ABSTRACT

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One of the mechanisms by which papillomaviruses establish persistent infection of the host is by tethering their genomes to host chromosomes during mitosis. This ensures maintenance and partitioning of the viral genomes to daughter cells after each cell division. Although studies have shown that the viral E2 protein links the viral genome to host chromosomes in several papillomaviruses, the exact molecular mechanism of this interaction has yet to be elucidated for the beta-papillomaviruses. The studies described in this dissertation aimed to characterize the interaction of the E2 protein of the human papillomavirus type 8 (HPV8), a type of beta-papillomavirus, with mitotic chromosomes. The E2 protein consists of a conserved N-terminal transactivation domain and a C-terminal DNA binding and dimerization domain that are linked by a flexible hinge. We have mapped a sixteen amino acid region in the hinge that, when linked to the DNA binding domain, is crucial and sufficient for chromosomal association. Further we have identified two residues in

this region, arginine 250 (R250) and serine 253 (S253) within a highly conserved RXXS motif that are required for HPV8 E2 chromosome binding. Additionally, we have shown that the S253 residue is phosphorylated. To gain insight into the regulation of the E2 chromosome binding function, we investigated the role of phosphorylation of S253. We have shown that S253 is phosphorylated by PKA in S-phase, which increases the half-life of E2 protein and modulates its interaction with host chromatin. Since E2 is also involved in transcriptional regulation and viral genome replication, we examined if mutating residues R250 or S253 affected the transcriptional activation or replication functions of the HPV8 E2 protein. Furthermore using a domain swapping approach, we also explored the role of the C-terminal domain in the HPV8 E2 chromosome binding function. Finally to establish the mode of interaction responsible for mediating HPV8 E2 chromosome binding, we employed both a proteomics approach and ribonuclease treatment techniques, to examine whether HPV8 E2 chromosomal association is mediated through protein-protein or protein-RNA interactions, respectively. Collectively, these studies have added to our current understanding of the interaction of HPV8 E2 protein with host chromosomes.

CHARACTERIZATION OF THE INTERACTION OF THE HPV8 E2 TETHERING PROTEIN WITH HOST CHROMOSOMES

By

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Dedication

This dissertation is dedicated to my spiritual guru, Mata Amritanandamayi and to my parents for all their love, guidance and support.

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expressed in words, for all the sacrifices they have made and always provided me with the best in life. Mom and Dad thank you for being such great parents and for inculcating the right values in me. You are my role models and I hope I can be a great parent to Govind just like you are. I owe everything I am today to you. I can also never thank my husband, Gireesh enough for always believing in me and encouraging me in all my endeavors. You are the pillar of my life. I am also blessed to have my son, Govind who has brought so much joy and happiness in our lives. Finally, I would like to thank God for showering grace on me in the form of all the different opportunities I have had in my life.

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Abbreviations

ACA	Anti centromere antibodies
ATP	Adenosine triphosphate
bp	base pair
BCA	Bicinchoninic acid
BPV1	Bovine papillomavirus type1
CBP	CREB binding protein
CBS	Chromosome binding sequence
CENP	Centromere protein
CIN	Cervical intraepithelial neoplasia
CREB	cAMP response element-binding
CTD	C-terminal domain
CTX	Cholera toxin
DAPI	4', 6'-diamidino-2-phenylindole
DBD	DNA binding and dimerization domain
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBNA1	Epstein Barr nuclear antigen 1
EBV	Epstein Barr virus
EDTA	Ethylene diamine tetraacteic acid
ER	Endoplasmic reticulum
EV	Epidermodysplasia verruciformis

FITC	Fluorescein isothiocyanate
HC	Hinge and C-terminal domain
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
HMG	High mobility group
HPV	Human papillomaviruses
h	hour
Kb	Kilobase
KAP1	Krab-associated protein-1
KSHV	Kaposi Sarcoma herpesvirus
LANA	Latency associated nuclear antigen
LCR	Long control region
LDS	Lithium dodecyl sulfate
MARS	Matrix attachment regions
MCB	Mitotic chromosome binding
MEME	Multiple EM Motif Elicitation
MHC	Major histocompatibility complex
NH	N-terminal domain and hinge
NLS	Nuclear localization sequence
NMR	Nuclear magnetic resonance
NP40	Nonidet P40
NRIP	Nuclear receptor interaction protein
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis

PBS Phosphate buffer saline

PCNA Proliferating Cell Nuclear Antigen

PCR Polymerase chain reaction

PCV Packed cell volume

PKA Protein kinase A

PML Promyelotic leukemia

PP2A Protein serine/threonine phosphatases 2A

PRMT5 Protein arginine methyltransferases 5

PV Papillomaviruses

RFC Replication factor C

RG Arginine glycine

RIPA Radio-Immunoprecipitation Assay

RLU Relative light units

RNA Ribonucleic acid

RNAse Ribonuclease

RNAi RNA interference

RPA Replication protein A

RS Arginine serine

SAFA Scaffold attachment factor A

SDS Sodium dodecyl sulfate

SR Serine arginine

SRPK1 Serine-arginine protein kinase 1

SSC Saline-sodium citrate

TAD	Transactivation domain
TAP	Tandem affinity purification
TBP	TATA box binding protein
TE	Tris EDTA
UBF	Upstream binding factor
URR	Upstream Regulatory region
UV	Ultra violet
VLP	Virus like particles
WDR5	WD repeat containing protein
WSTF	Williams syndrome transcription factor
WT	Wild type

Chapter 1

Introduction

Portions of the section 'cellular targets of PV E2 proteins' were adapted from sections that I had written for a book chapter to be published in March 2012.

Koenraad Van Doorslaer, Vandana Sekhar, Jameela Khan, and Alison A. McBride (2012) - Replication and Maintenance of Viral Genomes by Association with Host Chromatin, Small DNA Tumor Viruses - Caister Academic Press

Chapter 1: Introduction

Classification of Papillomaviruses

Papillomaviruses (PVs) are a group of small, ubiquitous, double stranded, non-enveloped DNA viruses that are the causative agents of cutaneous and mucosal infections of both humans and animals [24]. Currently over 214 different types of PVs have been characterized (http://pave.niaid.nih.gov/#home). They are classified into different genera based on their nucleotide identity across the conserved L1 gene [24]. All sequenced papillomaviruses have been classified into various categories in the following order: genus, species, type and variant [58]. Based on the sequence of the L1 genes, PV types are classified into a genus if they show at least 60% sequence identity. If they show less than 60% sequence identity, they are grouped into another genus. Within a genus, if the L1 gene sequence shares identity between 71-89 %, the viral genome is labeled as a type and if the sequence shares identity between 60-70% it is classified as a species. Further, if the L1 gene sequence differs less than 2%, the genome is labeled as a variant [58,24].

Papillomaviruses are thought to have co-evolved along with their natural hosts. To date there are 29 different genera of papillomaviruses that have been characterized, of which five are human papillomaviruses (HPVs) [24]. There are twenty genera of non-human mammalian PVs, three of avian and one genus of reptiles [24]. The five different genera of HPVs include the *Alpha, Beta, Gamma, Mu and Nu* papillomaviruses [58].

***Alpha* PVs-** Members of the alpha-PV genus has been studied quite extensively primarily due to their clinical relevance. This genus contains mucosotropic viruses

that fall into two categories: high-risk and low-risk, depending on their propensity to cause infections that can progress into malignant carcinomas. High-risk viruses are associated with the development of anogenital cancers like cervical cancer, while the low-risk types rarely cause malignancies and are mainly associated with benign genital warts (reviewed in [30]). In addition, high-risk viruses are also associated with approximately 20% of the human oral cancers [289]. High-risk types include HPV16, HPV18, HPV31, HPV33 and HPV45. Examples of low-risk types are HPV6 and HPV11 [289].

***Beta* PVs**- Members of the beta PV genus infect the skin and hence, are cutaneous papillomaviruses. The most widely studied members of this genus are HPV5 and HPV8. Studies described in this dissertation are focused mainly on HPV8. Beta PVs are usually associated with asymptomatic infections in healthy individuals; but in immuno-compromised individuals these viruses cause lesions on the skin that can progress to squamous cell carcinomas after decades of infection [143]. Clinical manifestations of beta PV infections are most commonly seen in patients with a rare form of inherited autosomal recessive immune disorder called epidermodysplasia verruciformis (EV) [86]. HPV5 and HPV8 have been detected in 90% of squamous cell carcinomas in EV patients (reviewed in [185]). Most of these patients harbor mutations in two genes namely, EVER1 and EVER2 that are part of the EV1 locus on human chromosome 17. These genes belong to the transmembrane channel-like gene family [208]. However, since not all EV patients have been found to carry these mutations, it is likely that additional genes and loci are involved in EV development

[6]. Members of the beta genus have also been implicated in certain types of non-melanoma skin cancers [80]. However, the mechanistic contribution of beta PVs in the development of skin cancers is still not clearly established [58]. One of main reasons for this disparity is that infections with beta PVs are extremely common in general populations such that they are thought to be a part of the normal 'viral flora' of the skin. Thus, the presence of beta PVs in the skin surrounding the skin biopsies confounds the analysis of distinguishing between beta PVs resulting in malignant conditions from those that are part of normal viral flora of the skin [85].

Unlike the genome of high-risk alpha HPVs, the genome of beta PVs is not integrated into the host genome; rather, it is maintained as an extrachromosomal element during infection [78]. The focus of this dissertation is to elucidate the mechanism by which beta PVs maintain their genome as extrachromosomal elements in infected cells. This process is believed to be essential to establish persistent PV infection of hosts. Moreover, some studies indicate that HPV5 and HPV8 are oncogenic [60,192]. The oncogenic potential of HPV8 was first identified with the development of skin tumors in transgenic mice while expressing HPV8 early proteins from a keratin promoter [221]. More specifically, expression of HPV8 E2 protein has been shown to induce formation of skin tumors in transgenic mice [203]. Thus, the beta PV genus constitutes a group of viruses that share many unique characteristics.

Gamma, Mu and ***Nu*** **PVs-** Apart from the alpha and beta PVs, members of the rest of the genera of HPVs including *gamma, mu and nu* PVs infect the cutaneous epithelia of humans causing primarily benign warts.

Diseases associated with papillomaviruses

Papillomaviruses are the etiological agents of a broad spectrum of diseases. They cause infections that range from asymptomatic infections, benign lesions like common warts and anogenital warts to malignant carcinomas.

Benign lesions

Benign lesions include proliferative epithelial lesions that are referred to as 'warts' when appearing on the skin and 'condylomas' when occurring on genital epithelia. Many low-risk PV types such as HPV6 and HPV11 are associated with benign lesions [99]. These lesions may either regress spontaneously due to immunological response or persist indefinitely. HPV1 is responsible for causing plantar warts on the feet of infected individuals [74]. However, some papillomaviruses like HPV2 can target both cutaneous and mucosal epithelia [44].

Cancers

Among the different factors that can trigger development of cancer, approximately 15% of human cancers are associated with viruses [89]. Dr. Zur Hausen won the 2008 Nobel Prize in Physiology or Medicine for his pioneering efforts in discovering the connection between HPV and cervical cancer. Cervical cancer is the second most common cause of cancer death in women [197]. In the absence of HPV infection, development of cervical cancer is extremely rare. However, only small fractions of high-risk HPV infections that are not cleared by the immune system progress into carcinomas. Although HPV infection is essential, it is insufficient in many cases for development of cancer. There are many additional

factors that contribute to HPV induced malignancies. These include hereditary factors, immune suppression and various environmental cofactors. Progression to cervical cancer can be categorized into different stages of cervical intraepithelial neoplasia (CIN). It begins with a low grade lesion (CIN1), which progresses over time into CIN2 and then to a severe dysplasia (CIN3), and finally into a carcinoma that may metastasize [67]. High-risk types HPV16 and 18 together with the types HPV31, 33, 39, 45, and 58 are detected in about 97% of cervical cancer cases [268].

Cervical cancer is not the only cancer associated with HPV infections. HPVs have also been shown to exist in cancers of the oral cavity such as tonsils, base of tongue and the soft palate and the larynx [59,225,231]. As mentioned in the beta PV section, some cutaneous HPVs such as HPV5, 8, 12, and 23 cause lesions that further develop into squamous cell carcinomas, primarily in EV patients [193]. It is believed that, given its potential links to cervical, vulvar, penile, anal, oral, laryngeal and non-melanoma skin cancers, HPVs may contribute to about 10% of cancer cases worldwide [197,287].

HPV vaccine and prevention

Two prophylactic vaccines have been introduced to block initial infection with some of the high and low risk HPV types in young girls and boys. One is a quadravalent vaccine called Gardasil® (Merck) for protection against high-risk HPV16, 18 and low-risk HPV6 and 11. The second is a bivalent vaccine, Cervarix™ (Glaxo SmithKline) that protects against HPV16 and HPV18. Both vaccines consist of virus like particles (VLPs) formed from oligomerization of the L1 capsid proteins,

however, the adjuvants in which the VLPs are carried and the VLP expression systems differ (269). Since the two available vaccines are not effective in preventing cancer progression in already infected individuals and do not target infections with other high-risk HPVs, it is important for women to continue undergoing regular Pap smear screenings as a means for early detection of HPV associated infections.

Genome organization of Beta Papillomaviruses

The HPV8 genome is a circular double-stranded DNA that is 7654 base pairs long (Figure1.1). It encodes four early proteins (E1, E2, E6 and E7) and three late proteins (E4, L1, L2). The genome contains two promoters. The early promoter, p175 located within the long control region (LCR) regulates the transcription of early genes E1, E2, E6 and E7 [113,237]. The second differentiation-dependent promoter, p7535 is located at the 5' end of the LCR and transcribes the late genes E4, L1 and L2 upon late epithelial differentiation [98,213]. There is a short non coding region of 500 bp known as the LCR or upstream regulatory region (URR) located upstream from the coding region. This contains the viral replication origin [78]. In addition, this region also contains viral transcriptional enhancers such as specific conserved motifs called M33 and M29 and a unique AP1 site [214].

