Casein kinase II phosphorylates bovine papillomavirus type 1 E1 *in vitro* at a conserved motif

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The E1 protein of bovine papillomavirus type 1 (BPV-1) is a phosphoprotein which specifically binds and unwinds the virus replication origin by ATP-dependent helicase activity. The E1 protein has been shown to be multiply phosphorylated *in vivo*, although the sites of modification are incompletely mapped. Examination of the predicted amino acid sequence of all available E1 proteins revealed strong conservation between amino acids 25 and 60 of a motif consisting of a serine residue followed by a stretch of acidic residues. This conserved motif resembled a phosphorylation consensus site for the ubiquitous cellular kinase casein kinase II (CKII). Biochemical and mutational analysis demonstrated that the BPV-1 E1 protein is an *in vitro* substrate for CKII at the serine within this conserved motif.

Introduction

The bovine papillomavirus type 1 (BPV-1) E1 protein is a nuclear phosphoprotein essential for *in vitro* and *in vivo* replication of DNA containing the virus replication origin (Blitz & Laimins, 1991; Mohr *et al*., 1990; Santucci *et al*., 1990; Sun *et al*., 1990; Ustav *et al*., 1991; Ustav & Stenlund, 1991; Yang *et al*., 1991*a*). The E1 protein binds the virus replication origin in a site-specific manner, unwinds it through an ATP-dependent helicase activity, and interacts with the virus transcriptional activator/replication enhancer protein E2 (Seo *et al*., 1993; Spalholz *et al*., 1993; Thorner *et al*., 1993; Wilson & Ludes-Meyers, 1991; Yang *et al*., 1991*a*, *b*, 1993). The biochemical activities and DNA replication functions of E1 resemble those of the simian virus 40 large T antigen (Clertant & Seif, 1984; Fanning, 1992; Fanning & Knippers, 1992; Seif, 1984). T antigen is known to be phosphorylated at multiple sites *in vivo* in a manner that regulates the DNA replication and transformation activities of the protein (Chen & Paucha, 1990; Fanning & Knippers, 1992; McVey *et al*., 1989; Prives, 1990; Schneider & Fanning, 1988). The locations and functions of phosphorylation sites on E1 are less well characterized than those of T antigen. Studies done with truncated forms of E1 indicate that both the N- and C-terminal regions are phosphorylated *in vivo*, a modification pattern similar to that of T antigen (Fanning & Knippers, 1992; Prives, 1990; Santucci *et al*., 1990; Sun *et al*., 1990). Thr-102 has been shown to be an *in vitro* substrate for p34 cdc2 kinase, but mutational analysis revealed no functional role for the modification *in vivo* (Lentz *et al*., 1993). No other specific phosphorylation sites have been identified or predicted for E1.

Complete evaluation of the biological function of E1 phosphorylation will require the identification and mapping of all phosphorylated amino acids present in this protein. To facilitate future mapping and functional studies, we examined the predicted E1 amino acid sequence for sites resembling consensus motifs of known cellular kinases. The E1 protein of BPV-1 contains two potential sites, Ser-48 and Ser-584, for phosphorylation by the cellular enzyme casein kinase II (CKII). Efforts were concentrated on characterizing the N-terminal region consensus motif because analysis of all available papillomavirus E1 sequences (bovine, human and other) revealed strong conservation in the region of amino acids 25–60 of a motif consisting of a serine followed by a stretch of acidic residues, which resembled a CKII phosphorylation consensus motif. In this study, the codon for Ser-48 within the putative BPV-1 E1 N-terminal CKII site was mutated to encode a non-phosphorylatable glycine, in order to map the *in vitro* CKII phosphorylation profile of the protein. We demonstrated that E1 was a substrate for purified CKII *in vitro* and that the predicted N-terminal CKII site was recognized by the enzyme.

Methods

■ **Construction of plasmids and mutagenesis.** The vectors for expression of various E1 fusion proteins in *E. coli* have been previously described (Wilson & Ludes-Meyers, 1991, 1992), except for pGE1700T/S48G, the construction of which was as follows. The 524 bp *Xma*I to *Bst*BI fragment (nt 945 to 1470) of the E1 open reading frame was

