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Analysis of Kinase Effects on Viral Replication of the Papillomavirus

Joshua R. Raynes
University of North Florida

ANALYSIS OF KINASE EFFECTS ON VIRAL REPLICATION OF THE PAPILLOMAVIRUS

by

Joshua R. Raynes

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CERTIFICATE OF APPROVAL

The thesis of Joshua R. Raynes is approved by: Date

Signature Deleted

6-27-06

Committee Chairperson

Signature Deleted

6-27-06

Committee Member

Signature Deleted

6-27-06

Committee Member

ACCEPTED FOR THE DEPARTMENT:

Signature Deleted

6/27/06

Department Chairperson

ACCEPTED FOR THE COLLEGE:

Signature Deleted

6.29.06

Dean

ACCEPTED FOR THE UNIVERSITY:

Signature Deleted

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Dean of the Graduate School

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Jules Henri Poincare once said "Science is facts; just as houses are made of stone, so is science made of facts; but a pile of stones is not a house, and a collection of facts is not necessarily science, " and never has this concept been more real to me than during my research and the completion of this work. My efforts in the laboratory could have easily constructed a metaphorical stack of stones, but I could have never forged this thesis without the help of so many outstanding individuals.

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ABSTRACT

Papillomaviruses are a genera of small tumor viruses in the *Papovaviridae* family, whose lifecycle and replication ability is directed by epithelial differentiation. During latency, papillomavirus DNA replication occurs synchronously with the host cell's replication by the activation of the E1 protein. To elucidate the effects upon viral replication, this study utilized chemical inhibition of several kinases predicted to phosphorylate, and subsequently modify the activity of, the papillomavirus' E1 protein. The amount of DNA replicated was observed via autoradiography following DNA extraction and southern blotting of BPV-transformed C127 cells. Sample extracts from cells exposed to specific chemical inhibitors of PKC, CDK, and DNAPK showed a consistent and significant decrease in viral DNA when compared to the DNA abundance of a control set of extracts. Extracts of cells subjected to inhibition of CK2 displayed an observable increase in replicated viral DNA. To ensure that the kinase modification was not effecting the growth or viability of the cells, a neutral red assay was performed and found no significant difference between control and chemically treated samples in cell viability or overall cell number. These findings, in conjunction with the differential viral DNA abundance, implicate that kinases PKC, CDK, CK2, and DNAPK, have a role in viral genome replication.

INTRODUCTION

Papillomaviruses are small (capsid, 55-nm; genome, 8 kbp) obligate intracellular parasites that infect epithelial cells of the skin and mucous membranes in a range of vertebrate hosts [1-5, 6, 10]. These double-stranded DNA tumor viruses are one of the two genera within the *Papovaviridae* family and the non-enveloped virions exhibit T=7 icosahedral symmetry [28, 30]. Entry of virus particles occurs via abrasive mechanism, which exposes the target basal cell layer. This exposure is followed by clathrin-dependent endocytosis and nucleocapsid L2 protein-mediated egress from late endosomes into cell cytoplasm [31]. The papillomavirus has a complex lifecycle which is closely linked to the differentiation status of the epithelial cells. During latency papillomavirus DNA replication can only occur during the host cell S-phase of the cell cycle [7, 10, 17, 24]. Following viral attachment, entry, and nucleocapsid disassembly, initial infection and viral latency are characterized by low viral genome copy number (50-200 per cell) and minimal gene expression, where only a select few genes are transcribed in low concentration. The viral genome exists as an extra-chromosomal episome and remains perpetually present in the stratum basale layer, allowing for the virus's persistent ability to relapse [32]. Papillomaviruses are non-lytic, and mature virions are shed only as the stratum corneum desquamates.

These viruses primarily infect the basal layer, or stratum basale, of stratified squamous (both keratinizing and non-keratinizing) epithelial tissue. The stratum basale cells, found just superficial to the dermis, are often characterized as the skin "stem cells," as they are the only layer of cells that undergo mitosis and subsequently the only layer in

which DNA is replicated. These viruses are termed oncogenic because they transform the infected cells in their efforts to maintain replicative status in the cell, producing unregulated cellular growth. As the infected cells divide and proliferate outward the virus triggers a hyperproliferation of nearby keratinocytes and benign skin tumors (warts), or papillomas, are often produced [10, 25]. These warts are most often manifested in the skin of the host's outer extremities, but specific strains of the papillomavirus regularly arise in the mucosa [8, 10]. These neoplasms are not typically life threatening for the host, but if the viral cycle does not regress or the host is exposed to environmental carcinogens, they may become malignant and metastasize to other locations in the body. Certain "high-risk" papillomavirus strains, those that readily induce modifications allowing for immortalization, are more known to develop into malignant cancers, while those of "low-risk" are rarely linked to malignancy [10, 17].

The viral genome contains three basic regions, each characterized by their relative functions. The first region consists of an upstream regulatory domain, containing sequences that control transcription and replication. This sequence is followed by a region containing many of the genes involved in the actual viral replication, transcription, and transformation, the early domain; and a structural region, the late domain [7, 11, 24-26]. The early region encodes for the early proteins, E1-E8, and the late contains two genes; L1, which forms the viral capsid and L2, a minor capsid component, which is also responsible for viral DNA packaging. Translation of the late proteins is necessary for the progeny virons to be able to evacuate the host cell and proliferate the infection cycle. This stage in the lifecycle only occurs in the outer epithelial layers (outer stratum spinosum and stratum granulosum), those most highly differentiated [12, 13, 17].

Like all DNA tumor viruses, the papillomavirus uses host cell proteins for their own DNA replication. They do, however, encode for a specific replication origin (ori) binding and viral DNA unwinding molecule: the E1 protein [1-5, 7, 8]. The E1 protein, which is a 593-681 amino acid polypeptide (approximately 67.5-76.2 kDa depending on the viral strain), has the largest and most sequentially conserved open reading frame in the viral genome [7]. For the virus to replicate correctly, the α-- helix rich E1 protein forms a hexameric or double-hexameric complex, facilitated by the activity of a heat-shock chaperone protein [12]. E1 contains binding domains for DNA, ATP, and the E2 protein, an auxiliary origin recognition protein. When E1 and E2 are coupled together, the complex, via its loop-helix motif, will locate and attach to the origin replication site of the viral genome. While the presence of E2 in the complex is not altogether necessary, the sequence specific recognition ability of E1 is enhanced when E2 is present in the pre-replication complex [7, 12]. Once bound to the origin, E1 causes the initial unwinding of viral DNA at the replication fork through its 3`→5` helicase activity. Unwinding is an ATP dependent process, which hydrolyses the ATP bound at the P-loop of the protein at a rate of one molecule per round of activity [7, 12, 14]. This activity occurs at the carboxy-terminal domain. This model has been demonstrated by studies with amino-terminus deleted E1 where the truncated protein still maintains replication functionality, albeit at a slower rate of viral DNA unwinding [1, 2, 7]. Strain comparison studies have revealed that the carboxy-terminal domain is the most highly conserved section of the protein's sequence [7].

E1 is the only papillomavirus protein with enzymatic function, which it must employ to unwind the viral template DNA. In the presence of unwound template the

protein must then recruit the cellular replication machinery to the viral DNA. Following recruitment, it is then up to the infected cell to complete replication. Given the virus' compact genome, most of the components needed for viral reproduction are not encoded for by the viral DNA, and so all other factors necessary for replication must be supplied by the host cell. The E1 protein has been shown to be responsible for soliciting host cell DNA polymerases (α and δ), replication protein A (RPA), proliferating cell nuclear antigen (PCNA), and topoisomerases [13, 14, 23]. The success of E1's ability to bind to all of the required replication elements, within the proper time frame, suggests a significant level of regulation of E1's multiple binding sites [7, 20]. Data has demonstrated that the viral replication protein E1 may have the potential to influence the host cell environment significantly, instead of waiting idly in a latent stage. Expression of E1 protein has been shown to decrease the duration of G1 phase and increase S phase, allowing for maximal viral DNA replication and may contribute to the virus' pathogenesis and persistence within the host cell [9, 10].

Unique to the papillomavirus is its ability to direct the activation of its DNA replication to occur synchronously with the host cell's replication [7, 10, 17]. The timing of this activation and subsequent stimulation of the E1 protein is critical for viral replication and therefore, exhibits differential regulation sites specific for timing and utilization of the viral genomic replication. Without the presence of a complex cell cycle regulation system of its own, the virus depends largely upon the phosphorylation of E1 protein to regulate viral DNA replication during host cell S-phase [7-14, 25]. This fact also makes the papillomavirus an excellent model system for studying some aspects of eukaryotic DNA replication mechanisms [1-5, 17].