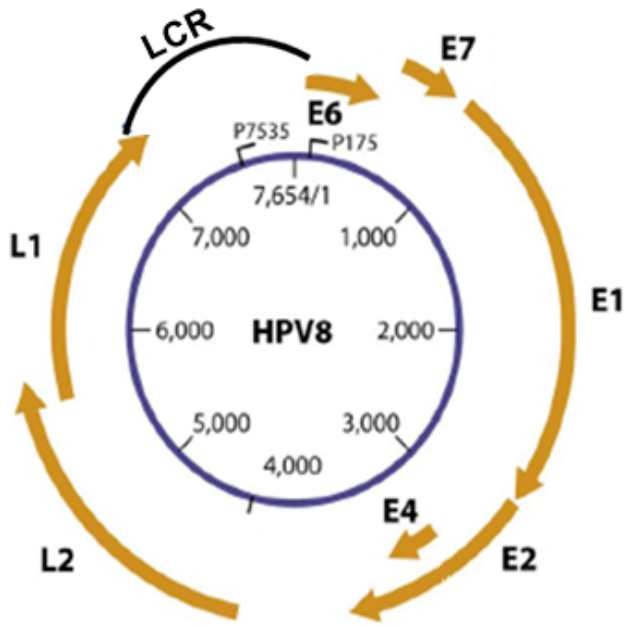


Figure 1.1
Genomic organization of HPV8. The genome is 7654 bp long coding for early proteins E1, E2, E6, E7 and late proteins E4, L1 and L2. The long control region (LCR) is shown and the early promoter, P175 and late promoter, P7535 are indicated. Modified from [144].

Proteins expressed by Papillomaviruses

Although the PV genome is small and encodes a limited number of viral proteins, these proteins interact with a multitude of cellular proteins to initiate an efficient, persistent infection that eventually results in the production of progeny viruses. The functions of the different papillomaviral proteins are described in detail below:

E1 protein

The PV E1 protein is an ATP-dependent helicase, approximately 70 kDa in size that binds to the viral origin of replication to initiate viral replication [280]. E1 consists of an N-terminal domain, a sequence specific DNA binding domain (DBD), an oligomerization domain and a helicase domain. During initiation of viral replication, both viral E1 and E2 proteins bind cooperatively to their respective binding sites within the replication origin [261]. The N terminal domain contains nuclear import and export signals that are important for shuttling the protein from the cytoplasm to the nucleus and vice-versa [62,118,149,158]. The helicase domain of E1 interacts with the N-terminal domain of E2 during the process of loading the E1 protein to the origin [218,234]. The E1 protein can bind to DNA either specifically via its DBD, which binds to E1 binding sites in the replication origin, or non-specifically via the helicase domain [46,218,234]. During initial binding, the presence of E2 in the complex prevents the non-specific binding of E1 by masking the DNA binding function of the helicase domain. But once the E2 protein dissociates, E1 converts to a double hexameric helicase ring encircling the DNA and can bind to DNA non-specifically [216,228,249,257]. The hexamers translocate bidirectionally in

the 3'-5' direction to unwind the DNA. The role of the oligomerization domain is to stabilize the interaction between the helicase domains by providing a rigid collar around the DNA [76]. E1 protein interacts with many cellular replication proteins such as replication protein A (RPA), DNA topoisomerase I, DNA polymerase α primase, proliferating cell nuclear antigen (PCNA), and polymerase δ and recruits them to the viral origin to proceed with viral genome replication [49,51,106,140,155,173,178].

E2 protein

E2 is a multifunctional protein that is required for viral DNA replication, regulation of viral transcription and is involved in viral genome maintenance. E2 protein consists of a conserved N-terminal transactivation domain linked by a highly flexible hinge region to a conserved C-terminal DNA binding and dimerization domain (CTD) [165][69,90,96].

N-terminal transactivation domain

The N-terminal domain is approximately 200 amino acids long and is a key regulator of viral transcription through its interaction with different cellular factors such as transcriptional factors and co-activators (reviewed in [167]). As mentioned previously, this domain is also involved in interaction with the E1 helicase domain to initiate viral DNA replication. The transactivation domain forms a cashew shaped structure consisting of three alpha helices in the N-terminal half linked by a fulcrum to a beta sheet region in the C-terminal region [11].

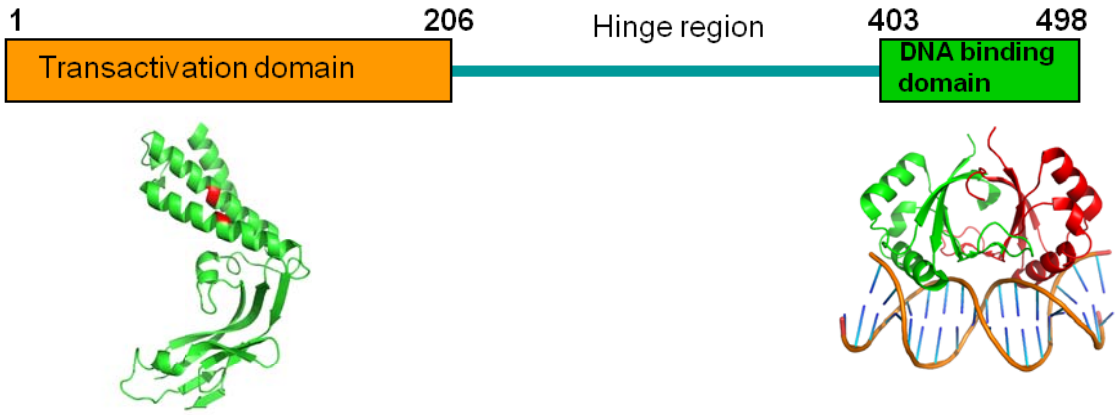


Figure 1.2
Schematic diagram of the full length HPV8 E2 protein
Schematic diagram representing the full length HPV8 E2 protein that consists of an N-terminal transactivation domain linked to the C-terminal DNA binding and dimerization domain by the flexible hinge region. The structures of the N-terminal and the C-terminal domains were reconstructed using PyMol (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC) [PDB ID-1R6K (HPV11 N-terminal domain) (105) and 2AYG (HPV6 CTD) [116,270]]. The N-terminal residues important for transcriptional regulation are highlighted in red. The dimer of the CTD is shown bound to an 18bp DNA target.

Different residues in the N-terminal domain are required for transcriptional regulation and replication. For instance, residues arginine 37 and isoleucine 73, located on the adjacent helices are important for transcriptional regulation in BPV1 E2 (Figure 1.2) [11] whereas, residue glutamate 39 of the E2 protein interacts with arginine 454 in the helicase domain of E1 for viral DNA replication [1].

C-terminal DNA binding and dimerization domain (CTD)

The CTD is approximately 100 amino acids long and is important for dimerization of the protein and binding to a 12bp palindromic consensus motif of $ACCN_6GGT$ in the viral genome [10]. It forms a dimeric beta barrel structure with each half of the barrel consisting of four beta strands and two alpha helices [110]. One of the alpha helices forms the recognition helix that recognizes specific DNA sequences in the viral genome [110]. This sequence specific interaction between the recognition helix and the DNA is mediated by both the backbone and the side chains of the alpha helix [110]. The E2 protein directly binds to the conserved sequences by inserting the recognition helix into the major groove of the DNA while the variable spacer regions in the minor groove are not contacted. Although the spacer sequence is variable, its conformation affects the relative affinity of the E2 proteins for the binding sites present on the viral genome [28,114]

Hinge region

The flexible hinge region of the E2 protein that links the N and the C-terminal domains is not well conserved and varies in size and sequence among the different

genera of PVs. However, within each genus the hinge contains certain functional elements that are conserved only among the related viruses. For example, the hinge of HPV11 E2 carries the nuclear localization signals [286], the HPV8 hinge carries elements required for mitotic chromosome binding and transcriptional regulation [235,248] and the BPV1 E2 hinge contains residues that contribute to E2 stability [201].

Interaction of the E2 protein with the E1 protein for initiation of viral DNA replication has been already described in the 'E1 protein' section. During transcriptional regulation of viral genes, binding of the E2 protein to binding sites of critical transcription factors such as Sp1 and TATA box within the LCR, results in transcriptional repression [97]. At high E2 levels, E2 protein occupies all the E2 binding sites and results in displacement of the TATA box binding protein (TBP) and Sp1 from their binding sites in the LCR. Thus, the E2 protein and the essential transcription factors, Sp1 and TBP, compete for their respective binding sites that are in close proximity to one another within the LCR and determine the fate of HPV transcription [23,54,255]. However, in addition to E2 occupying these binding sites, E2 repression also requires interaction with cellular proteins like Brd4 and other repressor complexes to repress transcription via its N-terminal transactivation domain [229]. Additionally, the E2 protein plays an important role in genome maintenance during persistent infection of hosts by linking the viral DNA to host chromosomes. Much of the initial studies understanding genome maintenance were carried out in BPV1. BPV1 E2 mediated mitotic tethering is carried out in association with a cellular partner identified as the Brd4 protein [281]. Mutations in the N-terminal

domain of BPV1 E2 protein (R37A and I73A) that abrogate Brd4 interaction also compromise E2's mitotic chromosome binding ability [19]. The C-terminal DNA binding domain of E2 is not absolutely required for the interaction with Brd4, but the dimerization function of this domain greatly increases the affinity of Brd4 for chromatin, most likely by enabling the assembly of higher order E2-Brd4 complexes [41].

Studies described in this dissertation aim to understand the mechanism by which HPV8 E2 protein interacts with mitotic chromosomes.

E6 protein

The E6 protein of high risk PVs is a small 18KDa viral oncoprotein containing two zinc finger domains [16]. Unlike E6 proteins of high-risk HPVs that consist of an additional PDZ (post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)) domain, which is involved in protein-protein interactions [182], beta E6 lacks a PDZ domain. One of the most important functions of E6 proteins is the induction of cellular proliferation and delayed differentiation of host cells. This is achieved through E6 binding and inactivation of the tumor suppressor protein p53 via its interaction with the cellular ubiquitin ligase E6-associated protein (E6AP) [222]. This interaction leads to the ubiquitination and subsequent proteosomal degradation of p53 [223]. Although E6 proteins from both high-risk and low-risk alpha HPVs bind p53, only high-risk E6 is capable of p53 degradation. However, the E6 protein of beta PVs such as HPV5 and HPV8 is unable to bind p53 [247]. It is not completely clear as to the mechanism of

p53 inactivation by beta E6 proteins. Instead, E6 proteins from multiple beta PVs have been shown to target the Bak protein for proteosomal degradation and in the process protect keratinocytes from UVB induced apoptosis [260]. Thus, this process is believed to contribute towards the progression of non-melanoma skin cancer. Additionally, HPV5 E6 expressing cells have been shown to be defective in repairing UV induced thymidine dimers [126]. Moreover, association of histone acetyl transferase p300 with E6 proteins from HPV5 and HPV8 results in its proteolytic degradation[117]. Further, the reduced levels of p300 in cells affect many downstream signaling events such as expression of differentiation markers. Thus, this process could delay the differentiation of cells [117]. Another speculated effect of p300 degradation is believed to be the inhibition of p53 transactivation [117]. This could be a mechanism by which beta E6 proteins affect p53 function. More recently, the HPV5 and HPV8 E6 proteins have been shown to downregulate the expression of interleukin-8 (IL-8), which can aid in progression of UV induced tumorigenesis [4]. In addition E6 proteins of these viruses can also immortalize rodent fibroblasts [75]. Studies of the beta E6 proteins indicate that they have different mechanisms that contribute to tumor progression compared to alpha E6 proteins.

E7 protein

In addition to the E6 protein, E7 is the other viral oncoprotein that is involved in cellular immortalization [20,179]. E7 is a small 11 kDa, acidic phosphoprotein containing a zinc finger domain and an LXCXE motif, which is necessary for binding to the cellular tumor suppressor retinoblastoma protein (pRB) [266]. E7 protein works

in conjunction with the E6 oncoprotein to delay host cell differentiation and induce immortalization by sustaining cells in an S-phase like state. The E7 protein of high-risk HPVs accomplishes this by binding to pRB and mediating its proteosomal degradation through the ubiquitin-dependent pathway. This results in the expression of S-phase genes and hence, maintains cells in a prolonged pseudo-S phase during which viral replication can take place [72]. The E7 protein from a beta PV type, HPV38 is capable of inactivating pRB and result in loss of regulation of cell cycle progression [40]. However, the E7 proteins of HPV8 and HPV5 weakly bind pRB proteins but do not degrade them and furthermore, cannot immortalize primary human keratinocytes [227,277]. Since, HPV5 and HPV8 are thought to be oncogenic; this suggests that beta E7 proteins might contribute to tumorigenicity via a different mechanism compared to the alpha PV E7 proteins. Additionally, the HPV8 E7 protein is speculated to play a direct or indirect role in viral DNA replication because of its ability to complement a E7 mutated BPV1 genome, which was observed to be deficient in viral DNA maintenance [122]. Thus, the above described studies indicate that the properties of beta E7 are different from that of the alpha E7 proteins.

L1 and L2 proteins

L1 and L2 are the major and minor capsid proteins, respectively, of PVs. PV capsids consist of 360 copies of L1, which are arranged as 72 pentameric capsomeres (pentamers) [47]. Each pentamer is made up of five monomers of the 55 kDa L1 major capsid protein [83]. These pentamers arrange to form an icosahedral lattice. The L2 minor capsid protein is located internally and consists of 72 molecules of the 74

kD minor capsid protein [38]. The L2 protein is multifunctional and involved in a number of functions such as genome encapsidation [190,224], interaction with L1 protein and capsid stabilization [83,125], and nuclear import of the HPV genome during infection [84].