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excised from pdBPV.1 DNA (ATCC #37134) and purified by agarose gel electrophoresis and Prep-A-Gene (Bio-Rad). The purified fragment was cloned into M13mp18 which had been digested with *Xma*I and *Acc*I within the multiple cloning region. The compatible cohesive ends generated by *Bst*BI (E1 fragment) and *Acc*I (M13 vector) ligated to form a *Taq*I site. The resultant recombinant, designated M13E1/524, was used to generate ssDNA by standard procedures (Sambrook *et al*., 1989). Site-directed mutagenesis of the M13E1/524 DNA was performed with the USB T7-GEN *In Vitro* Mutagenesis Kit according to the manufacturer's protocols. The oligonucleotide used in the mutagenesis changed BPV-1 nt 990 from A to G, which converts E1 codon 48 from serine to glycine. The mutagenesis reaction products were transformed into *Escherichia coli* SDM cells and individual plaques were sequenced directly with the dsDNA Cycle Sequencing system (Gibco BRL). A mutant phage was identified and designated M13E1/524M. Double-stranded phage DNA was prepared after infection of *E. coli* JM101 with the mutant phage stock and the DNA was amplified by PCR as previously described (Phalen *et al*., 1991) using primers from the M13 flanking sequences. Amplified DNA was digested with *Xma*I and *Taq*I and the 524 bp E1 fragment was purified as described above. The 524 bp mutant fragment was substituted for the corresponding wild-type fragment in the E1 expression vector, pGE1700T, to generate pGE1700T/S48G. The identity and sequence of pGE1700T/S48G were confirmed by direct DNA sequencing of the substituted region. The specific fusion products of the various vectors used in this study were as follows: pGE1700, RecA–E1–β-galactosidase (β-gal); pGE1700T, RecA–E1; pGE372, RecA–β-gal; pE1 series (pE1_{212}, pE1_{249}, pE1_{259}, pE1_{312}, pE1_{333}, pE1_{413}), OmpF–E1–β-gal where the E1 portion represents a specific subregion of the full-length E1 protein (see Fig. 4*a*); pFZ, Ompf–β-gal; pORF, no fusion product.

■ **Preparation and characterization of fusion proteins.** Whole cell extracts from *E. coli* cultures expressing control or E1 fusion proteins were prepared as previously described (Wilson & Ludes-Meyers, 1991) and the total protein content of the extracts was determined by the Bio-Rad Protein assay. Characterization of fusion proteins present in the extracts was done by electrophoresis of samples on denaturing SDS–polyacrylamide gels followed by Coomassie blue staining or Western blotting.

■ ***In vitro* phosphorylation with CKII.** Bovine testis CKII (obtained from D. Litchfield, University of Washington, Seattle, Wash., USA) was used for the majority of the experiments; where indicated, commercial rat liver CKII (Promega) was utilized instead. The anti-E1 (5996 and 5997) sera used for immunoprecipitations and Western blots have been described previously (Wilson & Ludes-Meyers, 1991, 1992). Reactions with the bovine testis CKII included 1–10 µl of extract (approximately 3–30 µg of total protein) incubated in a 30 µl volume containing 50 mM Tris–HCl pH 7·6, 150 mM NaCl, 10 mM $MgCl_2$, 17% (v/v) glycerol, 1 mM ATP, 1·2 µCi [γ-^{32}P]ATP (sp. act. 4500 Ci/mmol). The NaCl concentration was reduced to 50 mM when rat liver CKII was used, with EGTA included to 1 mM as recommended by the manufacturer. Reactions with rat liver CKII utilized 10 units of enzyme. All reaction components, for both enzymes, were assembled on ice and kinase activity was initiated by transfer to 37 °C. Inhibition reactions included heparin (0·5 µM) and were otherwise identical to the reactions described above. After 30 min of incubation, reactions were either terminated by addition of 30 µl of SDS sample buffer [75 mM Tris–sulfate pH 8·3, 2% (w/v) SDS, 1% (v/v) 2-mercaptoethanol] followed by a 5 min incubation at 95 °C , or were immunoprecipitated with 10 µl of serum and 100 µl of Protein A–Sepharose (Pharmacia) as previously described (Wilson & Ludes-Meyers, 1991). Washed immunoprecipitates were resuspended in 10 µl of SDS sample buffer and heated for 5 min at 95 °C. Immunoprecipitates were centrifuged for 1 min at 4 °C and 12000 ***g*** in a microfuge (Fisher Scientific) after heating. Supernatants were removed for analysis by electrophoresis on SDS–polyacrylamide gels. Following electrophoresis, gels were fixed in 10% (v/v) acetic acid, washed twice in water and dried for autoradiography. Dried gels were exposed to film for 1–3 days at −70 °C with an intensifier screen. Incorporation of radiolabelled phosphate into E1 protein increased for 20–30 min and was stable for at least 60 min (data not shown).