Protein phosphorylation is a common regulatory mechanism in cellular systems. Phosphorylation of proteins can yield a range of changes from subcellular localization, signal transduction, and modifications in stability and activity [22]. Several labs have dedicated years of study to the role of phosphorylation and the probable sites utilized in regulating E1 activities. Earlier studies have utilized various mutants of the E1 protein to elicit which residues of the protein must be phosphorylated for functional replication [2, 3, 4, 25, 33]. Substitution mutation of the bovine papillomavirus E1 protein at threonine 102 did not seem to significantly modify replication [4], but other mutants, such as serine 48 and serine 584, had significantly reduced or eliminated replication of the viral genome and were hypothesized to be possible phosphorylation sites of an activating nature [2, 25]. Yet, not all modification of suspected phospho-resides indicated an inhibitory activity on replication; alteration of residues serine 90 and serine 109 actually lead to an increase in replication activity [3, 33]. These studies provide support for the differential effects of certain kinases, but are limited in that site specific assays can only determine that site's activity, and not the cumulative action of phosphorylation of all targets of a particular kinase. Experimental modification of CDK within an in vitro system seemed to suggest that some level of E1 dependency exists on the kinase, but site specificity was not determined [26]. Recent mass spectrometric analysis of the E1 protein revealed a clear map of major E1 protein phosphorylation sites. This data revealed five new phosphoamino acid residues and confirmed two known sites [17]. Adding these sites to other previously established phosphorylation sites brings the total number of known phospho-regulation residues on the E1 protein to ten. Eight of the ten phosphorylation sites are within the amino terminal domain, the region of least conserved sequence

(Figure 1). While overall the region varies between viral strains, the phospho-residues themselves are highly conserved. The positioning of these regulatory sites in this domain follows logically in that the necessary regulatory mechanisms should be extremely specific to the host environment. Which kinases are responsible for modifying these sites, as well as their subsequent pathways, are still unclear.

The cyclic dependency of replication lead to the initial hypothesis that mitogen activated protein kinases (MAPK) and protein kinase A (PKA) are the phosphate donors [8], however recent experimental analysis does not seem to indicate that MAPK plays a significant role in phosphorylation. PKC, CDK, and CK2, all have been demonstrated to phorphorylate the E1 protein in vitro [21, 23, 26]. Of those sites identified by mass spectrometric (MS) analysis, five were found to be of the consensus of CK2 target substrates, one of CDK, and one of DNAPK, based upon computer prediction algorithms [17]. Those sites not confirmed by MS analysis, yet verified by other studies, include an additional site in the consensus for CDK and two sites of PKC [3, 4, 33]. Such predictions seem intuitive given that those kinases which are known to effect cell cycle progression and/or DNA modification are the most likely candidates of site phosphorylation, taking in account E1's role in viral replication.

Protein kinase C (PKC) is a family of isozymes, which are typically activated via receptor binding of hormones, growth factors, and signal transduction agents [15, 16]. Some forms of the kinase require calcium and/or phospholipids to stimulate activity. PKC has been shown to be necessary for optimal efficiency in production of viral progeny and upregulation of late gene expression in human papillomavirus [27, 41]. Inhibition of PKC signal transduction pathways has known effects on tumor suppression

and inhibition of rapid cell proliferation [15, 16], and is of key interest to this study, since the kinase is known to play a key role in epidermal differentiation [3, 40]. PKC substrates are typically serine/threonine residues with upstream or downstream basic residues, particularly arginine [27].

Cyclin dependent kinases (CDK) are a group of kinases largely responsible for proper cell cycle progression, specifically at the G1/S and G2/M phase boundaries [21-23, 26]. CDK has been demonstrated to have effects upon the nuclear export sequences (NES) and nuclear localization sequences (NLS) of human papillomavirus E1 [21], however its effects upon the conserved localization domain in BPV E1 has not been definitively assayed [7]. Target consensus sequence for CDK phosphorylated residues are comprised of serine or threonine amino acids followed immediately by a proline [21, 26].

Casein kinase II (CK2), like all of the kinases included in this study is a type of serine/threonine kinase. CK2 functions are much more multi-faceted than most other kinases studied [20]. The consensus sequence for all CK2 targets is dependent upon acidic residues, like glutamate or aspartate, downstream from the phospho-residue [18]. Its activities range from signal transduction, growth control, cell shape, and even spindle formation. CK2 has been found in almost all tissues of all eukaryotes, and is ubiquitous in its intracellular distribution [18-20]. High amounts of CK2 are found in cells exhibiting elevated activity levels, especially those which are rapidly proliferating, which strongly suggests a supportive role in replication. This enzyme is of particular interest in that it expresses constitutive activity, which is optimal for viral replication [18, 20].

DNAPK is the least studied kinase used in this analysis, but a few of its activities have been published. DNAPK has been shown to be activated by DNA double strand

breaks, aneuploidy, or detectable genome abnormality that then phosphorylates the p53 protein. This phosphorylation activates the well known tumor suppressor protein, which subsequently binds to and prevents any replication of the damaged DNA [29]. The kinase also has been demonstrated to interact with a variety of transcription factors including Ku70, cJun, cFos, Sp1, cMyc, and TFIID, and lead to the dissociation of RPA complex [29, 36]. The target phospho-residues of DNAPK must be closely followed by a glutamine residue [29]. Of particular interest to this study is the kinase's reported interaction with epidermal growth factor receptor, where the receptor's expression was decreased following inhibition of DNAPK [37].

This study serves to illuminate the overall effects of the predicted kinases on viral replication. Chemical manipulation of PKC, CDK, CK2, and DNAPK will provide details into the respective impact of the specific phosphorylation of the E1 protein by each kinase upon the virus. Whether the inhibitors and/or activators of the kinases lead to reduction or addition of detectable viral DNA should give clear indication of the specific role of the kinases in viral replication. Alteration of kinase function yields no effect, increase, or decrease of the concentration of viral DNA, as detected by autoradiographic film of DNA extracted from infected cells.

MATERIALS AND METHODS

Cellular Maintenance and Transfection. C127 cells (murine fibroblast) were maintained at 37°C with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) and

10% FBS supplemented with 100iu/mL penicillin and 100mg/mL streptomycin before harvest at late log phase. Cells were washed with PBS, and removed from plates by addition of 1mL of 0.25% trypsin/ 2mM EDTA, followed by rocking for 5 minutes. Cells were transferred to a sterile 50mL Falcon tube containing 5mL of 5mM BES buffered complete DMEM. The cell solution was kept on ice prior to counting. Cells were spun out of media at 5000 X g for 10 minutes and then resuspended in complete media/BES mixture to yield a concentration of 20 million cells/mL. BPV viral genome DNA was released from the pML vector via digestion with BamHI (Takara) and 1μg of DNA was added to eletroporation cuvettes containing 0.1X TE (1mM Tris, 0.1mM EDTA). Sheared, denatured salmon sperm was included as a carrier to final concentration of 50μg total (200μg/mL carrier in cell suspension). Approximately 5 million cells (0.25mL C127 cell suspension) were added to the DNA containing cuvette and the mixture was shocked at 250V and 975μF. The samples were incubated at room temperature for 10 minutes and each sample was transferred to a 15mL tube containing 10mL complete DMEM/BES mixture. Cells were pelleted at 750 X g for 10 minutes, resuspended in 8mL complete DMEM, and distributed on 8, 10cm diameter plates.

Focus Assay and Transformed Cell Removal. C127 cells that have successfully been transformed with an extrachromosomal episome of BPV DNA produce visible foci after 2-3 weeks. During this time cells are kept at 37°C with 5% CO_2 and media replaced twice weekly. After transformation, foci were marked on the plate, media removed, and plates washed with PBS. A sterile 3mm disk of filter paper, presoaked in 1X trypsin solution, was placed on each distinct focus for 1 minute. Each filter disk was then placed

in 3.5cm diameter plate, onto which complete DMEM was then added. Cells were maintained under the same conditions as previously described until they reached approximately 50% confluence. At this time cells were transferred to a 10cm diameter plate and either immediately used or stored in liquid nitrogen.

Cell Culture and Chemical Modification. Transformed cells were sustained as a monolayer culture in complete DMEM at 37°C with 5% CO_2 and were passaged to a 1:20 dilution at subconfluence into 10cm diameter plates. Two sets of samples for each kinase experiment were maintained, and a third for the PKC kinase trials to access the effects of the activator. The first set of plates, including one plate for times zero, 48, 96, and 144 hours (0, 2, 4, and 6 days, respectively), contained only those cells not exposed to chemical modifiers. These cells grown in complete DMEM and a small volume of DMSO (no more than 50μL per plate in any trial) served as a control group, where the volume of DMSO was equivalent to the volume of the chemicals in the experimental groups. Time point plates represented the length of time that the transformed cells remain exposed to the chemical modifiers. A second set was maintained as described above, with the addition of 5X IC_{50} of an inhibitor to a specific enzyme. Kinases of interest and their respective inhibitors are shown below:

Table 1. Summary of all kinases assayed in this study and the specific chemicals used to inhibit their activity.

Kinase	Chemical Inhibitor
PKC	Bisindolylmaleimide [BIM]
CDK	Roscovitine
CK2	2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole [DMAT]
DNAPK	4,5-Dimethyl-2-nitrobenzaldehyde [DMNB]

All inhibitors used are purchased from Calbiochem and reported as highly selective to their respective kinases and cell permeable. The third set of plates for the PKC trials was maintained under equivalent conditions, with the addition of 10μL of 10μM kinase activator, tetradecanoyl phorbol acetate (TPA; Calbiochem). Appropriate concentrations of chemical modifiers were determined from Calbiochem literature.