Papillomavirus lifecycle

Initial stage of infection

PVs have evolved to infect distinct niches in the cutaneous and mucosal epithelia of their host, thus strictly limiting the cellular targets available for infection. The life cycle of papillomaviruses is tightly coupled to the epithelial differentiation of the infected host keratinocytes (Figure 1.3). In uninfected epithelium, the basal layer contains cells that replicate continuously giving rise to transit amplifying cells [25]. These cells divide a limited number of times and then undergo differentiation as they move upwards through the epithelial layers following emergence of new cells from the basal layer. As the cells move upwards, the keratinocytes lose their nuclei and other cellular compartments and finally, cells are sloughed off the superficial cornified layers. During viral infection however, viruses infect the dividing basal cells of the epithelium through microabrasions or wounds by binding to the heparan sulfate proteoglycans on the exposed basement membrane [226]. This process is followed by a conformational change in the viral capsid proteins L1 and L2, which finally results in the internalization of the virus through an as yet unknown secondary receptor on migrating keratinocytes [226]. Following entry and uncoating of the viral capsid in

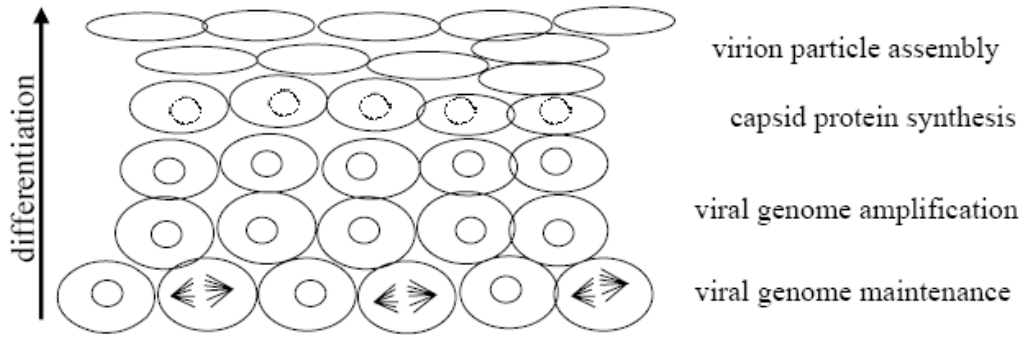


Figure 1.3
Diagram of the viral life cycle in a stratified host epithelium. The viral genome is maintained and partitioned in the lowermost dividing basal cells. Vegetative viral DNA replication occurs in the more differentiated layers and eventually cells from the outermost layer, containing the virus particles, are sloughed from the epithelium.

the late endosomes [130], the minor capsid protein L2, together with the viral genome, moves to the nucleus along the microtubule network [84]. It has been suggested that either the viral genome enters during nuclear membrane breakdown in mitosis [207] or is actively imported into the nucleus in complex with the L2 protein. Once inside the nucleus, it localizes close to the promyelotic leukemia oncogenic domains (PODs), possibly utilizing them for successful viral establishment [32].

Modes of replication

Upon infection, there is an initial establishment phase, wherein the incoming viral genome is translocated to the nucleus and replicated to a limited copy number to establish the infection. Following establishment, viral genomes are maintained as extrachromosomal elements that replicate in synchrony with host cellular chromosomes in the mitotically active basal cells. This stage of replication is classified as maintenance replication. For some papillomaviruses, it has been shown that the viral E2 protein maintains and partitions the viral genome by tethering it to the host chromosomes [123]. In addition to segregating the viral genome to daughter cells after each cell division, this process also ensures that the viral genome is retained within the nucleus of the infected cell. During the process of differentiation, the dividing cells of the basal layer migrate up to replenish the overlying, virus producing, differentiated cells. During the productive phase of infection called the amplification stage, in the upper layers of the stratified epithelium, replication switches to a vegetative mode. To ensure high amounts of viral DNA amplification for packaging into viral particles, viral proteins like E6 and E7 are expressed that

disrupt the normal process of cell differentiation and sustain cells in an S-phase like state as described previously [271]. Hence, these viral proteins delay the cell cycle exit of differentiating cells thereby, providing a cellular environment conducive to viral genome amplification. However, recent work indicates that viral DNA amplification occurs in the G2-phase of cell cycle [269] and the host DNA damage response is required for vegetative amplification [176]. Additionally during this phase, expression of E1 and E2 proteins result in a cellular DNA damage response that is believed to recruit the host DNA repair and replication machinery to viral DNA so that they can be utilized by the virus to replicate its genome [215]. Thus, progeny genomes are packaged into new viral particles.

Release of mature virions

Synthesis of the capsid proteins L1, L2 and assembly of virions occur in the terminally differentiated upper layers of the epithelium from where virus containing cells are shed. Thus being non-lytic viruses, papillomaviruses depend on the host epithelial cell differentiation program to release viral particles via the shedding of the upper epithelial squamous cells. The E4 protein is thought to play a role in viral egress by associating with keratin and resulting in collapse of the cytokeratin network [68].

Genome maintenance and viral persistence

One of the hallmarks of papillomaviral infection is its ability to cause persistent infection of the hosts. But one of the main challenges for the viruses

replicating their genome in dividing cells is to have a robust mechanism for viral genome maintenance. As described in the life cycle section, during the initial and maintenance stages of replication, the virus has to partition its genome in dividing cells to maintain them as low copy number episomes and retain them within the nucleus. In the case of PVs such as BPV1 the viral E2 protein acts as a molecular bridge and physically links their genomes to the host chromosomes, to ensure viral partitioning and nuclear retention following host cellular division. The earliest evidence of E2 protein's involvement in genome maintenance and physical interaction with host chromosomes came from observations wherein the BPV1 E2 protein and viral genomes were noted to be localized to discrete spots all over the arms of mitotic chromosomes [243]. Detailed studies in BPV1 established that the N-terminal domain of BPV1 E2, in association with the cellular protein Brd4, bound host chromosomes whereas the CTD of the E2 protein tethered the viral genome by binding to the E2BS on the viral genome [18,123,147,171,281]. In studies conducted in our laboratory, E2 proteins from different PVs were observed to bind to mitotic chromosomes in varying patterns [191]. This suggested that E2 mediated linking of viral DNA to host chromosomes could possibly be a common theme of genome maintenance among PVs. Unlike BPV1 E2, which was observed as small punctate dots all over chromosomes; the HPV8 E2 protein was observed to bind as large distinct foci on mitotic chromosomes. However, observation of HPV8 E2 protein in infected cells has been challenging. This is partly due to the low expression levels of E2 proteins in infected cells and partly due to lack of good antibodies against E2

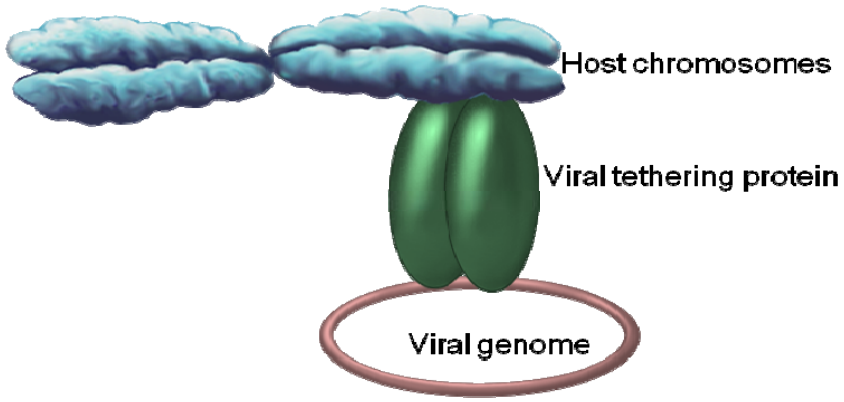


Figure 1.4
Model illustrating the tethering of viral genome to host chromosomes
Tethering the viral genome to host chromosomes is a common strategy employed by persistent viruses. Similar to the E2 proteins of PV, LANA of KSHV and EBNA1 of EBV also link the viral genome to host chromosomes.

proteins. Efforts to co-transfect and observe the viral genome and the E2 proteins in cells have not yielded positive results so far (Atasi Poddar, personal communication).

Nonetheless, tethering the viral genome to host chromosomes is a strategy employed by other persistent viruses such as the gamma herpesviruses EBV and KSHV. These viruses also express tethering proteins analogous to papillomavirus E2 protein. EBV encodes the EBNA1 tethering protein and KSHV encodes the LANA protein. EBNA1 consists of N-terminal and C-terminal domains linked by a central repetitive region composed of glycine and alanine residues. The chromosome binding regions of EBNA1 have been mapped to two short regions rich in glycine-arginine repeats. The C-terminal DNA binding and dimerization domain of EBNA1 is structurally similar to the CTD of E2 proteins [29]. Similarly, the CTD of LANA is also thought to have a structure similar to the E2 and EBNA1 CTDs [101,105]. The chromosome binding motifs of LANA are present in the N-terminal domain whereas the central domain consists of a non-conserved and long repetitive region. In addition, the LANA CTD by itself also binds to pericentromeric and telomeric regions on mitotic chromosomes [132].

Thus, utilization of similar strategies for genome partitioning and maintenance by different persistent viruses clearly highlights the essential role of this process in the viral life cycle.

Plasmid segregation in Prokaryotes and Eukaryotes

The process of segregation of genomic material is not limited to viruses. Segregation of low copy number plasmids can be observed in both prokaryotes and eukaryotes. To ensure efficient and faithful segregation of bacterial plasmids, a partitioning complex consisting of proteins called Par proteins is employed by bacteria [252]. ParA and ParB work in conjunction with the centromere like locus ParC to partition bacterial plasmids. The partitioning complex spatially localizes plasmids within the bacterial cell for proper segregation [252]. However, for partitioning of the yeast 2 micron plasmid that is present in almost all *Saccharomyces* yeast strains, the partitioning system consists of two encoded plasmid proteins, Rep1p and Rep2p and a cis-acting partitioning locus *STB* [94]. With respect to utilizing two proteins and a centromere like locus for segregation, both the bacterial and yeast partitioning system appear to be similar. However, there are no functional similarities between the two systems. Notably, one of the models to explain the segregation of yeast 2 micron plasmid proposes hitchhiking of the plasmid cluster, by means of tethering the duplicated cluster to the chromosomes via the cohesin complex [94]. Cohesin ensures attachment of duplicated plasmid clusters to the chromosomes by binding to *STB* locus, until the cell is ready to divide. During division, cohesin disassociates and the plasmids are segregated to daughter cells on the sister chromosomes [94]. This theme of hitchhiking on chromosomes is very close to the tethering mechanism of viral genomes to host chromosomes observed with papillomaviruses and gamma herpesviruses. There is also a second chromosome independent model for yeast plasmid segregation that although utilizes cohesin for

pairing plasmids, probably uses the mitotic spindle for the movement of the plasmids during division [94]. The features of chromosome segregation in higher eukaryotes vary slightly from those of yeast. In the eukaryotic system, cohesin is important for maintaining the sister chromatid pairing and unpairing mechanism and also helps differentiate sister chromatids from homologues during segregation. In addition to cohesin complex, another essential component of eukaryotic chromosome segregation is the condensin complex. Condensin complex is essential for preventing entanglement of chromosomes during segregation by maintaining the intramolecular compaction of DNA [115]. Finally cohesin undergoes proteolytic cleavage by enzymes such as separase, which results in chromosome sisters being separated and pulled away towards opposite cell poles by the mitotic spindle apparatus [183].

Although prokaryotes and eukaryotes employ distinct partitioning strategies for genome segregation, it is clear that the basic theme of transferring genetic information to progeny cells is evolutionarily conserved.

Cellular targets of PV E2 proteins

Although the E2 proteins from different PVs associate with host mitotic chromosomes to efficiently partition the viral genome to daughter cells, they interact with distinct chromosomal targets. This is mainly reflected by the fact that the E2 proteins from different PVs show different patterns of binding on the mitotic chromosomes [170,191,205,262]. The interaction of different viral tethering proteins with host chromatin could be direct, through binding to specific sequences on the host DNA, or could be mediated by association with cellular proteins or RNA binding.

Identifying different cellular protein partners that mediate the chromosomal binding functions of the various tethering proteins has been an area of intense research interest, especially because of their potential to be used as targets for novel antiviral therapeutics. The known targets of PV E2 proteins are described below.

Brd4

As described previously, BPV1 E2 mediated mitotic tethering is carried out in association with a cellular partner identified as the Brd4 protein [281]. Brd4 is a double bromodomain protein that binds to acetylated lysine residues on histones H3 and H4 [64]. Brd4 normally forms a diffuse coat around the mitotic chromosomes, but in the presence of E2 both proteins colocalize on mitotic chromosomes in punctate dots all over the mitotic chromosomes [19,124,170,171,282]. Brd4 is critical for E2 mediated transcription of all PVs [170,245,273], but is clearly not an essential cellular partner for genome partitioning in all the different PVs [124,170]. This is further supported by observations that R37A and I73A mutations that abrogate E2-Brd4 interactions, do not affect the mitotic localization of E2 proteins belonging to the alpha or the beta genus [170,205]. Notably, the EBNA1 and LANA proteins have also been shown to interact with Brd4 [152,283].

Ribosomal DNA (rDNA)

The HPV8 and HPV5 E2 proteins bind to the pericentromeric region of chromosomes as large distinct foci [191]. HPV8 E2 protein has been shown to associate with the ribosomal DNA loci present on the short arms of the acrocentric chromosomes where it colocalizes with the upstream binding factor (UBF) in mitosis

[205]. UBF is a transcription factor required for rDNA transcription by RNA polymerase I that remains bound to chromosomes during mitosis when transcription ceases [127,212]. During interphase, the HPV8 and HPV5 E2 proteins are seen localized both in a granular nuclear pattern and colocalized with SC35 speckles [141,235]. However, as cells transition from interphase to mitosis, there is disassembly of the nuclear membrane and nucleolus and the E2 protein is free to localize to rDNA. This spatial segregation during the different stages of the cell cycle would allow the E2 protein to actively participate in viral DNA replication and transcription during interphase and tether to host chromosomes for genome partitioning during mitosis. This separation might be essential considering the multitude of functions the E2 protein has to perform during these distinct phases of the cell cycle.

In the case of the alpha-PVs, under normal fixation conditions the alpha E2s are only observed on mitotic chromosomes during prophase and telophase. However, following a brief pre-extraction step that removes all the weakly bound nuclear proteins prior to fixation, the E2 proteins from alpha-PVs such as HPV11, HPV16 and HPV31 show a mitotic localization pattern similar to HPV8 E2. This suggests that the interaction of alpha E2s could be unstable and dynamic [191]. These E2 proteins might require a specialized cellular environment or additional cellular or viral factors to stabilize the binding of the E2 proteins to mitotic chromosomes.

Mitotic spindle, TopBP1, ChlR1 and MKlp2

Although HPV11, HPV16 and HPV18 E2 proteins have been reported to associate with the mitotic spindle during mitosis [55,262], it remains unclear how

these associations would result in the maintenance of the viral genomes within the host nucleus. In addition to being a transcriptional coactivator of HPV16 E2 [31], topoisomerase II-binding protein1 (TopBP1) also interacts and colocalizes with HPV16 E2 on mitotic chromosomes during late telophase [66]. The E2 proteins of BPV1, HPV11 and HPV16 interact with two proteins required for cellular chromosome segregation: ChlR1 (an ATP-dependent DNA helicase important for sister chromatid cohesion) [196] and mitotic kinesin like protein, MKlp2 (a motor protein required for cytokinesis) [284]. Although E2 and ChlR1 do not colocalize on mitotic chromosomes, it is believed that ChlR1 could load the E2 protein onto chromosomes [196]. On the other hand, the colocalization of MKlp2 and E2 has been observed in the mid-body during late mitosis [284].