■ **Purification of *in vitro*-phosphorylated RecA–E1.** RecA–E1 was phosphorylated *in vitro* with bovine CKII and electrophoresed as described above. The gel was exposed for autoradiography (4 h at 4 °C) without fixation or drying. Using the autoradiogram as a template, the portion of the gel containing the labelled RecA–E1 protein was excised. RecA–E1 was eluted from the gel slice by electrophoresis in an Elutrap device (Schleicher & Schuell) for 2 h at 200 V in 65 mM Tris–borate pH 8·3 with 1% (w/v) SDS. The eluted material was diluted with glass-distilled water and reconcentrated using a Centricon microconcentrator (Amicon). The dried pellet was extracted twice with 200 µl of cold acetone (5 min incubation on ice followed by centrifugation for 5 min at 4 °C and 12000 ***g***) and the product was dried for 10 min under vacuum. The dried pellet was resuspended in 400 µl of glass-distilled water. Recovery of radioactivity averaged 70% through this procedure.

■ **HPLC analysis of phosphoamino acids.** HPLC analysis was used to identify the types of amino acids phosphorylated by purified CKII *in vitro*. The purified RecA–E1 sample (10 µl) was supplemented with unlabelled phosphoserine and phosphothreonine standards, hydrolysed for 2 h in 0·5 ml 6 M HCl at 110 °C, dried to completion and redissolved in 25 µl glass-distilled water. The sample was derivatized with phenylisothiocyanate (PITC) according to the instructions provided by Waters Associates for use on the Pico-Tag system. The final sample was analysed by HPLC on a Waters Pico-Tag Reversed Phase Column using a modification of the usual Pico-Tag gradient. Using a Waters 840 Data Station and Pump Controller, the initial phase of the gradient went from 90% solvent A (acetate)–10% solvent B (acetonitrile) to 70% A–30% B in 5 min using curve 5. Elution at 5–10 min was with a linear gradient using curve 6 which ended at 49% A–51% B. Subsequently the gradient followed the normal Pico-Tag elution recommended by the manufacturer. The modification of the early elution phase greatly extends the resolution of the first 5 min of the gradient and allows phosphoamino acids to be separated. The eluted material was monitored by continuous micro-voltage detection and the corresponding fractions were collected for detection of radioactivity by Cherenkov counting. The hydrolysis and HPLC analysis were performed by the Biotechnology Support Laboratory at Texas A&M University.

■ **Analysis of predicted E1 amino acid sequences.** The nucleotide information for the sequence analysis of the E1 proteins of BPV-1 and 55 other papillomaviruses were obtained from GenBank. Predicted E1 amino acid sequences were determined from the nucleotide sequences using the SuperClone program (Coral Software). Secondary structure predictions and potential CKII sites were obtained using MacVector software (International Biotechnologies).

Results and Discussion

Two regions that appeared to be candidates for phosphorylation by CKII were identified by sequence analysis of the BPV-1 E1 protein, one in the N terminus and one near the C terminus. The N-terminal site contained a serine residue in a predicted β-turn motif followed by several acidic residues. This motif is characteristic of most known CKII phosphoryl-

FIBROPAPILLOMAVIRUSES

	10 · 20 · 30 · 40 · 50 · 60		
BPV1:	mandkgsnwdsglgcsyllteaecesdkeneepgagvelsvesdryd	**SQDED**	fvdnasvf
BPV2:	mandkgsnwdsalgcsyllteaecesdkeneepgagvelsvesdryd	**SQDED**	fldnasvf
DPV:	mdkenagssgvggdsfilfeaecsdtdsespaqge	**STDED**	lldnatavpgnhlelfqtqe
EEPV:	maetagssgqgggayicfeadcsdsdtevdspvqcsd	**SSDED**	lvdnanivpgnhlelfqt

CUTANEOUS PAPILLOMAVIRUSES

CRPV:	maegtdplddcggfldteadcldcdnleedltelfdadtvs	**SLLDDTD**	zgnslepfzh
HPV1a:	madnkgtendwflveatdceetleetslgdldnvscvsdl	**SDLLDE**	apzszgnslelfhk
HPV3:	mddtsgtegecseleraggwfmveaivdrrtgdtv	**SSDEDEEED**	ggedlvdfiddrpvgd
HPV7:	maddsgtedvgsgcsgwflveavvdkqtgdvv	**SEDEDED**	aiedsgydmvdfindtvvseh
HPV10:	mddntgteggacseseraggwfiveaivdrrtgdpi	**SSDDDEEEDE**	agedfvdfiddtrs
HPV15:	msddkgtydpkegcsdwfvleaecsdasldgdleklfeegtdtdi	**SDLIDNED**	tvzgnsr
HPV27:	medsegtdgteedgcraggwfhveaiithgqrqv	**SSDEDED**	ctetgedvdfidnrvpgdg
HPV41:	masrvsdtgngnenkenegtvasdhsearcsyilfeaec	**SDGGDDEE**	smedslvedlvdd
HPV49:	maddkgtdpkegcsewfidneadcsdlendleqlfdespksni	**SNLLNDEEDVE**	qgnsrd
HPV63:	mtdrgtnnddwyivdeaecrdddeseledledtynslfnrsesdi	**SDLLDD**	tqqsqgnsl