DNA extraction. The cells were maintained as described above until their relative extraction time points were reached (time points were chosen to allow significant time for differential viral DNA accumulation). Media was aspirated from the plates and the plates were then washed with PBS. Cells were lysed by addition of 800μL HIRT buffer (10mM Tris pH 7.8, 10mM EDTA, 0.6% SDS) , rocked for approximately 5 minutes, and the lysate placed in microfuge tubes. 200μL of 5M NaCl was then added to the suspension. The tubes were iced for 20 minutes and subsequently centrifuged at 13,500 X g for 20 minutes at 4°C (All later centrifugation also performed at 4°C). Supernant was transferred to a clean tube and 800μL of phenol/chloroform mixture (1:1) added. Following thorough vortexing, the tubes were then centrifuged at 13,500 X g for 7

minutes. The top aqueous layer was removed, added to 800µL chloroform, vortexed, and centrifuged briefly at 13,500 X g. 700µL of the aqueous layer was removed and added to 42µL 5M NaCl and 665µL isopropanol for storage at 4°C until all time points were collected.

Digest of Extraction Samples. Once all the time points of each kinase trial were collected, the samples were centrifuged at 13,500 X g for 10 minutes, the supernant aspirated, and the pellet washed with ice cold 70% ethanol. The pellet was air-dried before being resuspended in 10µL of 20µg/mL RNase and held at 65°C for 20 minutes. The suspension was allowed to cool at room temperature and added to 2.5µL of 10X H Eco buffer (Takara), 12µL of DI H_2O, and 0.5µL of Eco R1 enzyme (Takara). The mixture was digested overnight at 37°C.

Electrophoresis and Southern Blotting. 5µL of loading dye was added to each digested sample and the mixture was then separated via standard 0.8% agarose gel electrophoresis, followed by ethium bromide staining. Each gel was photographed to ensure that lanes contained DNA and for later comparison. Agarose gels were soaked in Southern Blot denaturation solution (1.5M NaCl, 0.5M NaOH) for 15 minutes and then resoaked for an additional 20 minutes to ensure single stranded DNA. DNA from agarose gels was transferred to nitrocellulose membranes in 10X SSC solvent (1.5M NaCl, 0.15M Na citrate). The transfer proceeded for 12-24 hours and the membrane was then autocross-linked via UV Stratalinker (Stratagene).

Hybridization and Autoradiography. A radio-probe was constructed by random-primed synthesis according to manufacture's instructions (Promega) from the isolated DNA of the BPV genome and P^{32} (dCTP). Before hybridization, the Southern blot membranes were washed in 2X SSC and placed in hybridization flasks with 7mL Rapid Hyb-buffer (Amersham Biosciences). The flasks were placed in a hybridization oven at 55°C for 20 minutes and then 65°C for 30 minutes. The constructed probe was brought to a boil and quick chilled before being poured into the hybridization flasks. Flasks were kept in the oven at 65°C for 2 hours, followed by subsequent washes of the membrane to eliminate background radioactivity. The radiolabeled membranes were sealed in saran wrap and placed on X-ray film in an autoradiography cassette. Exposed film was removed from the cassette and developed according to manufacture's instructions (Kodak).

Neutral Red Viability Assay. Viability of control and chemically treated samples were assayed by neutral red uptake (NR) of the transformed cells at various time points. BPV/C127 cells were maintained as previously described until NR assay time point was reached. One plate of each sample set represented times 0, 72, and 166 hours of exposure to the respective kinase inhibitor. As described previously, control samples were those plates that contained only DMSO dilutent, in place of an equivalent amount of chemical modifiers. At time points, media was removed from sample plates, and the plates then washed with PBS. A mixture of 1.0% Neutral Red dye (Life Technologies) in complete DMEM was added to the cell cultures and returned to incubator at 37°C with 5% CO_2 for an additional 4 hours. After initial observation, cells were removed from plates by addition of 1mL of trypsin, followed by rocking for 5 minutes. A portion of the

suspension was then placed on a standard hemocytometer where overall cell number and cell viability, assessed by neutral red uptake, was determined. Total cell number was counted on the four corner grids of the hemocytometer, with two suspension aliquots counted from each time point plate of control and inhibited samples. From these counts, average number of cells per mL of each time point plate was determined. Number of total viable cells was determined by counting of those cells which had clearly taken in the neutral red dye. Percent viability of each plate was then calculated by dividing the number of viable cells by the total number of cells observed.

RESULTS

Mass spectrometric and mutant analysis has provided a map of the major phosphorylation sites of the bovine papillomavirus' E1 protein (Figure 1). Eight serine and two threonine residues were determined to be phosphorylated. By computer algorithms and manual sequence analysis of these phosphorylation sites, those kinases that are the most probable donators of the phosphate groups were predicted [3, 4, 17, 33]. A composite summary of the known phospho-residues and their predicted kinases are shown in Table 1. A comprehensive analysis of enzyme impact upon viral DNA replication of the papillomavirus was performed in order to gain insight into the relative regulatory activity of these kinases. Cellular kinases PKC, CDK, CK2, and DNAPK were chemically inhibited and/or activated, and Southern blotting of DNA extracts supplied directly observable results of each kinase's effect on viral DNA abundance. Manipulation

of the kinases could either yield stimulatory or inhibitory effects upon viral DNA replication. Following cellular exposure to kinase inhibitors, viral DNA should be detected at minimal concentrations or not all at, if the kinase has a stimulatory effect upon E1 in vivo. Conversely, assuming a kinase exhibits an inhibitory effect upon E1,

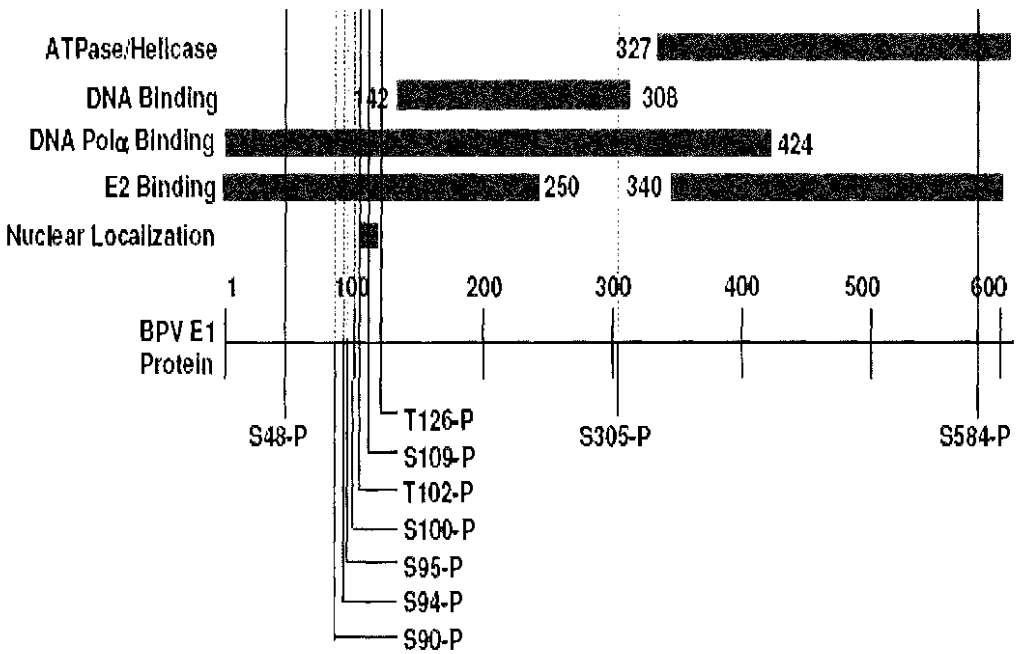


Figure 1. Positional phosphate map and functional domains of the BPV E1 protein. The 605 amino acid protein is represented on the lower horizontal line. The position of each of the phosphorylated amino acids is shown below the line. Functional domains in most cases were determined by deletion mutagenesis analysis as described in [7] and references therein. Modified from figure in [17].

and so overall viral replication, the sample set containing the kinase inhibitor will yield increased viral DNA on the southern blot. If the kinase only has a minimal regulatory

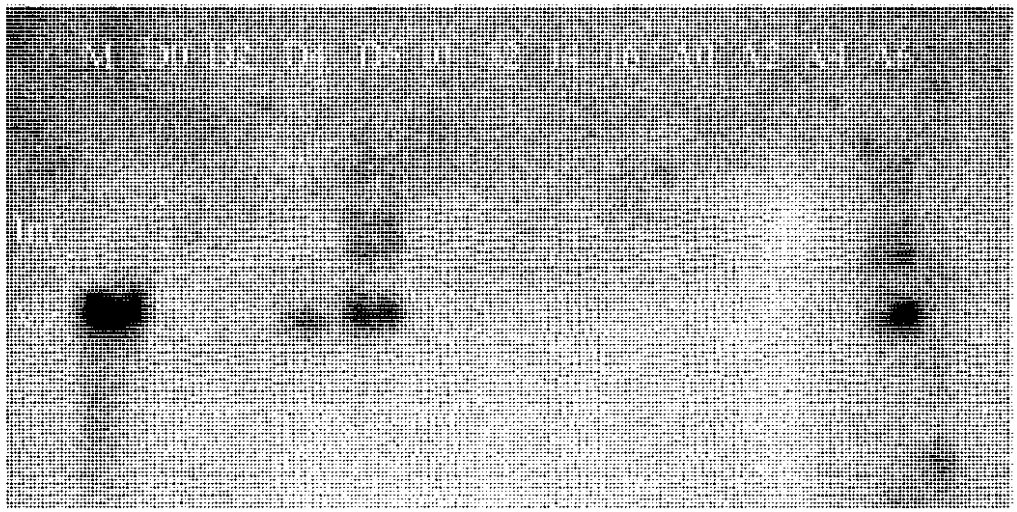
phosphorylation role, or if the prediction was incorrect and the kinase does not play a part in regulation of the E1 protein, manipulation of the kinase would exhibit no effect at all.