It is clear that the interaction of E2 proteins with mitotic chromosomes is complex and may involve multiple different interacting partners to aid in viral genome maintenance.

Rationale for the Dissertation

As described previously, several studies have shown that the viral E2 protein acts as a molecular bridge to tether the viral genome to host chromosomes in different papillomaviruses. However, the exact molecular mechanism of this interaction has yet to be elucidated for E2 proteins of beta-papillomaviruses. The main objective of this dissertation was to characterize the interaction of the E2 protein of the HPV8, a member of beta-papillomaviruses, with mitotic chromosomes. In an attempt to gain a

detailed understanding of this interaction, we have addressed the following questions in this dissertation:

1. What domains of the HPV8 E2 protein are required for interaction with mitotic chromosomes? As described previously, several studies have shown that different PV E2 proteins interact with mitotic chromosomes in varied patterns. Previous work from our laboratory has shown that the requirements for HPV8 E2 chromosomal interaction are different from the well characterized BPV1 E2. The initial objective of my study was to map the regions required for chromosomal interaction in the HPV8 E2 protein using deletion analysis. Further, using a mutagenesis approach I identified residues essential for chromosome binding. In addition, I have also investigated the role of the C-terminal domain in the chromosomal association function.
2. How is the E2 chromosome binding function regulated? To gain further insight into how the HPV8 E2 mitotic chromosome binding function is regulated, I have examined the role of HPV8 E2 phosphorylation. I have also identified the protein kinase responsible for E2 phosphorylation.
3. Is the HPV8 E2 chromosomal association mediated through protein-protein or protein-RNA interactions? To obtain a clearer understanding of how E2 associates with host chromosomes, proteomics and RNAse treatment techniques were employed. Using these approaches, I have examined whether the E2 chromosomal association is mediated through protein-protein or protein-RNA interactions.

Understanding the mechanism of interaction of the HPV8 E2 protein with mitotic chromosomes will enable us to devise strategies that can interfere with the process of E2 tethering the viral genome to host chromosomes and thus, disrupt HPV genome persistence in infected cells.

Chapter 2: Materials and Methods

Plasmids

DNA fragments encoding domains or sub-domain regions of the HPV8 or HPV5 E2 proteins were amplified from plasmids expressing either full length HPV8 or HPV5 E2 proteins using PCR primers listed in the table below (Table 1). The PCR amplified products were first cloned into the pTZ19U *in vitro* expression plasmid with the appropriate restriction enzymes. The pTZ19U plasmid contains the T7 promoter. Using restriction enzymes HindIII and BamHI, the inserts from pTZ19U were cloned in the pMEP4 expression vector that contains an inducible metallothionein promoter. The fragments encoding the C-terminal domains (CTD) of E2 proteins from HPV107, SfPV1 (CRPV), LANA-CTD and mutated LANA-CTD (LANA-SHP) were PCR amplified with the appropriate restriction sites using the primers listed in table 1. The PCR amplified products were first cloned into the pTZ19U in vitro plasmid with the appropriate restriction enzymes followed by cloning in the pMEP4 expression vector. The HPV8 C-terminal domain was expressed either with a FLAG tag fused upstream of residues 404 (C) or with an additional CBP (Calmodulin binding protein) and SBP (Streptavidin binding protein) tag cloned between the FLAG tag and CTD (Tag-C). The fragments encoding the CTDs of BPV1 E2 and HPV11 E2 were sub-cloned from the respective pTZ19U plasmids encoding respective full length E2 proteins using ClaI and BamHI sites. The HPV4 CTD was sub-cloned into pMEP4 vector from the pTZ19U plasmid using BamHI and EcoRI sites. The CTD fusion proteins of different PVs were generated by cloning the PCR amplified CTD encoding fragments into the ClaI and BamHI sites of

a pTZ19U plasmid carrying the fragment encoding the HPV8 240-255 hinge peptide. From the pTZ19U plasmid, the inserts were cloned in the HindIII and BamHI sites of the pMEP4 expression vector. All E2 proteins were expressed with an N-terminal flag epitope tag from an inducible metallothionein promoter in the pMEP4 expression vector.

Table 1 – List of primers used for cloning

Name	Sequence (5'-3')	Restriction Sites	Purpose
AM1383	TATAAGCTTCCACCATGGCTACAGCCACCTCTAGGCGA	HindIII,NcoI	Forward primer; amplifies aa 313 to 498 of HPV8 E2
AM1384	CCTTTGGATCCTTATAGACTGTCCAGGTTACCATAAGATGTATC	BamHI	Reverse primer; amplifies aa 313 to 498 of HPV8 E2
AM1502	TATAAGCTTCCACCATGGCCGCCGCCAAGACCCCCACC	HindIII,NcoI	Forward primer; amplifies aa 216-498 of HPV8E2
AM1503	TATAAGCTTCCACCATGGGCTCCACCACCGTATCCAGGTC	HindIII,NcoI	Forward primer; amplifies aa 286 to 498 of HPV8E2.
AM1504	TATAAGCTTCCACCATGGGCAGAACAAGACCGCAAAAAGAGCAGAG	HindIII,NcoI	Forward primer; amplifies aa 255 to 498 of HPV8 E2.
AM1505	TTATTATCGATTCTGCTGGACGGCCGTCTCC	ClaI	Reverse primer; apmlifies aa 216 to 255 of HPV8E2.
AM1506	TATAAGCTTCCACCATGGGTCAAACCGAAACCAAAGGACGAAGGTA	HindIII,NcoI	Forward primer; amplifies aa 240 to 498 of HPV8E2.
AM1507	TTATTATCGATTTTGTTGTGGCTGTTTTGCAGGGGACC	ClaI	Reverse primer; amplifies aa 216 to 240 of HPV8E2
AM1508	TTATTATCGATCCTGGTGCGGTGTCGCGACCTT	ClaI	Reverse primer; amplifies aa 216 to 272 of HPV8E2.
AM1509	TTATTATCGATGGATACGGTGGTGGAGCCAACG	ClaI	Reverse primer; amplifies aa 216 to 290 of HPV8E2.
AM1591	TTATTATCGATCGACTTGGAAGATTACTGGAA	ClaI	C-terminal domain of HPV-5 E2.
AM1592	CCTTTGGATCCTTAAAGACTGTCCAGGTTGCCAT	BamHI	C-terminal domain of HPV-5 E2.
AM1593	TTATTCCATGGCCACCCCCGCGACCCCCACCACC	NcoI	216-255 of HPV-5 E2 hinge.
AM1594	TTATTATCGATGGAGGGCCTCCGTCCGTACCT	ClaI	216-255 of HPV-5 E2 hinge.
AM1675	CATGGGTCAAACCGAAACCAAAGGACGAAGGTACGGGAGACGGCCGGACAGCAGAAT	NcoI,ClaI	240-255 region of HPV-8 E2 hinge S253D
AM1676	CCAGTTTGGCTTTGGTTTCCTGCTTCCATGCCCTCTGCCGGCCTGTCGTCTTAGC	NcoI,ClaI	240-255 region of HPV-8 E2 hinge S253D
AM1677	CATGGGTCAAACCGAAACCAAAGGACGAAGGTACGGGAGACGGCCGTCCGACAGAAT	NcoI,ClaI	240-255 region of HPV-8 E2 hinge S254D
AM1678	CCAGTTTGGCTTTGGTTTCCTGCTTCCATGCCCTCTGCCGGCAGGCTGTCTTAGC	NcoI,ClaI	240-255 region of HPV-8 E2 hinge S254D
AM1679	CATGGGTCAAACCGAAACCAAAGGACGAAGGTACGGGAGACGGCCGGCAAGCAGAAT	NcoI,ClaI	240-255 region of HPV-8 E2 hinge S253A
AM1680	CCAGTTTGGCTTTGGTTTCCTGCTTCCATGCCCTCTGCCGGCCGTTCGTCTTAGC	NcoI,ClaI	240-255 region of HPV-8 E2 hinge S253A
AM1682	CCAGTTTGGCTTTGGTTTCCTGCTTCCATGCCCTCTGCCGGCAGGCGTTCTTAGC	NcoI,ClaI	240-255 region of HPV-8 E2 hinge S254A
AM1683	CATGGGTCAAACCGAAACCAAAGGACGAAGGTACGGGGCACGGCCGTCCAGCAGAAT	NcoI,ClaI	240-255 region of HPV-8 E2 hinge R250A
AM1684	CCAGTTTGGCTTTGGTTTCCTGCTTCCATGCCCCGTGCCGGCAGGTCGTCTTAGC	NcoI,ClaI	240-255 region of HPV-8 E2 hinge R250A
AM1685	CATGGGTCAAACCGAAACCAAAGGACGAAGGTACGGGAGAGCACCGTCCAGCAGAAT	NcoI,ClaI	240-255 region of HPV-8 E2 hinge R251A
AM1686	CCAGTTTGGCTTTGGTTTCCTGCTTCCATGCCCTCTCGTGGCAGGTCGTCTTAGC	NcoI,ClaI	240-255 region of HPV-8 E2 hinge R251A
AM1687	CATGGGTCAAACCGAAACCAAAGGAGCAGCATACGGGAGACGGCCGTCCAGCAGAAT	NcoI,ClaI	240-255 region of HPV-8 E2 hinge RR246,247AA
AM1688	CCAGTTTGGCTTTGGTTTCCTCGTCGTATGCCCTCTGCCGGCAGGTCGTCTTAGC	NcoI,ClaI	240-255 region of HPV-8 E2 hinge RR246,247AA
AM1689	CATGGGTCAAGCAGAAGCAAAAGGACGAAGGGCAGGGAGACGGCCGGCAGCAAGAAT	NcoI,ClaI	240-255 region of HPV-8 E2 hinge with all the possible phosphorylation sites replaced by Alanines.
AM1690	CCAGTTCGTCTTCGTTTTCCTGCTTC CGTCCCTCTGCCGGCCGTCGTTCTTAGC	NcoI,ClaI	240-255 region of HPV-8 E2 hinge with all the possible phosphorylation sites replaced by Alanines.
AM1691	CATGGGTCAAACCCAAACCAAAGGACGAAGGTACGGGAGACGGCCGTCCAGCAGAAT	NcoI,ClaI	240-255 region of HPV-8 E2 hinge E242Q
AM1692	CCAGTTTGGGTTTGGTTTCCTGCTTCCATGCCCTCTGCCGGCAGGTCGTCTTAGC	NcoI,ClaI	240-255 region of HPV-8 E2 hinge E242Q
AM1693	CATGGGTCAAACCGAAACCGCAGGACGAAGGTACGGGAGACGGCCGTCCAGCAGAAT	NcoI,ClaI	240-255 region of HPV-8 E2 hinge K244R
AM1694	CCAGTTTGGCTTTGGCGTCCTGCTTCCATGCCCTCTGCCGGCAGGTCGTCTTAGC	NcoI,ClaI	240-255 region of HPV-8 E2 hinge K244R
AM1977	TTATTATCGATCGGCTTCGCGAGCTTATAACA	ClaI	CTD of CRPV E2 protein
AM1978	CCTTTGGATCCCTAAAGCCCATAAAAATTCCC	BamHI	CTD of CRPV E2 protein
AM1979	TTATTATCGATCGACTTGGAAGGTTACTGGAT	ClaI	CTD of HPV107 E2 protein
AM1981	TTATTATCGATGTGGAAGAGCCCATAATCTTG	ClaI	CTD of LANA from aa 933 to 1162
AM1982	CCTTTGGATCCTTATGTCATTTCCTGTGGAGA	BamHI	CTD of LANA from aa 933 to 1162

Plasmids used for transactivation and replication assays: Fragment encoding the HPV8 origin from 7626-188 bp of the HPV8 genome is cloned between HindIII and EcoRI of pUC18 plasmid. HPV8 E2 proteins including the full length wild-type, S253A, R250A, R250A/S253A and R431K/R433K mutated E2 proteins were expressed from pMEP4 plasmids. HPV8 E1 protein was expressed from a pMEP9 expression plasmid. BPV1 E2 was expressed from a pMEP4 expression plasmid.

Mutagenesis

Two sets of E2 point mutations were generated. One set was designed in the background of a 40 amino acid region from the HPV8 E2 hinge (residues 216-255) fused to the C-terminal DNA binding domain (CTD). The second set was designed in the background of a 16 amino acid region from the hinge (residues 240-255) fused to the CTD. DNA fragments encoding the respective amino acid substitutions were chemically synthesized (GenScript Corporation) and cloned in frame with the CTD into the pTZ19U plasmid and the pMEP-4 expression plasmid. All E2 plasmids were sequenced to confirm the mutations.

Cells

The following cells were used for experiments described in this dissertation:

Parental Cells	Description	Culture Media
CV-1	African green monkey kidney cells	DMEM +10% fetal bovine serum+ penicillin(100U/ml)+ streptomycin (100µg/ml)+ 2mM glutamine
C33A	HPV negative human cervical carcinoma cells	DMEM +10% fetal bovine serum+ penicillin(100U/ml)+ streptomycin (100µg/ml)+ 2mM glutamine
U2OS	Human osteosarcoma cells	DMEM +10% fetal bovine serum+ penicillin(100U/ml)+ streptomycin (100µg/ml)+ 2mM glutamine

Establishment of stable pMEP-E2 cell lines

CV-1 and C33A cell lines stably expressing E2 were generated by transfection of pMEP-E2 plasmids with Fugene 6 (Roche) according to manufacturer's instructions and selection with 200µg/ml or 80µg/ml hygromycin B (Roche), respectively. After 2 weeks, drug-resistant colonies were pooled and cultures were expanded.