MUCOSAL PAPILLOMAVIRUSES

HPV6b:	maddsgtenegsgctgwfmveaivqhptgtqi	**SDDEDEEVED**	sgydmvdfiddsnithns
HPV11:	maddsgtenegsgctgwfmveaivehttgtqi	**SEDEEEEVED**	sgydmvdfiddrhitqns
HPV16:	madpagtngeegtgcngwfyveavvekktgdai	**SDDENENDSD**	tgedlvdfivndndylt
HPV18:	madpegtdgegtgcngwfyvqaivdkktgdvi	**SDDEDE**	natdtgsdmvdfidtqgtfceq
HPV30:	maspegtddegggctgwfhveavvkkrtgdii	**SEDETEEDE**	gtasdldgfldnsnvittq
HPV33:	madpegtngagmgctgwfeveavierrtgdni	**SEDEDE**	taddsgtdllefiddsmensiq
HPV40:	madspgtedggagcsgwfvveavvdkqtgdav	**SEDEDEEDIED**	sgfdmidfidnsvvaee
HPV45:	madpegtdgegtgcngwffvetivekktgdvi	**SDDEDE**	tatdtgsdmvdfidtqlsiceq
HPV52:	medpegtegeregctgwfeveaiiekqtgdni	**SEDEDE**	naydsgtdlidfiddsninneq
HPV57:	medsegtdgtdedgcraggwfhveaiithgqsqv	**SSDEDEDETE**	tredldfidnrvpgdg

Fig. 1. Conservation in animal and human papillomavirus E1 proteins of a serine followed by a stretch of acidic residues which resembles a CKII site. Shown are the first 60 amino acids of a representative sample of E1 proteins from the three subgroups of papillomaviruses. The predicted CKII recognition motifs are boxed. HPV, human papillomavirus; DPV, deer papillomavirus; EEPV, European elk papillomavirus; CRPV, cottontail rabbit papillomavirus.

ation sites (Meisner & Czech, 1991; Pinna, 1990). The C-terminal site was less conserved and lacked a predicted β-turn, but was extremely rich in acidic residues flanking the serine at amino acid 584. Examination of the N-terminal regions of 55 animal and human papillomavirus E1 proteins revealed strong conservation of N-terminal sequences resembling a CKII phosphorylation consensus motif. Between amino acids 25 and 60, all E1 proteins examined possessed a serine residue followed by an acidic-rich region. Fig. 1 shows a representative sample of E1 proteins from the three categories of papillomaviruses. The conserved presence and location of such a motif among the E1 proteins of papillomaviruses with a broad range of host and tissue specificities may indicate biological significance of the site as a common regulator of E1 function.

To determine if the E1 protein was in fact a substrate for CKII, *in vitro* labelling experiments were performed using E1 fusion proteins expressed in *E. coli*. Whole cell extracts were prepared from cells expressing or lacking E1 fusion proteins. The presence and relative expression of the fusion proteins were confirmed by Coomassie blue staining and Western blotting (Fig. 2*a*, *b*). Extracts containing each of the fusion proteins were incubated in the presence of purified CKII and [γ-^{32}P]ATP, and products were electrophoresed on SDS–polyacrylamide gels which were dried and autoradiographed (Fig. 2 *c*). Little or no phosphorylation was detected in the pGE372 control extract that did not contain an E1 fusion protein, indicating that few, if any, *E. coli* proteins were substrates for CKII under these conditions (Fig. 2*c*, lane 1). Strong phosphorylation of at least one host cell protein was observed in other *E. coli* backgrounds (see Figs 4 and 5). The RecA–β-gal fusion protein expressed by pGE372 was not phosphorylated, indicating that neither the RecA nor β-gal portions of the fusion proteins contained CKII phosphorylation sites. When an extract containing the 75 kDa RecA–E1 fusion protein was incubated with CKII, a predominant phosphorylated product was detected that had the correct molecular mass for the fusion protein (Fig. 2*c*, lane 2). A similar experiment with an extract containing a RecA–E1–β-gal tribrid