Table 2. Composite of all identified phosphorylation sites on the bovine papillomavirus E1 protein. Included in the table are the locations of each specific phospho-residue in the sequence and the kinase that is most likely the phosphate donating enzyme for the site.

Identified Phos. Sites & Kinases	
Residue	Kinase
Serine 48	CK2
Serine 90	PKC
Serine 94	CK2
Serine 95	CK2
Serine 100	CK2
Threonine 102	CDK
Serine 109	PKC
Threonine 126	CDK
Serine 305	DNAPK
Serine 584	CK2

Protein Kinase C (PKC) Analysis. Residues serine 90 and serine 109 which are included in the phosphorylation site map (Figure 1) were not determined from mass spectrometry, but were confirmed by other studies, in which mutation analysis of the residues each yielded a significant change replication status [33 and 3, respectively]. PKC was the kinase determined to target both residues via manual sequence analysis and in vitro phosphorylation of wild-type and mutant enzyme.

A.



B.

Predicted Kinase Phosphorylation Sites		
Residue	**Sequence**	**Meth Pred**
Ser 90	LKRKVLG **S** SONSSGS	Mutant Analysis
Ser 109	TPVKRRK **S** GAKRRLF	Mutant Analysis

Figure 2. Regulatory impact of PKC phosphorylation of target residues of E1. (A) Autoradiographic film of Southern blot containing DNA extracted from BPV transformed C127 monolayer is shown. Included on film are lanes containing isolated BPV-1 genome for use as a marker (M), extracts from control samples (D0-D6), extracts from PKC inhibited samples (I0-I6), and extracts from samples exposed to PKC activator (A0-A6). All sample sets contain extracts taken at day 0 (before exposure to chemical modifiers), day 2, day 4, and day 6. Major visible bands represent the viral genome (Vg) at approximately 8kbp and a slower moving band of either viral DNA that has become integrated with cellular DNA or uncut viral genome (Int). (B) Two phosphorylation sites are predicted targets for PKC, namely serine 90 and serine 109. The surrounding sequence of the phospho-amino acid and method originally used to predict the residue as such a site are included in the table.

The effect of PKC phosphorylation upon the regulation of the E1 protein and its subsequent effects on viral DNA replication are directly observable on the

autoradiographic film (Fig. 2A). To verify that the radioactive probe of the viral genome successfully and specifically hybridized to the viral DNA extracted from the C127 monolayer and not to the cellular DNA, a control lane of isolated BPV-1 genome was included in all assays of each kinase. The control marker used was the same genome utilized in C127 transformation, which was comprised only of BPV DNA that was released from the storage vector pML by Bam HI digestion. As expected, the marker traveled as a distinct, single band and migrated to a point of approximately 8kbp on agarose gel electrophoresis (data not shown). Viral DNA replicated in vivo should also migrate to a similar location following electrophoresis, and so intact non-integrated viral genome will be visible in a band parallel to the marker band. To clearly view this band on the autoradiographic film the marker concentration must be within the same order of magnitude as the replicated viral DNA, so the marker is not visible on every film. However, aligning the film with the picture of the ethium bromide stained agarose gel allows each band's relative size and inferred composition to be determined where necessary. All bands labeled as Vg were determined to be 8kbp.

The DMSO control samples (Fig. 2A D0-D6) showed a clear increase in viral DNA load over the time course of the experiment, indicating continual viral replication as the cells divide. DNA concentrations on day zero and day two were not enough to be detected on the film. A single band, representing the replicated viral genome, is present beginning at day four (D4). By day six the band has increased in intensity enough to indicate at least a doubling in concentration of viral DNA. Two bands, each slower moving than the isolated viral genome are also present at D6. While cellular DNA alone should not hybridize with the radioactive probe, and therefore not be visible as a band on

the autoradiographic film, cellular DNA that has acquired the viral genome would be visible. Although the viral genome normally exists as an extrachromosomal episome, the observation of integrated DNA is not unheard of [34, 35], but it is not ideal for this study. These heavier bands could alternatively represent a portion of the viral DNA that was not cut by the restriction enzyme during digestion. The intact structure of the genome would cause it to migrate slower during electrophoresis.

Lanes labeled as I0-I6 contain DNA extracted from the BPV/C127 cells that had been exposed to the PKC inhibitor, Bisindolylmaleimide (BMI), which acts competitively for the ATP-binding site of the kinase. There is no distinct BPV DNA banding present at any time during the course of the trial in these inhibited samples. This does not indicate that no viral DNA is present, but instead signifies that the DNA concentration never accumulated to detectable levels. When compared to the control extracts (D0-D6) the significant difference in relative abundance of replicated viral DNA between normal and inhibited PKC conditions is obvious. Due to the complete absence of detectable viral DNA in the inhibited trials for PKC there was some concern that the DNA extraction had somehow failed, yet the I0-I6 lanes in ethium bromide stained agarose gel clearly contained DNA (data not shown). It seems clear that inhibition of the PKC enzyme has an effect upon viral replication, and so implicates PKC as a regulatory kinase of the E1 protein.

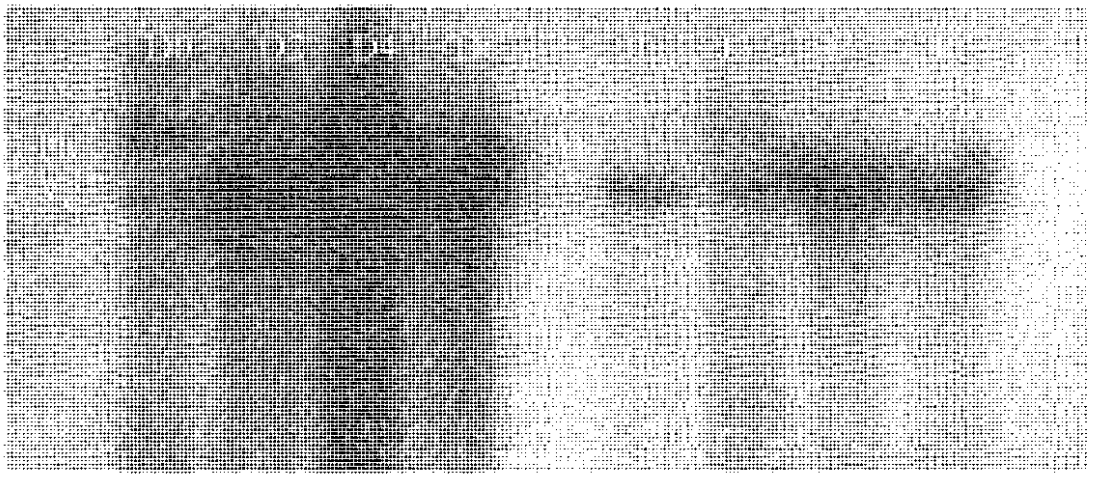
The remaining four lanes (A0-A6) hold DNA samples from cells maintained in media containing a general PKC activator, tissue plasminogen activator (TPA). Results appeared very similar to those in the control samples (D0-D6). Lane A0 and A2 appear to contain no detectable bands of the viral DNA. At day four in the trial viral DNA is at

detectable levels, as seen in lane A4. It contains a band of low intensity, which is barely visible on the film at 8kbp, identifying it as intact viral genome. This band is of considerably lower concentration than the band present at D4. Bands present in lane A6 are comparable with those seen in D6. The band of highest intensity, and so highest concentration, is at a migratory distance consistent with that of the viral genome. Just as in D6, A6 also contains two bands seen above the intact genome, representative of integrated DNA or uncut viral genome. Although the concentration of replicated viral DNA in A4 was lower than that in D4, the other extraction samples, particularly day 6, are similar enough not to label the activator exposed samples as possessing a significantly different amount of viral DNA. From this data it does not appear that stimulating PKC via TPA yields a change in viral replication and so does not alter the regulatory phosphorylation of the E1 protein. These findings do not discount PKC as a regulatory kinase, but may imply that there are limitations to the regulatory activity.

Cyclin-Dependent Kinase (CDK) Analysis. There are three identified potential target residues for cyclin dependent kinase (CDK) on the bovine papillomavirus' E1 protein (Fig. 3B), and two of these sites are found near E1's nuclear localization signal (T 102 and T126, Fig. 1). Threonine 102 was identified as a phosphorylation residue by prior mutation analysis with CDK as its most likely kinase, based upon manual sequence analysis and enzyme assay of the mutant E1 protein [4]. Although mutation of the residue did not reveal significant changes in viral replication, the residue could still have a regulatory status in latent replication or exhibit only transient phosphorylation. Threonine 126 was recognized as a phosphorylation site by mass spectrometry. CDK was predicted

as a potential kinase for the site by computer algorithm [17]. Serine 283 is also a predicted target for CDK phosphorylation based on sequence analysis, but was not included in the phosphorylation site map and has yet to be thoroughly assayed.

A.



B.