Immunoblotting

Cellular proteins were extracted in 2% SDS, 50mM Tris-HCl pH6.8, 10% glycerol and Complete® protease inhibitor (Roche). Protein concentration was determined using the BCA (bicinchoninic acid) protein assay kit (Pierce) according to manufacturer's instructions. For each sample, 12µg-20µg protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electro-transferred onto Immobilon-P membranes (Millipore). E2 proteins were detected with M2 anti-flag monoclonal antibody (Sigma) followed by horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Pierce). Immune complexes were detected on the membrane with the chemiluminescent reagent SuperSignal West Dura (ThermoScientific). Images were captured using the Kodak Image station system and quantitation was performed using Kodak MI software.

Immunoprecipitation and Immunoblotting

Cells extracts were prepared in modified RIPA buffer (20mM HEPES pH 7.0, 150mM NaCl, 1mM EDTA, 1% NP-40, 1% deoxycholate, 0.1% SDS) containing Complete® protease inhibitor cocktail (Roche) and PhosSTOP® (Roche). Protein concentration was determined using the BCA (bicinchoninic acid) protein assay kit

(Pierce). Equal amounts of total protein were immunoprecipitated using M2 anti-FLAG antibody beads (Sigma) at 4°C for 1h. Immune complexes were washed five times with RIPA buffer and E2 proteins were eluted in 50µl of Lithium dodecyl sulfate (LDS) sample buffer and separated on a 4-12% gradient polyacrylamide gel electrophoresis gel (Invitrogen). Proteins were electro-transferred onto Immobilon-P membranes (Millipore). E2 proteins were detected with M2 anti-FLAG monoclonal antibody (Sigma), phosphorylated E2 proteins with rabbit anti-RXXS motif antibody (Cell Signaling) followed by horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Pierce). Proteins were detected on the membrane with the chemiluminescent reagent SuperSignal Extended West Dura (Pierce).

In vivo phosphorylation assay

E2 protein expression was induced for 3h with 3µM $CdSO_4$ in medium containing 0.42mCi/ml [^{32}P]-orthophosphate or a combination of 0.2mCi/ml [^{35}S]-methionine and [^{35}S]-cysteine. Cells extracts were prepared in modified RIPA buffer (20mM HEPES pH 7.0, 150mM NaCl, 1mM EDTA, 1% NP-40, 1% deoxycholate, 0.1% SDS) containing Complete® protease inhibitor cocktail (Roche) and PhosSTOP® (Roche). E2 proteins were immunoprecipitated using M2 anti-flag antibody beads (Sigma). Immune complexes were washed five times with RIPA buffer and E2 proteins were eluted in 2% SDS-PAGE sample buffer and separated by SDS-PAGE. Gels were fixed, dried and autoradiographed. Quantitation was performed using PhosphorImager (Typhoon, Molecular Dynamics) and ImageQuant software (GE).

PKA inhibitor and enhancer treatments

CV-1 cell lines expressing E2 proteins were plated onto 10 cm plates so as to achieve 80-85% confluence at harvest. The following day cells were pretreated for 2h with PKA inhibitors, 10μM H89 (Calbiochem 371962) or 6μM KT-5720 (Tocris 1288) or PKA enhancers, 10μM forskolin (Calbiochem 344270) or 100ng/ml cholera toxin (Calbiochem 227036). Following pretreatment, E2 expression was induced with 3μM $CdSO_4$ for 3h in the presence and absence of the inhibitors or enhancers. Samples were prepared for immunoprecipitation and immunoblotting as described below.

Indirect immunofluorescence

CV-1 cell lines expressing E2 proteins were grown on Superfrost Plus slides. For cell cycle experiments, cells were synchronized in 2mM thymidine for 16h. The thymidine was removed to allow progression of the cell cycle and the cells were fixed at indicated time points in 4% paraformaldehyde for 20 mins at room temperature. E2 expression was induced with 3μM $CdSO_4$ for 3h before fixation. For mitotic synchronization, thymidine was removed and cells were cultured for an additional 9h, with E2 expression induced by the addition of 3μM $CdSO_4$ during the last 3h. Cells were fixed in 4% paraformaldehyde for 20 mins at room temperature and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 15 mins at room temperature. The E2 proteins were detected with monoclonal anti-FLAG M2 antibody (1:500; Sigma, F3165), UBF with rabbit anti-UBF antiserum (1:100; Santa Cruz Biotechnologies, sc-9131) and ACA with human anti-ACA antibody (1:500; Immunovision, HCT0100). Slides were stained with DyLight

secondary antibodies (Jackson ImmunoResearch). Slides were mounted in Prolong Gold (Invitrogen) containing 1μg/ml DAPI (4', 6'-diamidino-2-phenylindole). Images were collected with a Leica TCS-SP5 laser scanning confocal imaging system.

Protein complex purification for the proteomics study:

Preparation of Nuclear Extracts

Each of the E2 expressing C33A cell lines were plated on four, 15 cm dishes at a concentration of $8X10^6$ cells per dish and harvested after 48h following E2 induction with 3μM cadmium sulfate for 4 h. Cells were harvested using the protocol described below:

Buffer A (hypotonic buffer)	2X Buffer C	Buffer D
10 mM Hepes, pH 7.9 10 mM M KCl 1.5 mM $MgCl_2$ Complete protease inhibitor (Roche)	20 mM Hepes, pH 7.9 0.42 M NaCl 1.5 mM $MgCl_2$ 0.2 mM EDTA Complete protease inhibitor (Roche)	20 mM Hepes, pH 7.9 100 mM KCl 1.5 mM MgCl2 0.2 mM EDTA

Inhibitors used:
1 mM PMSF
1 mM Na_3VO_4
0.1 mM Na_2MoO_4
10M NaF

Cells on plate were washed with PBS twice and scraped into 10ml PBS. Cells were pelleted at 1,200 rpm, 4°C for 5 minutes. Pellets were resuspended in 5-10 PCV (packed cell volume) of ice cold Buffer A and incubated on ice for 10 minutes followed by centrifugation at 1,200 rpm, 4°C for 5 minutes. Pellets were again resuspended in 2-5 PCV of ice cold Buffer A and the cell suspensions were

homogenized by passing them through the homogenizer by 10-20 strokes. Contents of the homogenizer were transferred to centrifuge tubes. Suspensions were again centrifuged at 2000 rpm, 4°C for 5minutes. Pellets were resuspended in 1-2 volume ice cold 2X Buffer C and passed through the dounce homogenizer by 6 strokes. The suspensions were kept on a stir plate that was placed on ice for 30-45 minutes. The homogenized suspensions were then transferred to centrifuge tubes and centrifuged at 15000 rpm, 4°C for 30 minutes. Supernatants were collected into chilled tubes and dialyzed against Buffer D for 3h. The extracts were again centrifuged for 10 minutes at 4°C.

The nuclear extracts (1.6ml total volume) were divided into two fractions: one fraction was the input fraction and rest of the extracts was used for immunoprecipitation. For input fraction, 112µl of the nuclear extracts + 40µl LDS loading buffer + 8µl DTT were mixed together. For the immunoprecipitation assay the M2 anti-flag coated antibody agarose beads were used as described under the section immunoprecipitation and immunnoblotting. Following immunoprecipitation, the samples were eluted twice; once with flag peptide elution (150µl) and second time with sarcosyl elution (150µl). 12.5µl of each of the eluates were mixed and loaded on a NuPage 4-12% gradient polyacrylamide gel. 5µl of the input lysate for each of the sample was run on a separate NuPage 4-12% gradient polyacrylamide gel. The rest of the immunoblotting protocol is described under the section Immunoprecipitation and Immunoblotting.

Table 2- List of antibodies used for the proteomics study

Antibody	Company	Dilution	Species
UBF	Santa Cruz Biotechnologies, sc-9131	1:1000	Rabbit
WSTF	Sigma, W3641	1:1000	Rabbit
WDR5	Abcam, ab22512	1:1000	Rabbit
PRMT5	Bethyl laboratories, A300-850A	1:1000	Rabbit
Matrin 3	Abcam, ab51081	1:1000	Rabbit
SAF-A	Abcam, ab10297	1:1000	Mouse
SRPK1	BD Biosciences, 611072	1:1000	Mouse
TRIM28	Abcam, ab22553	1:1000	Mouse
U5 116	Proteintech group, 10208-1-AP	1:1000	Rabbit
Nucleophosmin	Abcam, ab24412	1:1000	Rabbit
PP2A	Cell Signaling technology, 2038	1:1000	Rabbit
Flag	Sigma, F3165	1:10000	Rabbit

RNAse A treatment followed by Immunofluorescence

Preparation of RNAse A:

RNAse A powder (Roche, 109169) was dissolved at a concentration of 10mg/ml in 0.01M sodium acetate pH 5.2. The solution was then boiled at 100°C for 15 minutes, followed by slow cooling to room temperature. pH was adjusted with addition of 0.1 volumes of 1M TrisHCl pH 7.4.

RNAse A treatment of E2 expressing cells: CV-1 cell lines expressing E2 proteins were grown on Superfrost Plus slides so as to achieve 80-85% confluence. For mitotic synchronization, thymidine was removed and cells were cultured for an additional 9h, with E2 expression induced by the addition of 3μM $CdSO_4$ during the last 3h. Cells were permeabilized with 0.1% Triton X 100 and 0.1% Tween 20 for 15 minutes. RNAse A treatment was carried out with the addition of 1mg/ml RNAse A in PBS onto slides for 10 minutes at room temperature. Following RNAse A treatment, cells were washed twice with PBS for 3 minutes at room temperature. Cells were then fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature. Following fixation, slides were immunostained with M2 anti-flag (1:500) and rabbit anti-fibrillarin (1:500) antibodies as described in the protocol in the immunofluorescence section. For E2 expressing cells that were to be stained with SYTO RNASelect stain, cells were permeabilized with 0.1% Triton X 100 and 0.1% Tween 20, treated with RNAse A for 10 minutes at room temperature followed by fixation with chilled methanol at -20°C for 10 minutes and chilled acetone at -20°C for 2 minutes. Following fixation, slides were immunostained with M2 anti-flag (1:500) and SYTO RNASelect stain according to the protocol described in the indirect immunofluorescence section. Slides were mounted in Prolong Gold (Invitrogen) containing 1μg/ml DAPI (4', 6'-diamidino-2-phenylindole). Images were collected with a Leica TCS-SP5 laser scanning confocal imaging system.

Replication assays

Transfection of U2OS cells and extraction of DNA: Using the Amaxa Nucleofector kit V, U2OS cells were transfected with 2μg plasmids according to the

manufacturer's instructions. Three days post transfection cells were harvested and low molecular weight DNA was extracted using the following protocol:

Extraction of the low-molecular weight DNA by HIRT extraction:

Solution I	Solution II	Solution III	HIRT digestion buffer
50mM glucose 25mM Tris-Cl (pH8) 10mM EDTA (pH8)	1%SDS 0.2N NaOH	3M KOAc (pH5)	20mM Tris (pH8) 100mM NaCl 10mM EDTA 0.2% SDS 200µg/ml. proteinase K.

Three days post transfection, cells were trypsinized and resuspended in 200µl of Solution I and then lysed by adding 400µl of Solution II made fresh before each extraction. Samples were incubated on ice for 5 minutes. Lysed samples were neutralized by addition of 300µl of Solution III, followed by incubation on ice for 10 minutes. Following neutralization, samples were centrifuged at 4°C for 10 minutes. The nucleic acids were collected and precipitated with 0.6 volumes of isopropanol and incubated at -20°C overnight. Precipitated DNA was centrifuged and resuspended in 200µl of HIRT digestion buffer. The resuspended solution was incubated at 37°C for 30 minutes followed by incubation at 50°C for 30 minutes. Samples were purified by a phenol\chloroform extraction, followed by a chloroform\isoamyl alcohol extraction using Phase Lock Gel tubes (Eppendorf). Pelleted DNA was resuspended in 200µl of TE and reprecipitated with 0.3M sodium acetate and 2.5 volumes ethanol. Finally, DNA was washed in 70% ethanol, resuspended in 50µl TE containing 20µg/ml RNAse A, followed by incubation for 20 minutes at 68°C.

The Hirt extracted DNA (25µl) was digested with the appropriate restriction enzymes including DpnI. DNA samples were digested overnight at 37°C. DNA samples were separated on a 0.8% agarose gel overnight at 25V.

Southern Blotting:

Denaturing Buffer	Transfer Buffer	Pre-hybridization buffer	Hybridization buffer	Neutralization buffer	Wash buffer
3M NaCl 0.4M NaOH	3M NaCl 8mM NaOH	3X SSC, 2% SDS, 5X Denhardts 200µg/ml ssDNA	3X SSC, 2% SDS, 5X Denhardts 200µg/ml ssDNA	1M Sodium phosphate buffer pH6.8	0.1% SDS 0.1X SSC

DNA samples were transferred onto Nytran SPC membranes (Whatman) using the TurboBlotter system, according to manufacturer's instructions. Following transfer for 4h at room temperature, the membrane was cross-linked with a Stratalinker UV Crosslinker (Stratagene) at 1200µJ/cm2. The probe was prepared by digesting either the full length HPV8 genome with the appropriate enzymes (BamHI) to release the complete HPV8 genome from the pUC9 cloning vector or by digesting HPV8 ori plasmid (EcoRI) to linearize it. The digested DNA was purified using QIAquick Gel Extraction Kit. The purified DNA was then radiolabeled with ^{32}P dCTP (3000 Ci/mmol) using the Random Primed DNA labeling Kit (Roche). Unincorporated nucleotides were removed by passing the samples through the Illustra ProbeQuant G-50 Micro Columns (GE Healthcare). Meanwhile, membranes were incubated in pre-hybridization buffer at 68°C for at least 1 hour before hybridization. $1x10^6$ counts/ml of either the HPV8 radiolabeled genomic DNA or the radiolabeled HPV8 ori plasmid were added to the hybridization buffer and incubated in a

hybridization oven at 68°C overnight. The next day, the membranes were washed until no background signal was detected and exposed to MS film (Kodak) or a Phospho imager screen and analyzed using Typhoon (Molecular Dynamics).

Luciferase assay for E2 transactivation:

Transfection and preparation of cell lysates:

CV-1 cells were plated onto 60mm dishes in triplicate at a density of 2.5 x 10^5 cells per dish. The following day different amounts of the E2 expression plasmids ranging from 0ng, 25ng, 100ng, 250ng and 1000ng were co-transfected with 1000ng of luciferase reporter plasmid pBS1073 using 6µl of Fugene 6 reagent, according to manufacturer's protocol. Cells were harvested at 48h post transfection. After washing once with 1X PBS, the cells were extracted in 500µl of 1X luciferase cell culture lysis buffer. Samples were centrifuged at 12,000g for 2 minutes at 4°C. Supernatants were transferred to new tubes that could either be stored at -70°C or could be used for assaying using a luminometer.