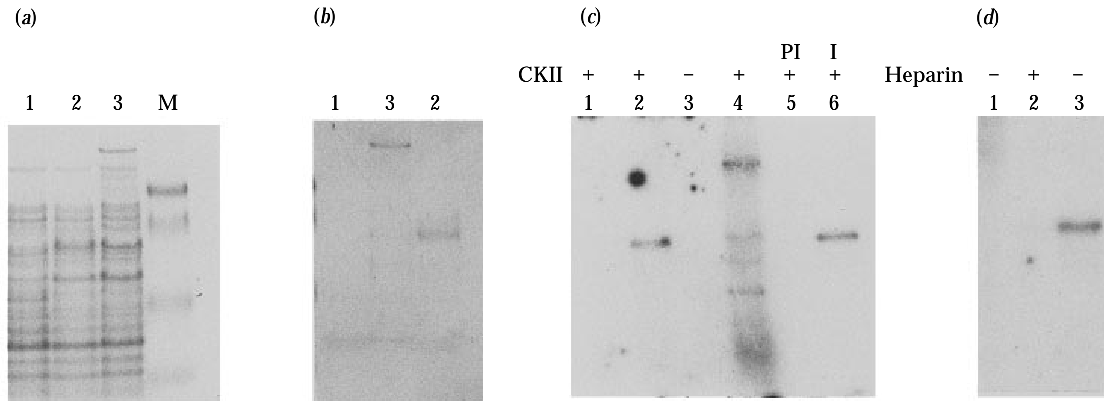

Fig. 2. *In vitro* phosphorylation of bacterially expressed E1 fusion proteins in the presence of purified CKII and [γ-^{32}P]ATP. Aliquots of the bacterial extracts (*E. coli* strain MC1061) were electrophoresed on a 15% SDS–polyacrylamide gel and visualized by either Coomassie blue staining (*a*) (lane 1, RecA–β–gal; lane 2, RecA–E1; lane 3, RecA–E1–β-gal) or Western blotting with anti-E1 serum (*b*) [lane 1, RecA–β-gal; lane 2, RecA–E1–β-gal (190 kDa); lane 3, RecA–E1 (75 kDa)]. (*c*) Extracts (lane 1, RecA–β-gal; lanes 2, 3, 5 and 6, RecA–E1; lane 4, RecA–E1–β-gal) were incubated with (+) or without (−) CKII as described in Methods. Phosphorylated products corresponding in size to the anti-E1 immunoreactive proteins were obtained only in the presence of both CKII and the E1 fusion proteins. The phosphorylated product in the RecA–E1 extract was immunoprecipitated by anti-E1 antiserum (lane 6), but not by preimmune (PI) serum (lane 5). (*d*) Phosphorylation of RecA–E1 (lanes 1–3) was inhibited by the inclusion of heparin (0·5 μM) added to reactions prior to CKII (lane 2). As in (*c*), there was no phosphorylation observed in the absence of CKII (lane 1).

fusion yielded a phosphorylated product with the molecular mass of the tribrid protein (190 kDa) (Fig. 2*c*, lane 4). The 75 and 190 kDa phosphorylated products were not produced in reactions using the control extract, consistent with these being the respective E1 fusions and not host proteins. Immunoprecipitation with an anti-E1 serum was used to confirm the identity of the phosphorylated product in the RecA–E1 reactions. The 75 kDa phosphorylated product was efficiently precipitated with the anti-E1 serum but not with the matched preimmune serum (Fig. 2*c*, lanes 5 and 6). No phosphorylated 75 kDa protein was immunoprecipitated from control extracts (data not shown). Its molecular mass, immunoreactivity and absence from control extracts all indicated that the phosphorylated 75 kDa protein was the RecA–E1 protein. Phosphorylation of E1 was achieved in whole cell extracts where E1 was a minimal component of the total protein. The ability of E1 to be specifically phosphorylated among a high background of *E. coli* proteins indicated that E1 was an efficiently recognized substrate for CKII.

To verify that the observed phosphorylation was catalysed by the exogenously added CKII and not a host enzyme or autophosphorylation by the fusion protein, two controls were performed and the results were as follows. First, no phosphorylation was detected in the absence of CKII (Fig. 2*c*, lane 3) and second, phosphorylation of RecA–E1 was reduced over 90% by the addition of heparin (Fig. 2*d*, lane 2), a known inhibitor of CKII (Pinna, 1990). These results indicate that the observed phosphorylation of RecA–E1 was due to the exogenously added CKII and not to an endogenous *E. coli* activity or to autophosphorylation by E1. A similar lack of intrinsic kinase activity has been reported previously for the E1 protein (Lentz *et al.*, 1993).