Predicted Kinase Phosphorylation Sites		
Residue	**Sequence**	**Meth Pred**
Thr 102	NEANRVL T PLQVQGE	Mutant Analysis
Thr 126	SGSEASE **T** PVKRRKS	Mass Spectrometry
Ser 283	FWFKSSL **S** PATPKHG	Sequence Analysis

Figure 3. Regulatory impact of CDK phosphorylation of target residues of E1. (A) Autoradiographic film of Southern blot containing DNA extracted from BPV transformed C127 monolayer is shown. Included on film are lanes containing extracts from control samples (D0-D6) and extracts from CDK inhibited samples (I0-I6). All sample sets contain extracts taken at day 0 (before exposure to chemical modifiers), day 2, day 4, and day 6. Major visible bands represent the viral genome (Vg) at approximately 8kbp and a slower moving band of either viral DNA that has become integrated with cellular DNA or uncut viral genome (Int). (B) Threonine 102, threonine 126, and serine 283 are the phosphorylation sites which are predicted targets for CDK. The surrounding sequence of the phospho-amino acid and method originally used to predict the residue as such a site are included in the table.

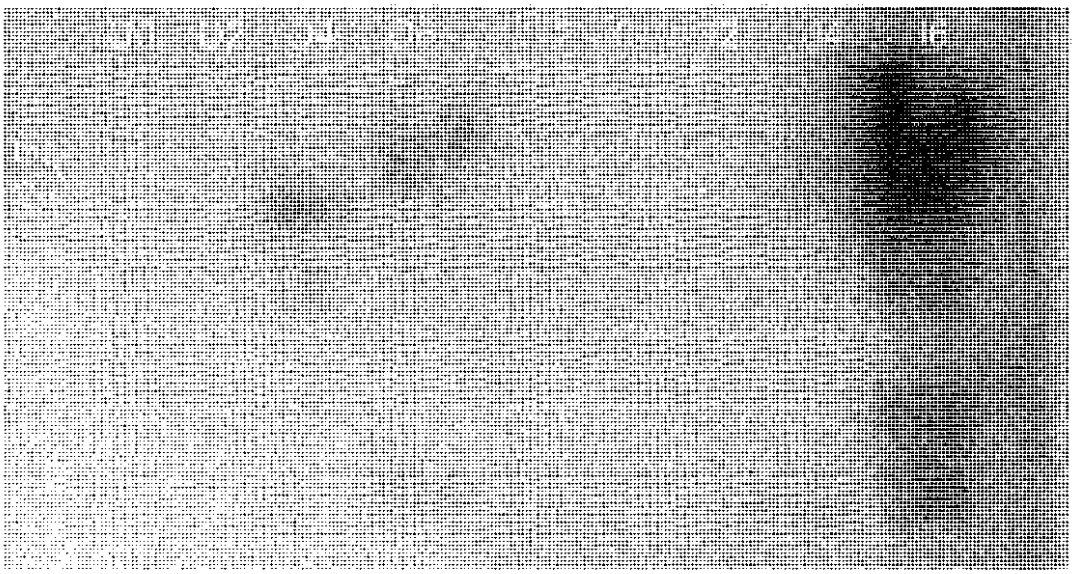
Regulatory replication activity of CDK was assayed by comparison of a control set of sample extracts with an inhibited set of extracts. The autoradiography of these extract samples is shown in Figure 3A. Lanes D0-D6 contain the DNA extracted from the BPV/C127 cells that had been maintained in complete DMEM and a small volume of DMSO dilutent to control for the volume of inhibiting chemical added to the inhibited set. The presence of viral DNA (Vg) is detectable at the first time point extract (D0) and accumulates over the course of the experiment, up to day 6 (D6). The band of intact genome is clear and distinct on all time points. A single band, heavier than the intact viral genome (Vg) is present in D0 and most likely represents a portion of the viral DNA that had integrated with the cellular DNA. There does appear to be a decrease in concentration of viral DNA from D4 to D6. While the cellular environment was controlled for and consistent with all time plates in all trials, some random differences in cell growth and replication between each plate could be anticipated and is the most likely explanation for the unaccountable decrease in viral DNA replication from these two time points.

Lanes I0-I6 contain the extracts from those plates exposed to the CDK inhibitor, Roscovitine, which competitively inhibits the kinase by blocking the ATP-binding domain. As with the control samples (D0-D6), the inhibited samples exhibit detectable amounts of viral DNA as early as the first time point (I0). It is clear that the amount of viral DNA increases over time, with no deviation. Comparison of the control and inhibited samples displays the obvious difference in detectable amount of replicated viral DNA. Extracted DNA from the CDK inhibited samples shows a consistently and significantly lower concentration of viral DNA (Vg) than the control samples. This

observation implicates a regulatory role for CDK in viral replication. Specifically, from the notable decrease in viral DNA present when the kinase is inhibited, it appears that under normal conditions in latent replication CDK phosphorylation of the BPV E1 protein serves to stimulate the synthesis of viral DNA. While these results cannot definitively confirm or deny that any target residue (T 102, T 126, or S 283) acts as a regulatory phosphorylation site, it does provide evidence that CDK targets some residue on the E1 protein to increase viral DNA replication.

Casein Kinase II (CK2) Analysis. The enzyme CK2 has over 300 known substrates and is a well-documented viral replication inducer [18], so it was not surprising that CK2 was the predicted kinase for so many phosphorylation sites (Fig. 4B). To study CK2's role in BPV E1 function, cells were treated as previously described with 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT). Like the autoradiograph of the PKC assay, the concentration of replicated viral DNA only reaches detectable levels on the film at days four and six (D4-D6 and I4-I6 of Fig-4A). The control extracts collected at day four (D4) show a clear, distinct band of intact viral genome (Vg). D4 also contains a small band, lighter than the intact genome at approximately 6kbp (Frag) which is most likely a portion of integrated or intact replicated genome that somehow became fragmented. D6 has a noticeably higher concentration of viral DNA that is spilt into at least three bands. The Vg band is very subtle and barely visible on this film, especially when compared to the denser band of integrated DNA in the D6 lane just above it. The third D6 band is a very small portion of (intact or integrated) viral genome fragment of only 2.2kbp. It is unclear at this time what lead to the fragmentation of the control

A.



B.

Predicted Kinase Phosphorylation Sites		
Residue	**Sequence**	**Meth Pred**
Ser 48	VESDRYD **S** QDEDFVD	Mass Spectrometry
Ser 94	VLGSSQN **S** SGSEASE	Mass Spectrometry
Ser 95	LGSSQNS **S** GSEASET	Mass Spectrometry
Ser 100	NSSGSEA **S** ETPVKRR	Mass Spectrometry
Ser 584	LIDEEED **S** EEDGDSM	Mass Spectrometry

Figure 4. Regulatory impact of CK2 phosphorylation of target residues of E1. (A) Autoradiographic film of Southern blot containing DNA extracted from BPV transformed C127 monolayer is shown. Included on film are lanes containing extracts from control samples (D0-D6) and extracts from CK2 inhibited samples (I0-I6). All sample sets contain extracts taken at day 0 (before exposure to chemical modifiers), day 2, day 4, and day 6. Major visible bands represent the viral genome (Vg) at approximately 8kbp, a slower moving band of either viral DNA that has become integrated with cellular DNA or uncut viral genome (Int), and smaller bands of fragmented viral genome (Frag). (B) Serine 48, serine 94, serine 95, serine 100, and serine 584 are the five phosphorylation sites which are predicted targets for CK2. The surrounding sequence of the phospho-amino acid and method originally used to predict each residue as such a site are included in the table.

samples in this trial, however the film does show the accumulation of viral genome that is necessary for the inhibited comparison.

Casein kinase II (CK2) phosphorylation activity was modified via ATP-competitive inhibition by 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT). The DNA extracts of those BPV/C127 cells exposed to this chemical modification are shown in lanes I0-I6. Viral DNA is only detectable on lanes I4 and I6 on this autoradiographic film. Due to the intense banding of I6, much of the banding in I4 is partially occluded, yet two bands can be clearly identified. The band representing the intact viral genome (Vg) contains a concentration of DNA, which appears very similar to that seen in D4. There is also a light band of relatively low concentration viral DNA fragment (Frag). The fragmented DNA is approximately the same size as the fragment band seen in lane D6. The extracts taken from BPV/C127 cells exposed to DMAT for 144 hours (D6) had the highest intensity band of any of the bands in any trial. Several bands are present in this lane. The concentration of the intact viral genome and integrated DNA bands are of great intensity and almost appear as one large band. Lane I6 also contains several smaller bands of DNA. Even though there is no great difference between D4 and I4, lanes containing extracts from day 6 on the film clearly show an intense increase in replicated viral DNA of the inhibited samples over the control samples. As intensity of banding is based upon concentration of viral DNA present in the extract, it appears as though I6 has roughly a four fold increase in concentration of replicated DNA. This observation readily categorizes CK2 as a participant in regulatory activity of the bovine papillomavirus. Based upon these results, it appears that the specific nature of CK2's involvement in regulation is an inhibitory one. Under normal conditions CK2

