40 T-antigen. 

Random-choice replication of extrachromosomal bovine papillomavirus (BPV) molecules in heterogeneous, clonally derived BPV-infected cell lines. 

The cellular DNA polymerase alpha-primase is required for papillomavirus DNA replication and associates with the viral E1 helicase. 

The bovine papillomavirus type 1 E1 and simian virus 40 large T antigen share regions of sequence similarity required for multiple functions. 

Two distinct regions of the BPV1 E1 replication protein interact with the activation domain of E2. 

The bovine papillomavirus type 1 E1 and simian virus 40 large T antigen, with human replication factor A. 

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