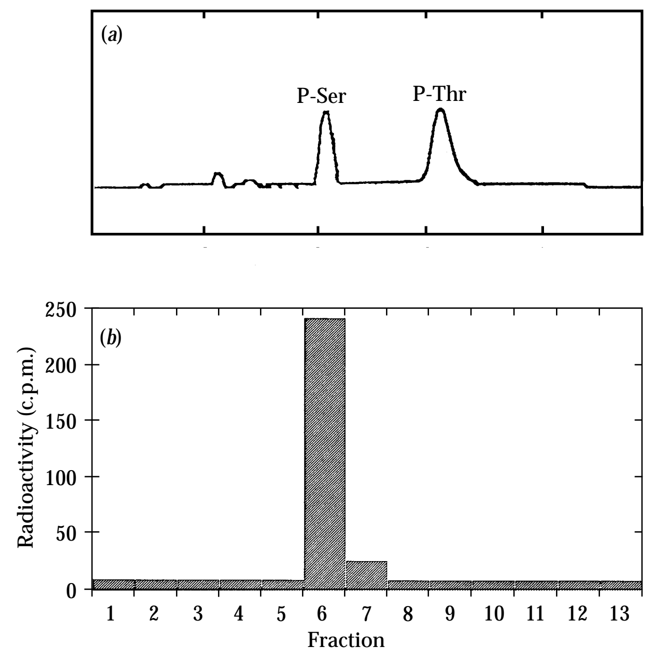

Fig. 3. Phosphoamino acid analysis of CKII-phosphorylated RecA–E1. RecA–E1 was radiolabelled *in vitro* by reaction with CKII, purified and hydrolysed as described in Methods. The hydrolysed sample was supplemented with unlabelled phosphoserine and phosphothreonine standards and was fractionated by HPLC. (*a*) The portion of the HPLC chromatograph showing the elution of the phosphoserine (P-Ser) and phosphothreonine (P-Thr) standards. (*b*) The radioactivity present in the HPLC fractions corresponding to the chromatograph in (*a*).

Phosphoamino acid analysis was performed on full-length E1 phosphorylated *in vitro* by purified CKII to determine the nature of the target amino acids (Fig. 3). While CKII can