Luciferase assay:

10ml of the luciferase assay buffer was added to the lyophilized luciferase assay substrate and equilibrated to room temperature before use. 100µl of the luciferase assay reagent was dispensed into luminometer tubes, one tube per sample. A Zylux Femtomaster FB12 luminometer was used to measure luminescence. Samples were read in triplicate and the readings were recorded as relative light units per second or RLU/second.

Dimerization assay

Co-immunoprecipitation assay:

E2 proteins were either translated independently or co-translated in vitro in the presence of [^{35}S] methionine, PerkinElmer Easy Tag L- [^{35}S] methionine, using the TnT coupled reticulocyte lysate systems (Promega). The incorporation of [^{35}S] for the different in vitro translated products was determined using the ImageQuant software. For the immunoprecipitation step, equivalent counts of single translated and co-translated proteins were used in a total volume of 24µl. From the 24µl of the mix, 2µl of each mix was kept for input lane. To the remaining 22µl of the mix, 78µl of NET-200 buffer was added to make up the final volume to 100µl. For immunoprecipitation, 40µl of M2 bead slurry per protein sample was aliquoted into a microfuge tube (total 400µl for 10 reactions), followed by centrifugation at 8500g for 30 seconds. The pelleted beads were washed twice with 1ml of NET-200 buffer and made up to original volume of 400µl with NET-200 buffer. 40µl of the resin was aliquoted into a new microfuge tube for each of the protein samples to be tested. The lysate mix was added to each of the tubes and incubated at 4°C for 1 hour on a rotator. Following incubation, the resin was washed three times with 1ml NET-200 buffer, spinning at 8500xg for 30 seconds each time. The samples were eluted in 50µl of 2X SDS sample buffer without DTT by heating at 100°C for 3 minutes. After adding 50mM DTT, 25µl of the eluted samples were separated on a 4-12% NuPage gradient polyacrylamide gel.

Identification of Putative E2 binding sites (E2BS)

The URR sequences of all the members of the alpha (75 types), beta (44 types), gamma (25 types) and delta (10 types) genera of PVs were obtained from the Papillomavirus Episteme (PaVE) database website (http://pave.niaid.nih.gov/#home). The sequences were loaded into the Multiple EM Motif Elicitation (MEME) software to create a consensus motif sequence for E2 binding site within each genus based on the criteria of looking for palindromic sequences with a minimum motif width of 6 residues and maximum motif width of 15 residues. Identified putative E2BSs were displayed as a sequence logo for each of the genus to compare the differences between the E2 binding consensuses between each of the PV genus. The sequence logo indicates the conservation of the nucleotides within the binding site.

Calculation of percent similarity among different PV E2 CTDs:

A fasta file containing the amino acid sequences of the HPV11, BPV1, SfPV1, HPV4, HPV107, HPV5 and HPV8 E2 CTDs was loaded into the BioEdit program (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The sequences were aligned using ClustalW. To calculate the percent similarity, each pair of sequences was aligned using the pairwise alignment option. Within the pairwise alignment option, the percent of similarity between the selected sequences was calculated using the option calculate similarity and/or identity.

Chapter 3

The following chapter is reproduced from a previously published manuscript:

Sekhar, V., Reed, S. C., and McBride, A. A. Interaction of the betapapillomavirus E2 tethering protein with mitotic chromosomes. J Virol 84(1). 2010.

Contributions: I wrote the manuscript and performed most of the experiments. Reed, S. C performed the SR speckle localization and part of the deletion mapping experiment and McBride, A. A. performed one of the in vivo HPV8 E2 phosphorylation assays and performed the alignments

Chapter 3: Interaction of the Betapapillomavirus E2 Tethering Protein with Mitotic Chromosomes

Introduction

The life cycle of papillomaviruses is coupled to the epithelial differentiation of the host keratinocytes. Infection is initiated when viruses enter the basal cells of the epithelium, through microabrasions or wounds. Upon infection, the viral genome replicates to a low copy number and is maintained as an extrachromosomal element that replicates in synchrony with the host cellular DNA. For some papillomaviruses, the viral E2 protein maintains and partitions the viral genomes by tethering them to the host chromosomes [18,123,147,243]. This mechanism ensures that the virus retains its genome within the nucleus of the infected cell and partitions its DNA to daughter cells after each cell division. This process helps maintain a persistent infection of the host; the dividing basal cells provide a reservoir of infected cells that migrate up to replenish the overlying, virus producing, differentiated layers. In the productive phase of infection, genome amplification, synthesis of the capsid proteins and assembly of virions occurs in the differentiated upper layers of the epithelium from where virus containing cells are shed.

Human Papillomavirus type 8 (HPV8) and type 5 (HPV5) belong to the betapapillomavirus genus. They infect the cutaneous epithelium of humans and cause asymptomatic infections in healthy individuals [108]. However, in individuals with epidermodysplasia verruciformis (EV), a rare, inherited immune disorder, infection results in flat wart-like papules on the skin, which become cancerous after decades of

infection [86]. The betapapillomaviruses have also been implicated in non-melanoma skin cancers [81].

The HPV E2 protein is multi-functional; it is involved in initiating viral DNA replication and regulating viral transcription, in addition to maintaining the genome as an extrachromosomal replicating element. The full length E2 protein consists of an N-terminal domain (approximately 200 amino acids) linked by a flexible hinge region to a CTD (approximately 100 amino acids) [168]. The E2 protein regulates viral transcription by binding to a 12bp palindromic sequence with a consensus motif of $ACCN_6GGT$ that is present in the LCR of the viral genome [10]. The N-terminal domain is important for the transcriptional regulation function of E2, interaction with the E1 replication protein, as well as interaction with many cellular proteins that are required for the transcriptional regulation of viral genes [177,209,233]. In BPV1 E2, the N-terminal domain associates with mitotic chromosomes in complex with the Brd4 protein [19,171,281].

A study from our laboratory analyzed the mitotic binding phenotype of the E2 proteins from thirteen different papillomaviruses, belonging to six different genera [191]. Most of the E2 proteins associated with mitotic chromosomes, but it was observed that the mitotic binding pattern varied among the E2 proteins from different genera. BPV1 E2 was detected as small speckles over the arms of all mitotic chromosomes. In contrast, the HPV8 E2 protein showed a distinct pattern of large foci bound to the pericentromeric region of chromosomes [191]. Our laboratory has shown that the HPV8 E2 protein associates with the rDNA loci present on the short arms of human acrocentric chromosomes and colocalizes with upstream binding

factor (UBF) [205]. UBF is a transcription factor required for rDNA transcription by RNA polymerase I [127]; it remains bound to chromosomes during mitosis even though transcription is silenced [188,212]. Studies in our laboratory have shown that the domains required for mitotic chromosomal association of HPV8 E2 are different from those required for BPV1 E2 binding; the hinge and CTD are sufficient and essential for chromosomal association [205]. Unlike BPV1 E2, the HPV8 N-terminal transactivation domain is not required for binding. Substitution of residues R37 and I73 in the transactivation domain abrogates Brd4 binding and mitotic chromosome binding of BPV1 E2 but an identical mutation does not abrogate chromosome association of HPV 8 E2 [205].

Other persistent DNA viruses have developed a similar strategy for genome maintenance and partitioning to enable them to persist in the host. Gammaherpesviruses KSHV and EBV encode DNA binding proteins LANA and EBNA1, respectively, which bind to specific sites in the viral DNA and tether the genome to host chromosomes. In addition to sharing the common function of genome tethering, these proteins also play an important role in viral genome replication and transcriptional regulation [88,148]. Structurally, all three proteins form, or are predicted to form, a similar dimeric beta-barrel C-terminal DNA binding structure, despite having no sequence homology ([101] and (reviewed in [56]). Short peptide sequences have been identified in LANA and EBNA1 that mediate binding to mitotic chromosomes [15,43,132,160]. In this study we have defined specific residues within the HPV8 E2 hinge region that mediate the characteristic HPV8 E2 mitotic chromosomal binding pattern. We show that a highly conserved motif, RXXS, is

required for this association, and that the serine within this motif is phosphorylated *in vivo*. Furthermore, this sequence has similarities to the chromosome binding regions of the herpesvirus EBNA1 and LANA proteins.

Results

Regions in the hinge of the HPV8 E2 protein required for Mitotic Chromosomal Interaction

Both the hinge and the CTD are essential and sufficient for HPV8 E2 mitotic chromosome binding [205]. The hinge region of the betapapillomavirus E2 proteins is unusual in composition compared to the E2 proteins from other genera of papillomaviruses. The hinge is not well conserved with other papillomaviruses but within the beta genus, the hinge regions have similar length and sequence composition. The beta PV E2 hinge regions are longer than those of most papillomavirus E2 proteins and are rich in RG and RS dipeptide motifs. The amino acid sequence is shown in Figure 3.1. The E2 hinge regions are thought to be unstructured, and this has been experimentally shown for HPV16 E2 [91]. The regions of the EBV EBNA1 protein required for mitotic chromosome binding contain A-T hooks [232]. The amino acid sequence of the HPV8 E2 hinge region has stretches of sequence with similarities to A-T hook motifs but there is no exact match with the A-T hook consensus motif (PRGRP). In this study we have defined the sequences from the hinge that are essential for the characteristic HPV8 E2 foci on mitotic chromosomes.

Figure 3.1
Amino acid sequence of the HPV8 E2 protein hinge**.** Amino acids 206 to 403 from the hinge region of HPV8 E2 are shown. SR and RS dipeptides are shown in red and the 16 amino acid chromosome binding region mapped in this study is underlined.

We have previously shown that HPV8 E2 is observed in distinct foci in the pericentromeric region of mitotic chromosomes in both African Green monkey CV-1 and human C33A cells [191]. We have mapped this localization in human C33A cells to the rDNA loci on the short arm of acrocentric chromosomes [205]. In CV-1 cells approximately three UBF foci are observed and the HPV8 E2 protein associates with these regions (data not shown). However, there are additional pericentromeric foci in CV-1 cells that do not overlap with UBF and we are currently characterizing these additional pericentromeric regions. All of the HPV8 E2 proteins used in this study associated with mitotic chromosomes in a similar pattern; they completely co-localized with UBF and rDNA foci in C33 cells and they associated with both UBF positive and negative foci in CV-1 cells.

To identify regions within the E2 hinge required for mitotic chromosome association, E2 proteins were expressed in CV-1 cells The localization of each protein was analyzed by indirect immunofluorescence using an antibody against an N-terminal FLAG epitope tag. A series of E2 proteins were expressed that contained an intact C-terminal domain (404 to 498) fused to various sequence segments from the hinge region (see Figures 3.2A and 3.2B). As shown in Figure 3.3, they were

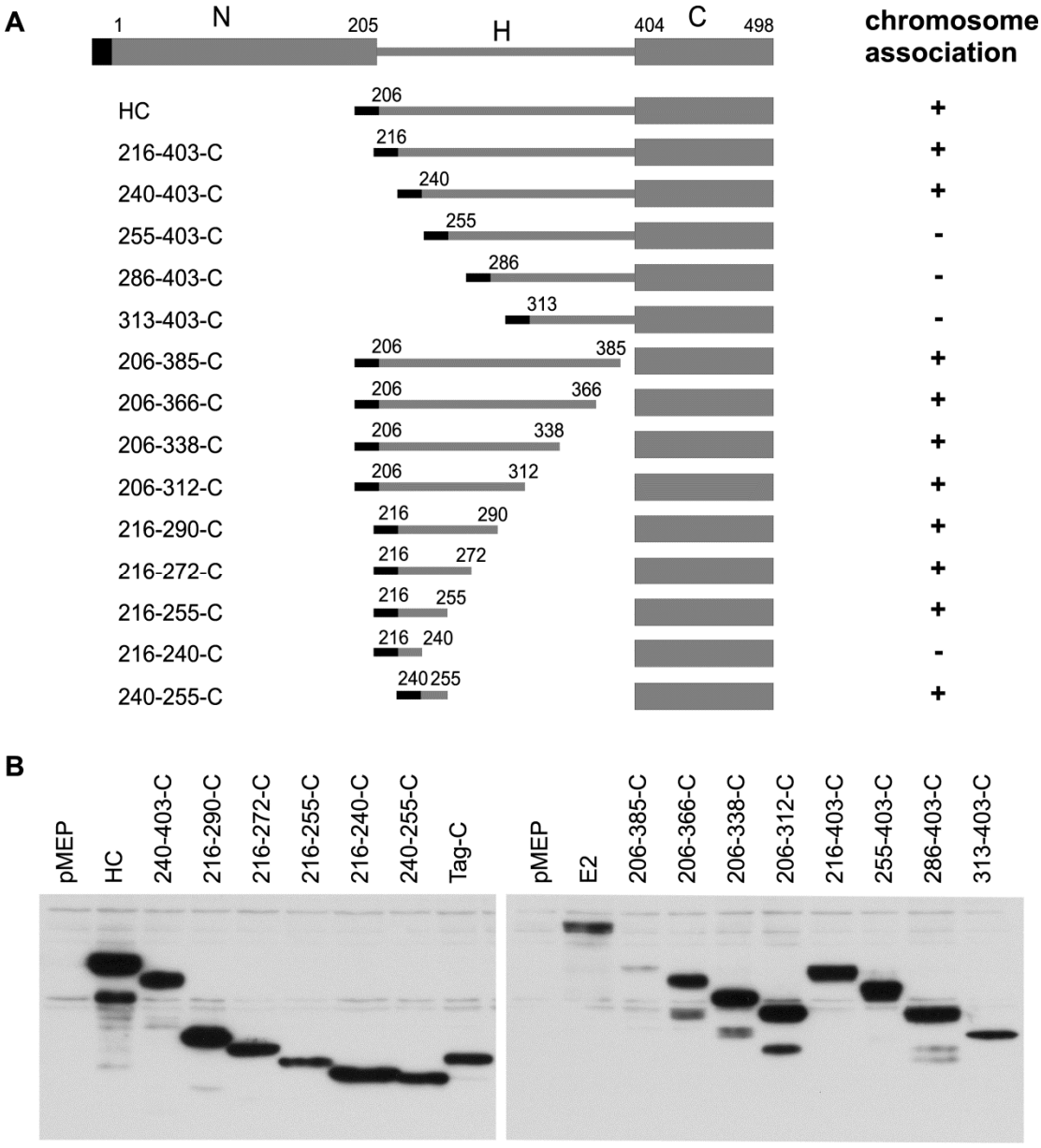


Figure 3.2
Truncated E2 proteins with deletions in the hinge examined for mitotic localizations. A. Schematic representation of E2 proteins truncated in the hinge region and their chromosome binding phenotype. Truncated E2 proteins were generated as fusion proteins with the indicated hinge sequence fused to the CTD. The FLAG epitope is shown at the N-terminus as a black box. B. Immunoblot analysis of the E2 proteins, as detected with an anti-FLAG antibody.

examined for their ability to bind to mitotic chromosomes in the distinct foci characteristic of HPV8 [191]. The N-terminal region of the hinge was truncated at amino acid positions 216, 240, 255, 286 and 313. Deletions up to position 240 had no effect on chromosomal association but further deletion abrogated the characteristic HPV8 E2 binding foci. Deletions were also generated to remove C-terminal portions of the hinge with endpoints at residues 385, 366, 338, 312, 290, 272, 255, and 240. In this case, deletion up to residue 255 had no effect on chromosome association but further deletion to 240 eliminated binding. Correspondingly, a small region from the hinge consisting of residues 240-255 fused to the C-terminal domain (240-255-C) was able to associate with mitotic chromosomes (see Figure 3.2 A and 3.3). The percentage of positive E2 expressing mitotic cells showing the foci binding pattern is shown in table 3.1. Furthermore, the 240-255-C mitotic foci co-localized with UBF in a similar fashion to the full-length E2 protein. Thus, a 16 amino acid region, when fused to the CTD, can mediate mitotic chromosomal binding.