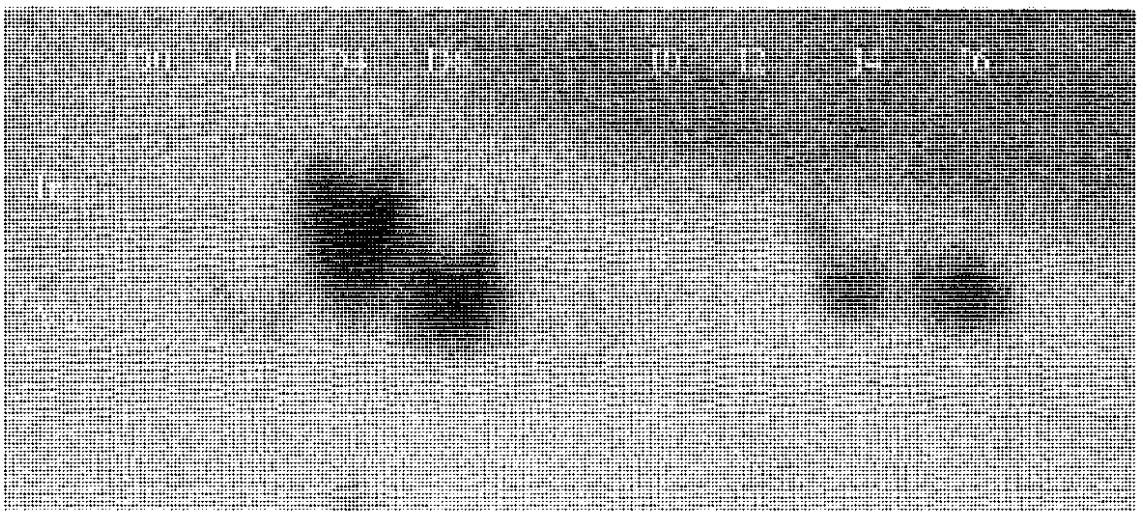
would therefore be expected to phosphorylate BPV's E1 protein and lead to a decrease in the synthesis of the viral DNA during latent viral replication. These results are especially encouraging for this study by showing that chemical modification of CK2 results in an increase in viral replication.

DNA Protein Kinase (DNAPK) Analysis. The enzyme DNAPK is the predicted phosphorylating agent of only one residue of the bovine papillomaviruses E1 protein, which was determined by mass spectrometry and computer algorithm (Fig. 5B). Serine 305, one of the two residues near the functional carboxyl terminus of the protein, is the only predicted target for DNAPK phosphorylation. The autoradiographic film displaying the extracts from the BPV/C127 plates of the DNAPK assay are shown in Figure 5A. As with all the previous films, lanes labeled as D0-D6 contain the radio-probed time point extracts of the control samples; those samples maintained only in complete DMEM and a volume of DMSO equal to that of the chemical inhibitor added to the other samples. DNA samples taken two days following the experimental set-up (D2) are the first to shown viral DNA which had been synthesized to a detectable concentration. The successive time point extractions collected at days 4 and 6 (D4 and D6) show a clearly observable progressive increase in concentration. Given the significant increase in band intensity of the intact viral genome (Vg) between days two and four, it can be asserted that the viral DNA concentration, at least, doubled. It is not as clear to assess the concentration increase of viral DNA between D4 and D6, due to the extremely dark banding of both samples. D4 appears to contain an area of radio-labeled DNA just above the 8kbp position of the intact viral genome, and while the boundaries of two distinct

bands cannot be discerned, it seems apparent that D4 possesses a region of intact viral genome close in proximity to a sample of viral DNA that has become integrated with a portion of cellular DNA or an uncut portion of intact viral genome (Int). The clear distinction of these areas is further complicated in this film by the curvature in the control bands, which is most likely due to an uneven migration of the extract samples during electrophoresis.

The inhibited sample extracts of the DNAPK assay are contained in lanes labeled I0-16. These samples were maintained under normal conditions as previously described, except for the addition of an IC_{50} concentration of 4,5-Dimethoxy-2-nitrobenzaldehyde (DMNB), a highly selective competitive inhibitor of DNAPK's ATP binding site. The presence of replicated viral DNA was not detected in the inhibited extracts until I4. The lane labeled as I4 possess one distinct band at 8kbp, representing the intact viral genome. The relative intensity of the band is just slightly more dramatic than that seen in the Vg band of the D2 extracts. I6 contains a single band of viral DNA, the intensity of which is clearly increased from I4 but not quite the concentration of D4 or D6. When comparing the bands of viral DNA present in the control and inhibited samples, it is clear that viral DNA is present at an earlier time point and accumulates to a much higher concentration in the control samples than the inhibited ones. Because replication of both sample sets unmistakably increased over time, at very different rates, the outcome of the DNAPK assay would support prediction of the kinase's function as a regulatory enzyme in

A.



B.

Predicted Kinase Phosphorylation Site		
Residue	**Sequence**	**Meth Pred**
Ser 305	AQTTLNE **S** LQTEKFD	Mass Spectrometry

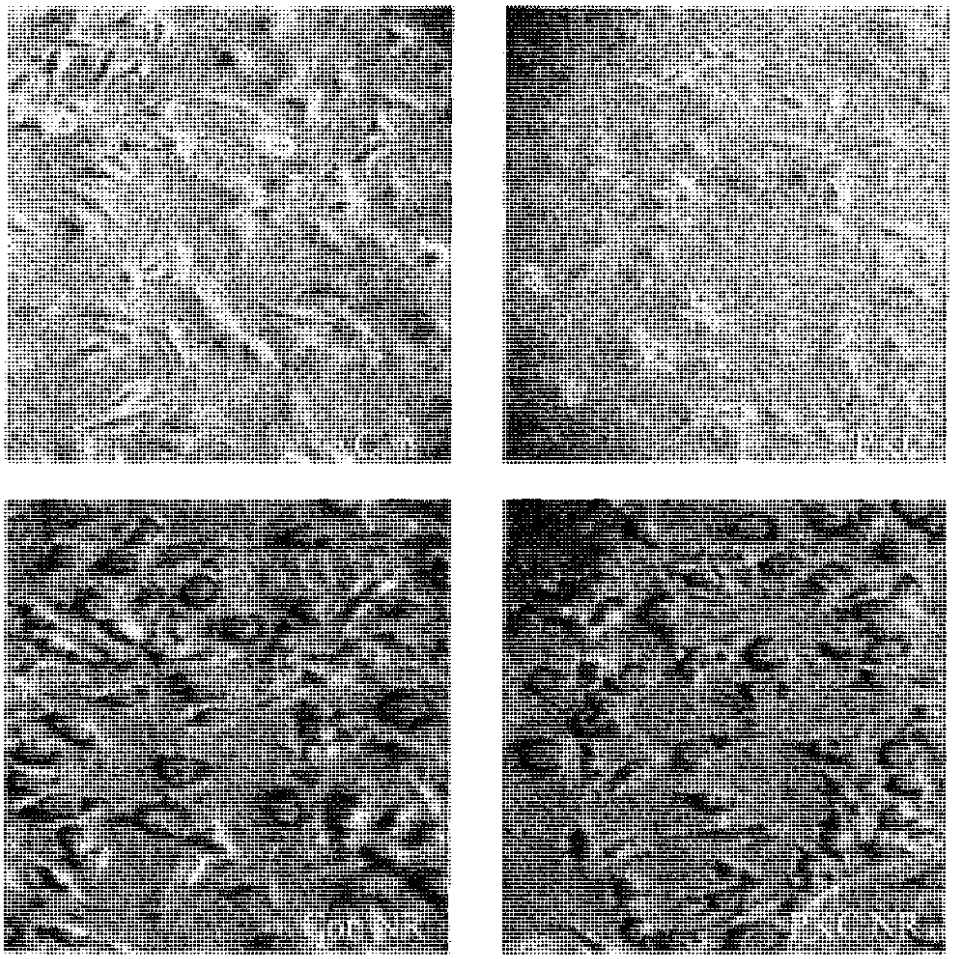
Figure 5. Regulatory impact of DNAPK phosphorylation of target residues of E1. (A) Autoradiographic film of Southern blot containing DNA extracted from BPV transformed C127 monolayer is shown. Included on film are lanes containing extracts from control samples (D0-D6) and extracts from CK2 inhibited samples (I0-I6). All sample sets contain extracts taken at day 0 (before exposure to chemical modifiers), day 2, day 4, and day 6. Major visible bands represent the viral genome (Vg) at approximately 8kbp and a slower moving band of either viral DNA that has become integrated with cellular DNA or uncut viral genome (Int) (B) Serine 305 is currently the only phosphorylation site which is predicted target for DNAPK. The surrounding sequence of the phospho-amino acid and method originally used to predict the residue as such a site is included in the table.

replication mechanism. Inhibition of DNAPK had noticeable effect on the concentration of viral DNA synthesized, and taking in to account this decrease in replicative activity; DNAPK could specifically be designated as a stimulatory kinase of E1 function.

Neutral Red Viability Assay. Under control conditions the BPV/C127 cells continuously grow and reach confluence from a 1:20 passage of monolayer cells in 4-6 days and exhibit the characteristic fibroblast spindle shape (Fig. 6A, top left). All plates were observed via light microscopy before addition of neutral red mixture, to ensure cells were consistently proliferating throughout the plates and to survey the cellular morphology of each trial's cells. There was no observable difference in visible density or cellular shape between the control plates and any of the inhibited samples. All plates possessed uniform distribution over the 10cm diameter dishes at each time point and no modified morphology was observed for any of the inhibited trials. The top right image in figure 6A shows a PKC inhibited plate at day three; this image is representative of all the inhibited plates, as there was no difference between them. The similarity seen between the plates suggests that no significant changes in cellular maintenance or structure had occurred due to the kinase inhibitions.