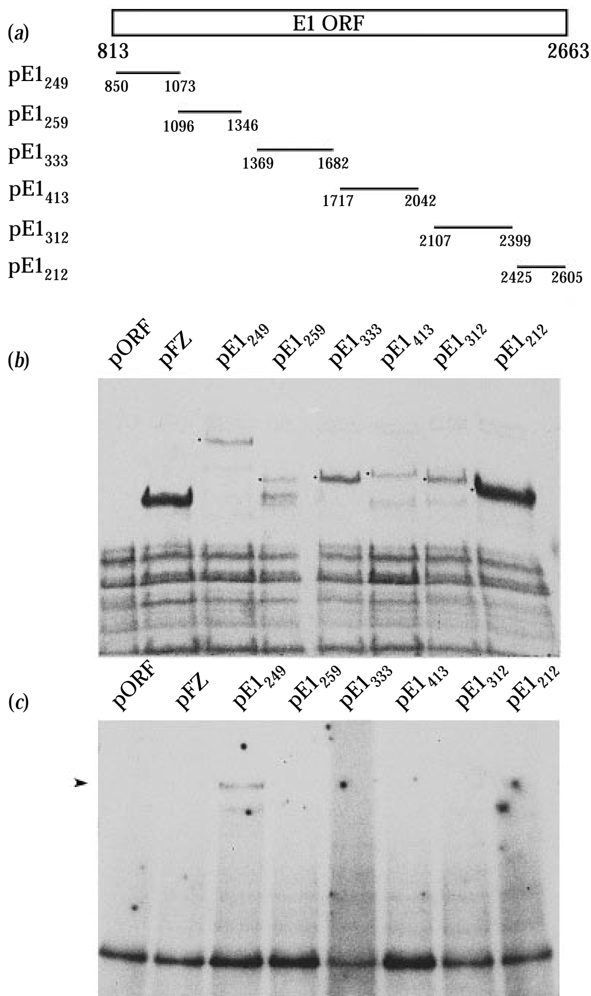

Fig. 4. Localization of a CKII phosphorylation site to the N-terminal region of RecA–E1. (*a*) Diagram of the region of BPV-1 E1 expressed from each of the pE1 series plasmids. The rectangle at the top shows the entire E1 open reading frame and below is the portion of the E1 sequence present in each clone. Numbers below each fragment refer to the BPV-1 nucleotide sequence. (*b*) Coomassie blue-stained 10% polyacrylamide–SDS gel of the pE1 series extracts (*E. coli* strain TK1046), the control pORF (no fusion protein) extract and the pFZ (RecA–β-gal) extract. The full-length OmpF–E1–β-gal fusion proteins are marked with an asterisk; shorter truncated forms are also visible with some fusions. Note that the $pE1_{249}$ product had an anomalously retarded migration as previously described (Wilson & Ludes-Meyers, 1991). (*c*) CKII phosphorylation of the extracts shown in (*b*). Phosphorylation and electrophoretic analysis of the pE1 extracts were performed as for the RecA–E1 extracts in Fig. 2 except that the pE1 samples were analysed on a 10% polyacrylamide–SDS gel.

potentially phosphorylate serines and threonines (Pinna, 1990), only phosphoserine was detected in RecA–E1. The absence of phosphothreonine was consistent with the sequences of the predicted CKII sites in E1, which had only serines available as the phosphate acceptors.

The general location of CKII phosphorylation events on RecA–E1 was determined using the previously described pE1 series of plasmids (Wilson & Ludes-Meyers, 1992). Each of the six pE1 plasmids expresses a non-overlapping portion of the E1 open reading frame as an OmpF–E1–β-gal fusion protein (Fig. 4*a*). Whole cell extracts were prepared for each pE1 plasmid as well as two control plasmids, pORF and pFZ. SDS–polyacrylamide gel analysis followed by Coomassie blue staining indicated that there were comparable amounts of soluble E1 fusion protein expressed in the extracts from each of the pE1 plasmids except $pE1_{212}$, which was expressed at a slightly higher level (Fig. 4*b*, lane 8). Control plasmids pORF and pFZ expressed no fusion protein and an OmpF–β-gal fusion, respectively (Fig. 4*b*, lanes 1 and 2).

Incubation of the pE1 series and control plasmid extracts with CKII resulted in phosphorylation of the $pE1_{249}$ fusion protein (Fig. 4*c*, lane 3). The $pE1_{249}$ fusion protein contains E1 amino acids 1–75, encompassing the N-terminal putative CKII phosphorylation site. None of the other five E1 fusions or the OmpF–β-gal fusion protein were phosphorylated under these conditions (Fig. 4*c*, lanes 4–8). Since these fusion proteins were present in the extracts in amounts equal to or greater than the $pE1_{249}$ fusion protein, failure to detect their phosphorylation indicated that they were not efficient substrates for CKII. Ser-584, which was contained within the $pE1_{212}$ fusion protein, was not detectably phosphorylated even though there were large quantities of this protein in the extract. However, all of the E1 fusion proteins tested that contained Ser-584 (RecA–E1, RecA–E1–β-gal and OmpF–$E1_{212}$–β-gal) lacked the authentic C terminus of E1. Owing to the expression plasmid construction method, each fusion protein was truncated following E1 amino acid 586, partially disrupting the acidic residue-rich region downstream of the potential phosphate acceptor serine, which may prevent effective utilization of this site by CKII. However, examination of other papillomavirus E1 proteins revealed less conservation of the C-terminal potential CKII site. While the presence of an acidic region near the C terminus was uniformly conserved, 18% of E1 proteins examined lacked a serine or threonine within five amino acids preceding the acidic region. The absence of an available serine or threonine in the C terminus of a significant number of E1 proteins suggests that phosphorylation may not be critically involved at this site. It remains to be determined whether the C-terminal site can in fact be utilized by CKII.

Phosphorylation of the $pE1_{249}$ fusion protein was consistent with utilization of the N-terminal predicted CKII site; however, there were seven other serines in the E1 portion of this fusion protein. To determine if phosphorylation actually occurred at Ser-48, a serine to glycine mutation was engineered at this position in the context of the RecA–E1 protein. Western blotting indicated that the S48G mutant E1 protein was expressed at equivalent levels to the wild-type RecA–E1 protein (data not shown). However, phosphorylation of the mutant fusion protein was significantly reduced (Fig. 5).