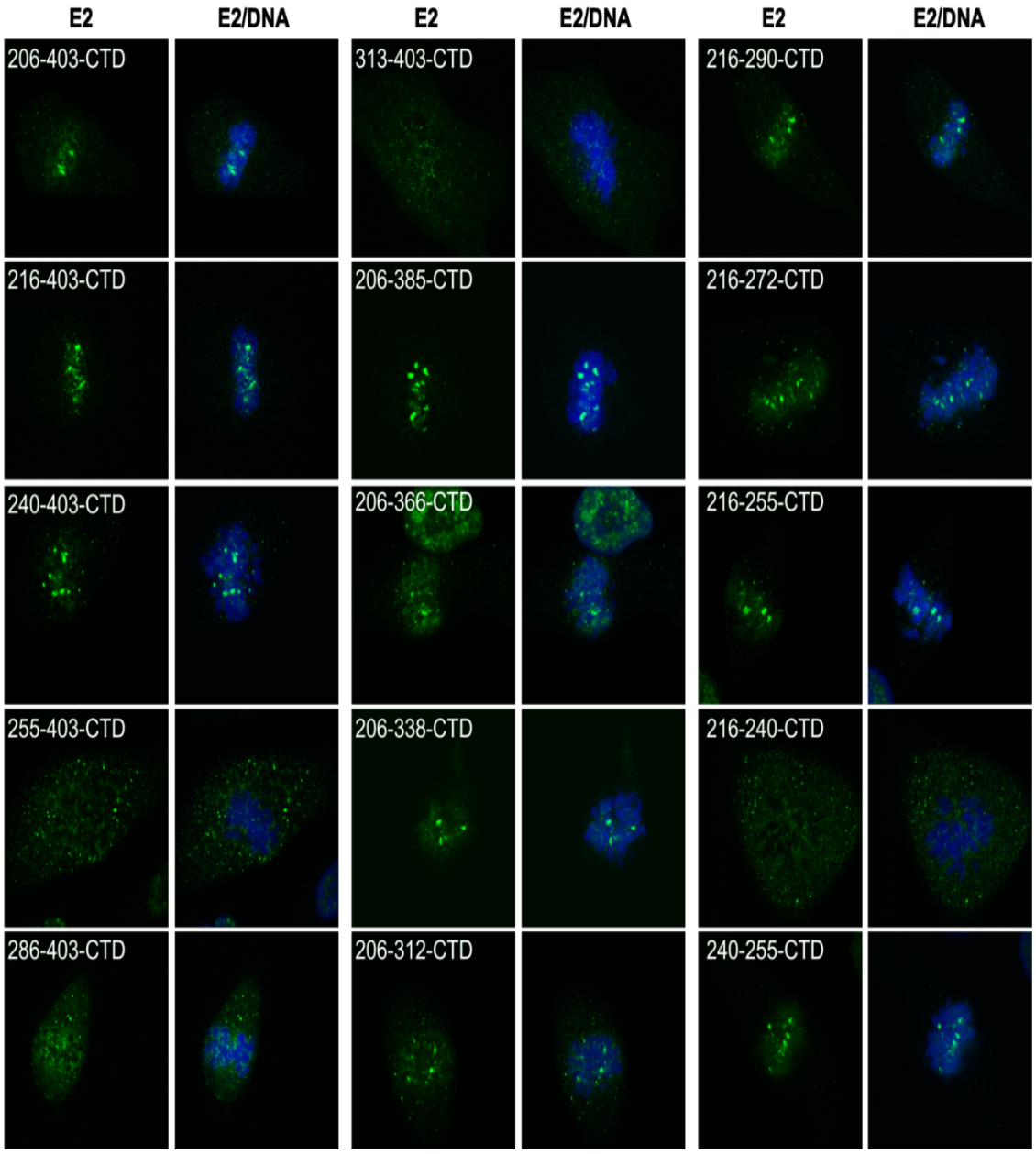


Figure 3.3
Chromosome binding phenotype of E2 proteins with deletions in the hinge.
Immunolocalization of truncated HPV8 E2 proteins in mitotic CV-1 cells. E2 proteins, as detected with a FITC-labeled antibody, are shown in green. Cellular DNA was stained with DAPI (blue). For mitotic localization of HPV8 E2 CTD alone, refer to figure 5.4 in chapter 5. The percentage of E2 expressing mitotic cells and the percentage of positive E2 expressing mitotic cells showing the foci binding pattern are shown in Table 3.1.

Table 3.1- Percentage of positive E2 expressing mitotic cells showing the foci binding pattern

E2 Protein	N (# mitotic cells examined)	% cells expressing E2	% E2 positive cells with E2 foci on mitotic chromosomes
E2	41	46.3	89.5
E2HC	40	55.0	95.5
216-404-C	29	75.9	100
240-404-C	41	56.1	82.6
255-404-C	54	38.9	0
286-404-C	42	40.5	0
313-404-C	30	36.7	0
216-240-C	35	34.3	0
216-255-C	35	82.9	93.1
216-272-C	27	85.2	73.9
216-290-C	40	72.5	100
240-255-C	37	43.2	81.3
207-312-C	36	69.4	100
207-338-C	34	79.4	100
207-366-C	44	75.0	100
207-385-C	34	88.2	100

The HPV8 E2 Proteins Localize to Nuclear Speckles, but this does not correlate with Mitotic Chromosome Binding

Proteins from the SR family of splicing factors contain multiple RS motifs and localize to nuclear speckles, where they are intimately involved in transcription and splicing (reviewed in [154]). The E2 protein from the betapapillomavirus HPV5 localizes to these speckles [141] and this localization is dependent on the hinge region. We found that the HPV8 E2 protein is also localized to the nuclear speckles in interphase cells, as shown by colocalization with SC35, a marker for nuclear speckles (see Figure 3.4). To determine which domains of the HPV8 E2 protein are required for this colocalization, FLAG-tagged E2 proteins containing various combinations of the N-terminal (N), hinge (H) and C-terminal domain (C) were expressed and analyzed for colocalization with the SC35 protein. As shown in Figure 3.4, the hinge region was required for this localization but it was not sufficient. The hinge region alone (H) did not associate with nuclear speckles and a combination of the N-terminal domain and hinge region (NH) resulted in only weak colocalization with SC35. In fact, the absence of the C-terminal domain resulted in nucleolar localization of these E2 proteins. However, a protein consisting only of the hinge and C-terminal domain (HC) gave strong nuclear speckle localization. Therefore, a function of the C-terminal domain, such as DNA binding or dimerization, might be important to augment this localization. Notably, the same two domains are required for mitotic chromosome association.

The E2 proteins with truncations in the hinge region were also analyzed for their ability to localize to nuclear speckles to determine whether this was dependent

on the multiple SR dipeptide motifs and to determine whether this association correlated with mitotic chromosome localization. The sequence of amino acids that was found to be important for mitotic chromosomal association (residues 240 to 255) contains one SR dipeptide motif (see Figure 3.1). Deletion of the C-terminal half of the hinge in the background of the full-length E2 protein resulted in only a minimal decrease in nuclear speckle localization (N-206-312-C; see Figure 3.5). However, a dramatic decrease was observed when the N-terminal half of the hinge was deleted (N-313-403-C and 313-403-C). Progressive truncation of the N-terminal half of the hinge resulted in decreased nuclear speckle binding. In the absence of the N-terminal transactivation domain this resulted in a progressive increase in nucleolar localization. However, neither half of the hinge was sufficient to localize the proteins to nuclear speckles, despite the presence of multiple SR sequences. We postulate that either a minimal number of SR motifs are required for this localization, that specific sequences from both halves of the hinge are required or that the deletion endpoint at residues 312/313 interrupts a crucial element. In interphase, the minimal chromosomal binding E2 protein, 240-255-C, was localized throughout the nucleus and nucleolus in a diffuse staining pattern and did not localize specifically to nuclear speckles. Therefore, nuclear speckle association does not correlate with mitotic chromosome binding.

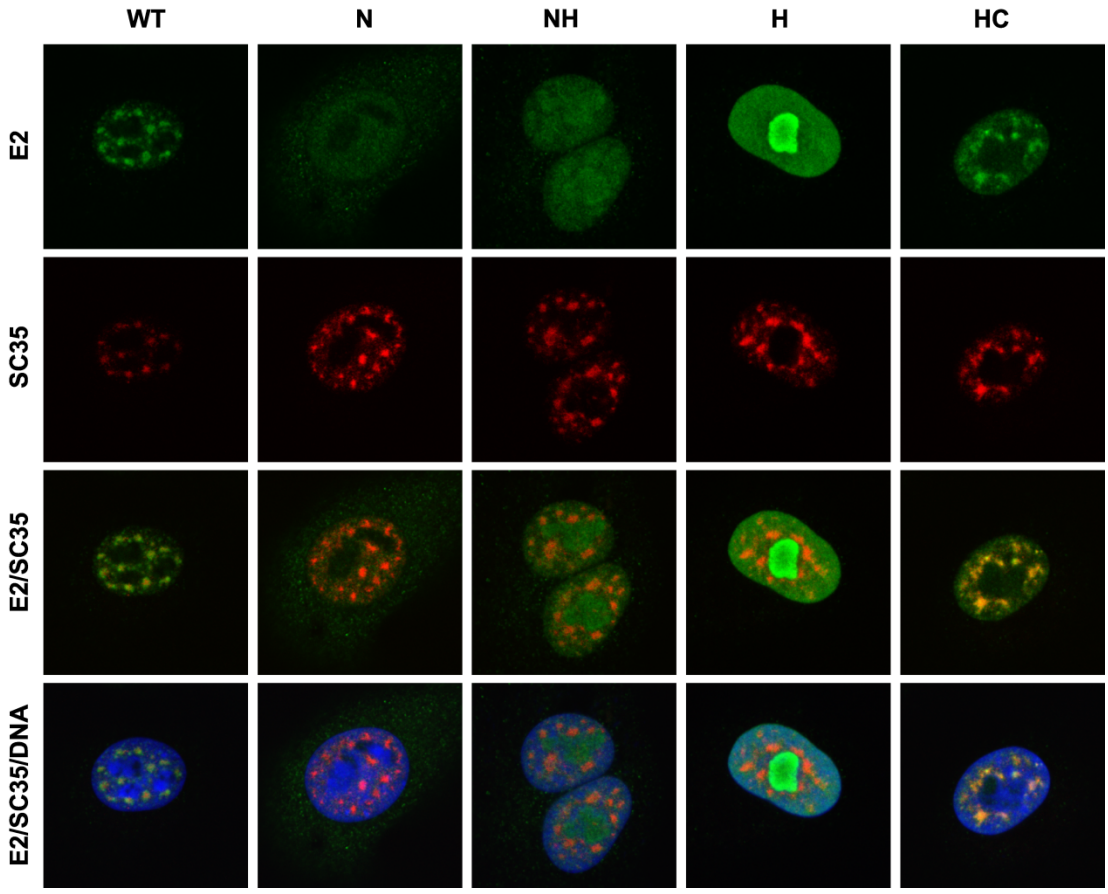


Figure 3.4.
Colocalization of E2 and SC35 in interphase cells. CV-1 cells expressing full length E2 (WT), or the N, NH, H or HC E2 domains were assayed for E2 and SC35 localization by indirect immunofluorescence. The E2 protein, as detected by a FITC-labeled antibody, is shown in green; the SC35 protein, as detected by a Texas Red antibody, is shown in red; colocalization of these proteins appears as yellow in the merged images. Cellular DNA was stained with DAPI (blue). The interphase localization of HPV8 E2 CTD is cytoplasmic.