Neutral red (NR) dye was used to aid in the assay of the BPV/C127 cells' overall health and proliferation. Neutral red uptake is a procedure commonly used to test for chemical cytotoxicity, based upon the experimental chemical's effects on cellular metabolism. The weakly cationic dye easily passes through the cellular membrane and is collected inside lysosome vesicles through a process which is dependent upon intact plasma and lysosomal membranes and active metabolism of the cell [38 and 39]. Cells which have experienced any damage or cessation in active metabolism will not be able to accumulate the bright red dye in the lysosomes, as an active, healthy cell would be able to, and so can be readily distinguished.

A.



B.

Percent Viability of C127 Cells at Times of Chemical Exposure			
	Day 0	**Day 3**	**Day 6**
Control	100*	99.991	99.977
CDK	100*	99.989	99.975
CK2	100*	99.989	99.965
DNAPK	100*	99.995	99.969
PKC	100*	99.997	99.967

C.

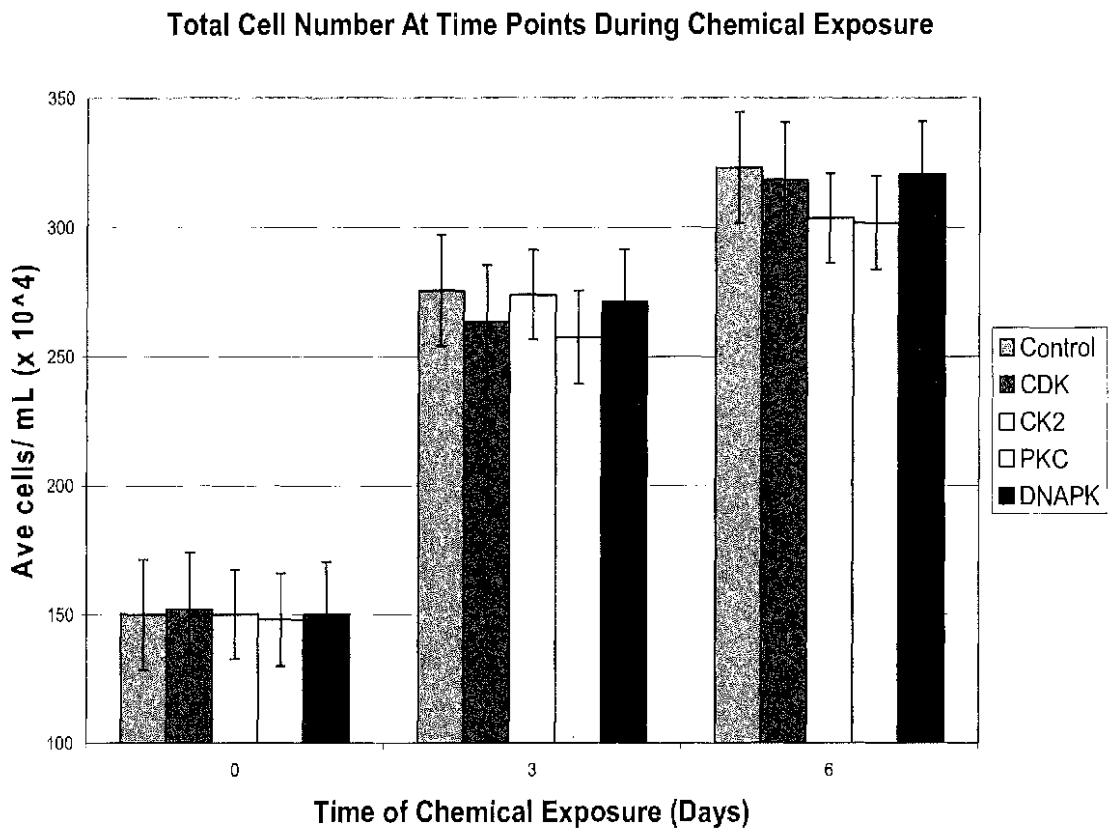


Figure-6. Viability and proliferative effects of chemical kinase modification on the BPV/C127 cells. (A) Cellular monolayer of C127 transfected with BPV-1 genome is shown in all fields. Top left image is a control sample (Con) of cells and top right is a plate of cells post addition of the PKC inhibitor, BMI (PKC). Bottom images are the same plates exposed for four hours to 1mL neutral red per 100mL complete DMEM mixture before observation (Con NR & PKC NR). All plates were observed through light microscope at day 3 of experiment, digital images taken at 100X magnification. Neutral red images were red enhanced for image color contrast. (B) Table includes calculated viability of cells taken at days 0, 3, and 6. All numbers reported as percent viable cells (number of viable cells/total number of cells observed). Viability was determined by each cell's ability to actively uptake the neutral red dye. * - Indicates that no cells were determined to be unviable (no non-colored cells seen). (C) Graphic representation of hemocytometer cell counts of control and inhibited samples at three different time points during experiment. Day zero counts were taken just before addition of chemical dilutent or modifier. Counts were also taken midway through (Day 3) and at the end (Day 6) of the chemical exposure.

The NR uptake assay was conducted as previously described with cell count, viability, and overall morphology observed on three separate time points for control and all types of inhibited samples. The PKC activator, TPA, was not included in this portion of the study due to the chemicals inability to produce any differentially observable effects from the control samples. Following each plates four hour exposure to the NR mixture, cells were again observed through a light microscope. There was no generally detectable difference between NR uptake of the cell plates at any time point. All viable cells that successfully accumulated the dye exhibited an expected change in morphology. The cells had a more swollen, rounded appearance, and a ring of red dye was seen around the nucleus. All plates demonstrated this cellular change indicating consistent uptake of the dye between the control and inhibited samples (Figure 6A, bottom left and bottom right images), but closer quantitative investigation is necessary to ensure that kinase inhibition is not significantly modifying cellular health and proliferation.

To acquire accurate cell viability and number counts, the cells were removed from the plates and a portion of the cellular suspension was placed on a hemocytometer. There were two plates used for each time point of each sample set and the counts were repeated eight times for each plate to ensure statistical significance. During each count total cell number and number of cells that had not accumulated the NR dye to a clearly discernable level were determined. Day zero plates did not contain a single cell which had not observably accumulated the dye (Fig. 6B). This was not surprising given that the cells had just recently been passaged and so were at a low density with plenty of fresh DMEM and no chemicals had yet been added. Total cell number was calculated for each

sample set and the average total cell numbers all fell within 148-152 x 10^4 cells/mL with a standard deviation within each set no more than +/- 18 x 10^4 cells/mL (Fig. 6C).

Midway through the maximum time of chemical exposure, day three, cells were again assessed for total number and viability. All plates were at approximately 70% confluence prior to NR exposure and cellular removal. Average total number of cells present varied more than those in day zero, but all averages remained with +/- 17 x 10^4 cells/mL of each other, an amount which is statistically insignificant (Fig. 6C). Closer observation of the cellular viability showed relatively few cells did not possess a detectable level of NR. There was a slight variation in amount of NR within the cells; however the vast majority of the cells viewed, exhibited the same relative concentration of NR as the cells seen in day zero. Those few cells with only a minimal amount of NR were counted as non-viable. Total number of non-viable cells remained less than .1% of the total cells observed (Fig. 6B).

By day six of chemical exposure, all plates were at or very near absolute confluence. The average number of total cells was highest in the control plates, closely followed by the DNAPK and CDK inhibited sets. The PKC and CK2 inhibited sample sets' average total number of cells were noticeably less than the other three sets, but remained within one standard deviation of their averages (Fig. 6C). The variation between the inhibited and control trials were not enough to declare any statistically significant difference among the assays. While the number of non-viable cells increased from the amount counted in day three, approximately 2 x 10^4 cells/mL, to 10 x 10^4 cells/mL, the percent viability assessment of the cells at day six was somewhat surprising. Despite the cellular density, lack of fresh media, and exposure to chemical

inhibitors for six days, the total number of cells raised enough for the percentage of viable cells to stay at around 99.9% for all sample plates, (Fig. 6B).

The collective results of the NR viability assay and total cell number counts, yield excellent support for the results from the viral replication assays. Kinase inhibition by the chemical modifiers BMI, Roscovitine, DMAT, and DMNB, do not appear to significantly alter cellular health or proliferation of the BPV/C127 cells. No difference in gross cellular structure, cell viability, or cell growth was determined to be considerable enough to declare that a change had occurred in cellular replication. Had then been a great difference in total cell number of any inbibited sample from the control, the integrity of the results of that kinase inhibitor's effect on viral replication would be in question, but because there was no significant difference in these variables, alteration of viral replication most likely resulted from the decrease in kinase phosphorylation of the bovine papillomavirus' E1 protein.

DISCUSSION

The results of these experiments provide a clearer picture of differential phosphorylation effects and add to the body of known information about regulation, in general, by establishing the effects of phosphate addition to the papillomavirus replication system. Relying upon chemical modification of specific kinases, this study has yielded evidence which assigns a regulatory role in viral replication of the bovine papillomavirus to the kinases PKC, CDK, CK2, and DNAPK. By analysis of the

concentrations of viral DNA between extracts from control and kinase inhibited BPV/C127 cells, specific activities were assigned to these kinases. This data in conjunction with the lack of impact these chemicals had upon cellular proliferation, maintenance, and viability suggests that PKC, CDK, and DNAPK phosphorylation of the BPV E1 protein lead to an increase in viral replication activity, while CK2 phosphorylation of this same protein causes a decrease in viral DNA replication. All kinases assayed in this study were predicted based upon manual or computer analyzed consensus sequence comparison of the identified phosphoresidues [2-4, 17, 25, 33]. Each enzyme has also been reported to have some role in cell cycle regulation and/or DNA metabolism, so it is not surprising that the results of this study implicate them as regulators of viral replication. This study only accounts for those kinases which regulate E1 during the latent state of infection, when the protein would be present in the undifferentiated cellular strata of the host. The kinases and their relative effects do not address vegetative replication.