While the above results were consistent with Ser-48 being the phosphorylation target of CKII *in vitro*, there must be at

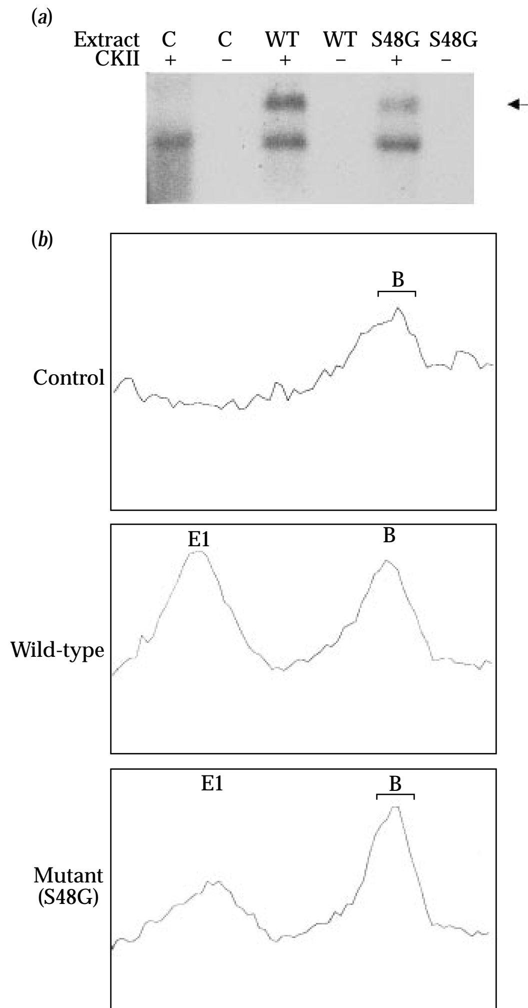

Fig. 5. Analysis of phosphorylation of the Ser-48 mutant RecA–E1 protein. (*a*) Extracts from *E. coli* TB1 cells expressing pGE372 (lanes C), pGE1700T (lanes WT) and mutant pGE1700T-S48G plasmid (lanes S48G) were prepared and incubated with (+) or without (−) rat liver CKII. Equivalent samples from each CKII reaction were electrophoresed on a 10% Tris–glycine gel. The arrow indicates the position of the E1 protein. (*b*) The amount of radiolabelled material in (*a*) was evaluated using a Molecular Dynamics PhosphorImager. Shown are the scans for the three sample lanes with CKII in the region of the gel containing the RecA–E1 protein (denoted E1) and the cellular background protein (denoted B).

least one additional target site to account for the residual phosphorylation. In our original examination of the N-terminal region of E1, a second possible CKII site was noted at Ser-26 (V. Wilson, unpublished observations). Ser-26 is not likely to be a strong CKII target site in the wild-type E1 because the acidic region following Ser-26 is disrupted by a basic residue (Lys-28). The presence of basic amino acids within the CKII recognition region typically reduces substrate utilization by CKII (Pinna, 1990). However, the ability of CKII to impart a low level of phosphorylation to the Ser-48 mutant protein indicates that there must be an additional CKII phosphorylation site or sites in E1. Studies are in progress to determine if Ser-26 is the site responsible for the residual phosphorylation observed with the Ser-48 mutant.

The studies presented here confirm that a predicted CKII site at Ser-48 in the BPV-1 E1 protein is an authentic target for CKII *in vitro*. The strong conservation of the Ser-X-X-Glu motif and the related Ser-Glu/Asp-Leu-Leu/Ile-Asp motif in all E1 proteins indicates a possible biological role for these sites that may depend on use of these sequences *in vivo* as CKII phosphorylation sites. CKII phosphorylates a variety of cellular proteins involved in cell growth and proliferation, suggesting that CKII may be an important regulator of cell cycle functions (Meisner & Czech, 1991). As we have recent data that the BPV-1 E1 protein perturbs the host cell cycle and affects host cell growth (Belyavski *et al*., 1994*a*, *b*, 1995, 1996), the potential phosphorylation of E1 by CKII *in vivo* may be critical for these effects. Assays with the Ser-48 mutant in the context of the complete BPV-1 genome show defective replication and reduced numbers of transformed foci compared to wild-type BPV-1 (G. D. McShan & V. G. Wilson, unpublished results), a finding consistent with the hypothesis that Ser-48 has a role in the regulation of E1 functioning *in vivo*.

We thank Dr David Litchfield for supplying purified bovine CKII. The phosphoamino acid analysis was performed by the Texas A&M University Biotechnology Support Laboratory. We thank Gennie Schuller for excellent technical support. This work was funded by PHS Grant CA5699.

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Received 31 May 1996; Accepted 3 September 1996