By predicting the kinases of five newly identified sites and two already suspected sites the data gathered from mass spectrometric analysis served as an excellent starting point for this study, but not every phospho-residue, and so not every possible kinase, was definitively identified. It remains possible that some sites which are only rarely or briefly phosphorylated were missed in the analysis [17] and of course the kinases which recognize these sites would be overlooked. Therefore, it was of interest to this study to include those kinases which, based upon alternative analytic means, are predicted to phosphorylate other phospho-residues. Assays of serine 90 and serine 109 have strongly implicated these residues as phosphorylation sites [3, 33]. This data,

together with kinase's function in epidermal differentiation, provided enough support for PKC's role as a regulator to include it in the kinase assays.

Inhibition of PKC in a monolayer of BPV/C127 cells lead to a dramatic decrease in the concentration of viral DNA that was extracted from the cells. This finding in addition to the kinase's effects on repressed tumor suppression, rapid cell growth [15, 16], and a reported role in regulation of epithelial differentiation [3, 40], provides strong support for the enzymes stimulatory action in viral replication. The coupling of a regulator of skin differentiation status and viral DNA replication into the same kinase would serve as a great means for the virus to link its lifecycle with its host replication activity. The replication inducing activity of the kinase in this study contrasts with its activity observed in earlier studies. Serine 90 and serine 109 site specific studies found that a serine/alanine mutant of E1, which was unable to be phosphorylated at those residues, replicated more efficiently than the wild type E1 [3, 33]. Utilizing a system similar to that used in this study, it was reported that the inability of E1 to be phosphorylated by PKC lead to an increase viral replication activity. What lead to the dissimilar results of these studies is unclear at this time. However, it is quite possible that phosphorylation of one PKC residue versus phosphorylation of two or more causes different effects upon replication. It is important to note that the results of this study cannot definitively assert that the kinases predicted to target the identified phosphorylation sites are indeed acting on these sites. These results serve only as a comprehensive analysis of the kinase effects, not the effects of any site specific phosphate addition. A degree of overlap does exist between sequence consensuses of the kinases, so it remains possible, although unlikely, that the kinases are targeting a site

other that those predicted for it or targeting a residue missed in previous assays as a phosphorylation site.

CDK, a major regulator in cell cycle progression, has been shown to phosphorylate threonine 102 in vitro; however mutation study of the site, like those performed on S 90 and S 109, did not appear to significantly alter viral replication. Yet, based upon this study's autoradiographic data, cyclin dependent kinases (CDK) appear to donate their phosphate groups in a mechanism which promotes viral DNA replication. At this time CDK's other predicted residues, threonine 126 and serine 283 have not been analyzed. It may be that T 126 or S 283 site is the key replication regulator and mutation would cause a change in viral replication like that seen in this study or that all three sites must be phosphorylated to effect replication regulation, but further investigation of this site is needed to determine its function. While the E1 protein of the bovine papillomavirus does not appear to express the CDK target domain of nuclear export, as the human group does [21], the significant decrease in concentration of viral DNA replicated in those cells exposed to the enzyme's inhibitor signifies that CDK phosphorylation does have some effect on E1 activity. CDK has been reported as having stimulatory and inhibitory functions in replication of the cellular genome [36]. The dynamic status of this kinase may suggest that the enzyme differentially acts upon the regulatory E1 protein, based upon concentration, cellular activity, or other metabolic factor, in which case the results of the CDK assay in this study are only one facet of the kinase's total activity.

Several characteristics of the enzyme CK2 made it of great interest to this study; including its published effects upon replication of other viruses, high activity in

proliferating cells, and constitutive nature [18, 20]. This kinase has an incredibly diverse group of known targets of both cellular and viral nature with a wide range of function. Like CDK, it may be that this enzyme can lead to several different effects and this study has only elucidated one of those functions in the viral lifecycle. The CK2 assay clearly indicates that phosphate addition by this kinase serves as an inhibitory factor in viral replication. Of those residues predicted as targets for CK2, only serine 48 and serine 584 have been specifically analyzed. Mutation assay of the serine 48 residue suggested a stimulatory role for CK2 in viral replication, where substitution of the serine amino acid for alanine blocked phosphorylation at this site and yielded a dramatic decrease in the concentration of viral DNA replicated [25]. The S 584 residue, found in the ATPase domain on the carboxyl end of the E1 protein (Fig. 1), was studied by mutation assay and results showed a clear reduction in viral replication [1]. This kinase activity predicted by both mutant studies is opposite what was discovered by this study. However, as previously mentioned the mutation analysis experiments only analyze the function of a single residue's phosphorylation, while this study looks at the kinases' overall effects. The three other residues, S 94, S 95, and S 100, identified as targets for this kinase have yet to be studied. Specific study of these other residues may yield more definite explanations of this kinase's activity.

Results of the DNAPK assay provided evidence of the stimulatory action of the kinase's function in viral replication of the bovine papillomavirus. Inhibition of the kinase lead to a significant decrease in concentration of viral DNA replicated that was extracted from the BPV/C127 cells. This activity is not necessarily consistent with the known tumor suppressive functions of DNAPK, but is in line with the kinase's described

epidermal growth factor receptor activation [29, 37]. Activity of serine 305, a residue in the DNA binding domain of E1 [17], to date, has not specifically been analyzed.

While the information yielded from the autoradiographic films of the kinase trials are encouraging and certainly sheds some light on the respective regulatory roles of the kinases studied, this data alone cannot assert that the differential concentrations of viral DNA replicated is solely due to the alteration of E1 protein phosphorylation by those kinases. The very nature of the virus lifecycle further complicates this matter. All the kinases included in this study are supplied by the host cells, which the virus takes advantage of, and obviously the kinases' primary responsibly would be to carry out cellular functions. Any modification of these kinases, could hypothetically lead to an alteration of those cellular functions, such as; cellular replication, cell cycle progression, growth, cellular maintenance, etc. Therefore, if the chemical modifications used in the previously described experiments were causing great changes in the health or growth of cells exposed to the inhibitors then any changes in concentration of viral DNA would simply be an artifact of that change, and not due to changes in kinase regulation of the E1 protein. In other words, reduction in cellular replication, growth, and overall health, would directly lead to reduction of viral replication, which is so highly dependent upon the cellular machinery. To support any results derived from the replication assay experiments, any effects on cellular metabolism must be accounted for. The lack of impact upon overall cellular health and viability as assessed by the neutral red assay strongly dissociates the differential DNA abundance between the control and treated samples from a simple corruption of cellular activity. Instead, such results illustrate that

any differences between the relative viral DNA amounts are due to the modification in activity of the kinases and their effects upon the viral replication system.

This study utilized a cellular system similar to the native cells in the host epithelium. The cellular monolayer of C127 exhibited exponential growth, like the stratum basale layer of bovine epithelium undergoes during differentiation. Both experimental and native systems consist of ectoderm derived mammalian cells and possess the same kinases. There may exist some variation in replication activity of these kinases between the two cells types, but effects are believed to be subtle, given the reported consistencies of the enzyme actives between similar cells [15-21, 29]. The BPV transformed C127 cell monolayer system used in this study has been employed successfully by several other experiments with the papillomavirus [1-5, 33]. Our results support a general model for regulation of E1 function and viral DNA replication by the cellular kinases of the host organism.

Of immediate interest would be to utilize this system to gain a comprehensive understanding of these kinases effects on viral replication of the human papillomavirus (HPV). A phosphorylation site map of HPV, like that of BPV seen in figure 1, is not available at this time, but given the similarity of human and bovine epithelium and their native kinases, it would be reasonable to believe that comparable kinase effects would be observed. A clear image of the exact mechanisms by which this virus uses the cellular enzymes to regulate the replication of its viral life cycle will aid in the study and possibly the treatment of this virus. A virus which has grave effects upon the human population, including the approximately 70 strains of which have been attributed as a factor in the

development of cervical cancer, the second most common cancer in women worldwide [11].

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VITA

Joshua R. Raynes

Born, Savannah, Georgia.

Married, June 18 2004 in St. Augustine, Florida to Rachel R. Raynes.

Education

June 2000 -Graduated with honors from Nease High School. St. Augustine, Florida.

May 2004 –Graduated Summa Cum Laude with Bachelor's of Science in Biology, minor in Philosophy, from University of North Florida. Jacksonville, Florida.

June 2006- Completed degree requirements for Master's of Science in Biology from University of North Florida. Jacksonville, Florida.

Employment History

Aug 2005 to May 2006- Graduate teaching assistant at University of North Florida, Jacksonville, Florida.

Aug 2000 to Jan 2006- Certified Pharmacy Technician at CVS pharmacy, Jacksonville, Florida.

May 2004 to Aug 2005- Research Assistant at University of North Florida, Jacksonville, Florida.

June 2004 to Dec 2004- Neurology Intern at Jacksonville Neurological care center with Dr. Carlos Gama, Jacksonville, Florida.

Mar 2003 to June 2004- Patient care technician at St. Luke's Hospital Emergency Department, Jacksonville, Florida.

Publications

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