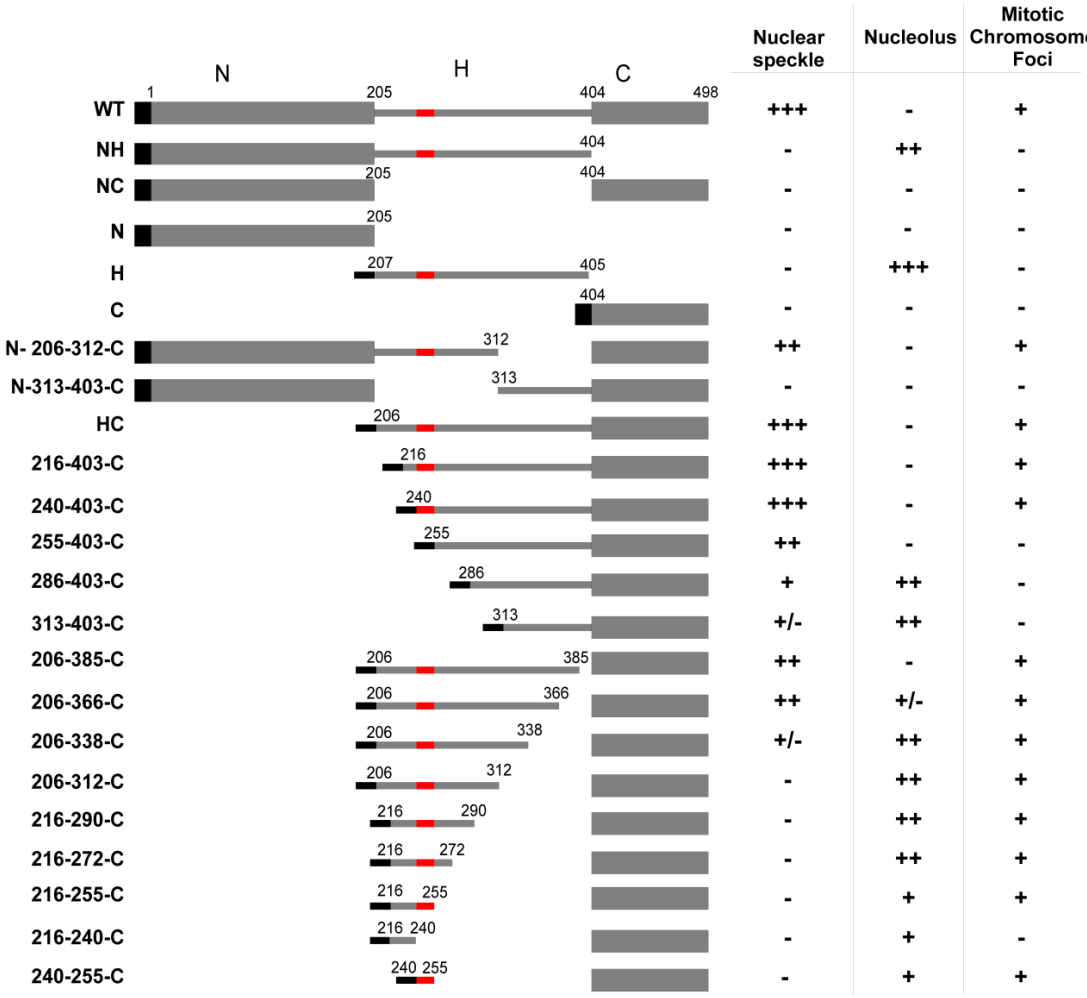


Figure 3.5
Summary of the intracellular localization of the HPV8 E2 Proteins.
All E2 proteins used in this study are shown with their intranuclear localization indicated to the right. Nuclear speckles were determined by colocalization with SC35: -, no apparent colocalization with SC35; +/-, minimal; +, low; ++, moderate; and +++, high colocalization with nuclear speckles (all of the E2 proteins colocalized with SC35). Nucleolar localization was determined by colocalization with UBF: -, minimal nucleolar localization; +, equal detection in nucleus and nucleolus; ++, moderate and +++, high amounts of protein accumulated in the nucleolus relative to nucleus. The 16 amino acid chromosome binding region mapped in this study is shown in red.

The Chromosome Binding Region is functional in other BetaPapillomavirus E2 Proteins

To confirm whether the pattern of chromosome binding seen with HPV8 E2 is common to other beta-papillomavirus E2 proteins, we examined the association of HPV5 E2 with mitotic chromosomes. As shown in Figure 3.6, the mitotic localization of HPV5 E2 was similar to HPV8 E2, with E2 detected as large distinct foci on the chromosomes. These foci also overlapped with the Pol I transcription factor, UBF (data not shown). To test that the chromosome binding region mapped in HPV8 E2 was also functional in HPV5 E2, we expressed an HPV5 E2 protein carrying a forty amino acid region similar to the mapped chromosome binding region of HPV8 (216-255) fused to the C-terminal DNA binding domain. The HPV5 216-255 C protein was also observed to associate with mitotic chromosomes (see Figure 3.6D) and to colocalize with UBF (data not shown). We also carried out experiments in which the C-terminal domains and 40 residue hinge chromosome binding regions from each E2 protein were exchanged (see Figure 3.6B). The pattern of mitotic binding was the same for each of the E2 fusion proteins (see Figure 3.6D). Hence, from this study we can conclude that the mitotic binding pattern observed with the HPV8 E2 protein is also observed in other beta-virus E2 proteins and involves a similar region of the hinge region along with the DNA binding domain. Figure 3.6A shows a comparison of the 216 to 255 regions of HPV5 and 8. Notably, the smaller 16 amino acid chromosome binding region (240-255; underlined in HPV8) is the largest invariant region within the 40 amino acid peptide.

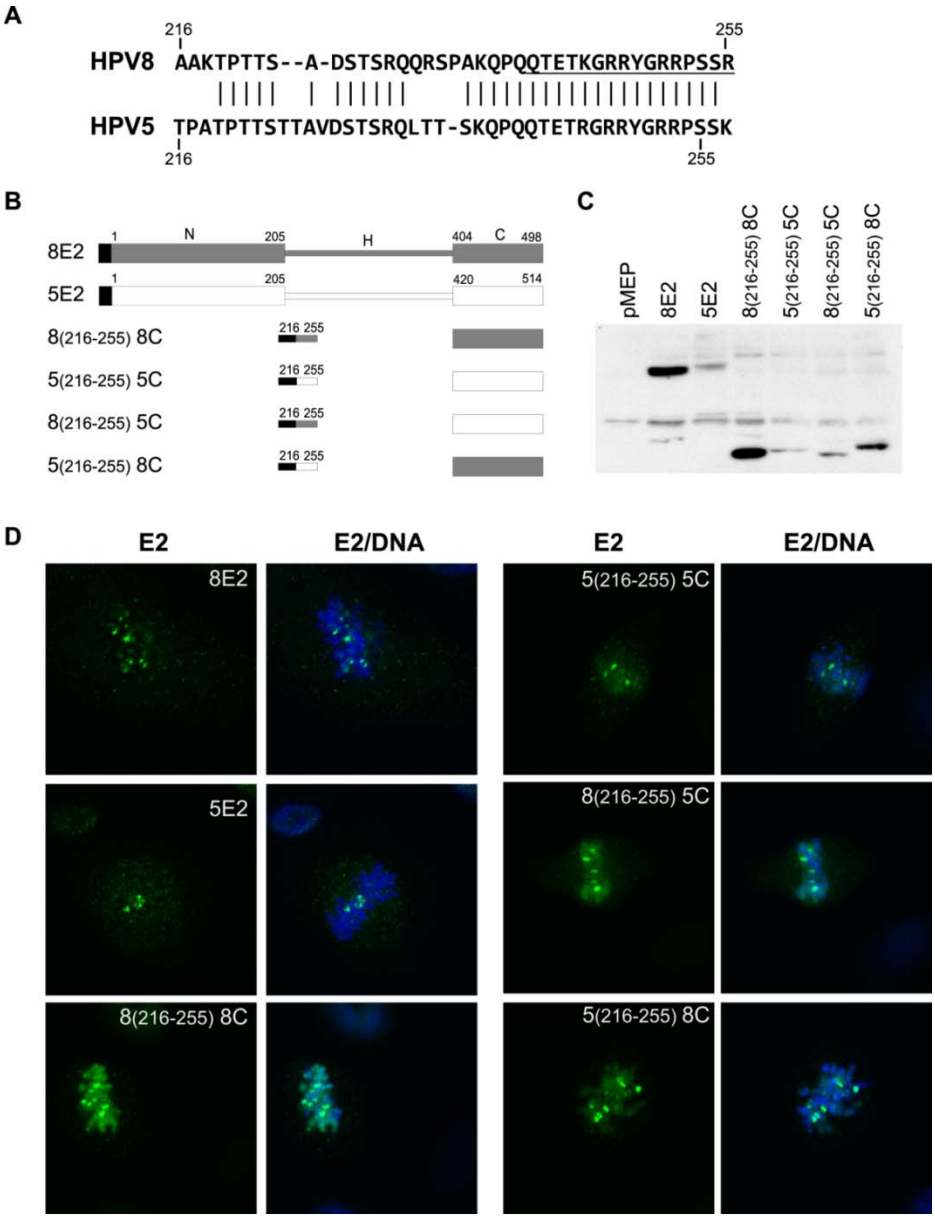


Figure 3.6
HPV5 E2 protein shows a similar mitotic binding pattern to HPV8 E2 protein.
A. Homology between the HPV8 and HPV5 216-255 hinge regions. Underlined region indicates the chromosome binding region in HPV8 E2.
B. Schematic representation of the HPV8 and HPV5 E2 fusion proteins. The black box represents the flag-tag.
C. Immunoblot analysis showing the expression levels of the full length, truncated and fusion E2 proteins of HPV8 and HPV5. E2 was detected using an anti-FLAG antibody.
D. Immunolocalization of the HPV8 and HPV5 full length, truncated and fusion E2 proteins in mitotic CV-1 cells. E2 proteins, as detected with a FITC-labeled antibody, are shown in green. Cellular DNA was stained with DAPI (blue).

The Chromosome Binding Region of the Beta Papillomaviruses is highly conserved

Elements sufficient for mitotic binding of HPV8 and HPV5 E2 map to the same hinge region of the proteins. To assess whether this region is also conserved among the five different species of beta-papillomaviruses, the predicted E2 sequences from 29 different beta-viruses, belonging to species 1, 2, 3, 4 and 5, were analyzed using the Clustal W alignment program (see Figure 3.7). The alignment showed that the 16 amino acid chromosome binding region contains a small stretch of the most highly conserved residues in the E2 hinges of all beta-papillomaviruses. Hence, we have mapped the region of E2 required for mitotic chromosomal association to a short peptide that is highly conserved in all beta HPVs. The fact that this region has been conserved throughout evolution in an otherwise divergent region of the E2 protein strengthens its functional importance in the viral life cycle.

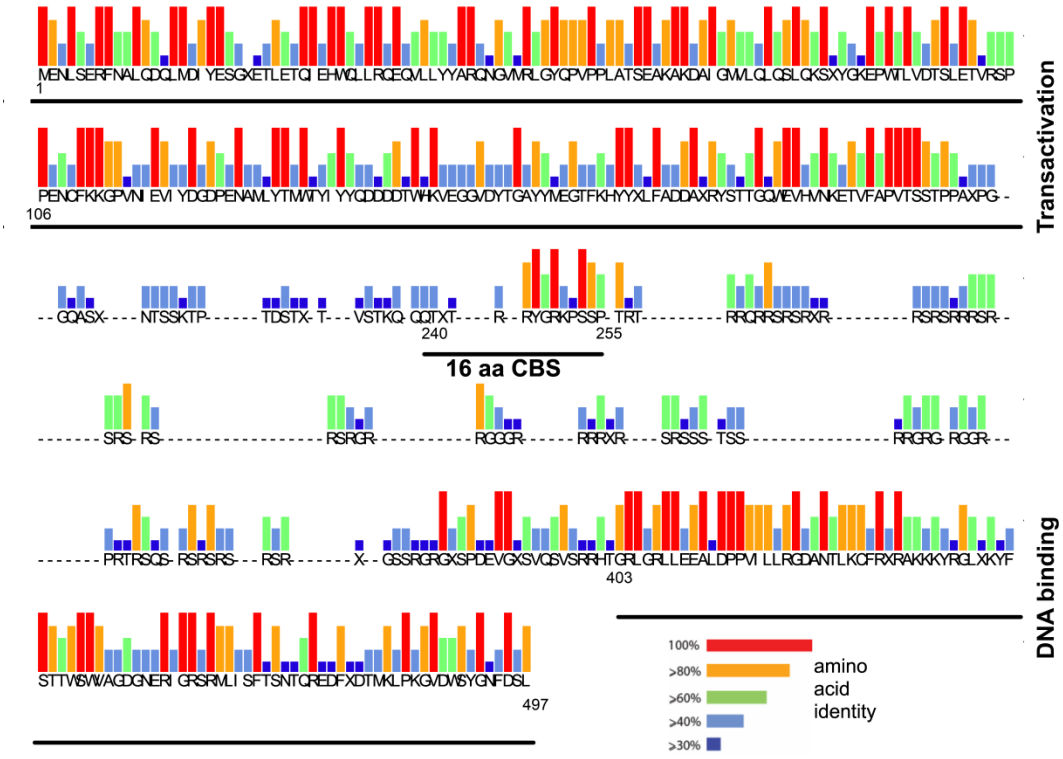


Figure 3.7
Sequence analysis of the Chromosome Binding Region
Clustal W alignment of 29 papillomaviruses E2 proteins belonging to the five different species of the beta genus. The numbers represent the amino acid residue numbers of the HPV8 E2 protein. Residues arginine 250, serine 253 and tyrosine 248 are 100% conserved in these 29 sequences. The 16 amino acid chromosome binding sequence (CBS) is underlined. The transactivation domain and DNA binding dimerization domain are indicated.

An RXXS motif in the hinge is crucial for mitotic chromosome binding

To further map specific residues in the hinge important for mitotic chromosomal interaction of the HPV8 E2 protein, we generated specific amino acid substitutions. These studies were initiated at the point when we had mapped the chromosomal binding region to the forty residues between amino acids 216-255 of the hinge. The point mutations were generated in a background of these forty residues fused to the C-terminal domain. This region contains a few residues that are highly conserved among all beta-papillomavirus E2 proteins and also contains number of putative consensus sites for different modification enzymes such as CK2, Aurora kinases and protein arginine methyl transferases (PRMTs). Key residues were substituted with alanine to eliminate sites of possible post-translational modifications, such as arginine methylation (R250/251) and serine/threonine phosphorylation (T219, SP233/234 SS253/254), and to change conserved residues (P220A, D225, QQ239/240, Y248, GG245/249 and P252). The sequences of these mutations and expression level of the mutated E2 proteins are shown in Figures 3.8A and B, respectively.

The mutated 216-255-C E2 proteins were tested for their ability to bind mitotic chromosomes by immunofluorescence (Figure 3.8C). Only two of the mutated E2 proteins, RR250/251AA and SS253/254AA, showed a loss of the chromosome binding phenotype. Neither protein was observed in the characteristic, distinct HPV8 chromosomal foci. All of the other mutated E2 proteins exhibited the wild-type pattern of mitotic localization. Since, the full length E2 protein colocalizes with UBF

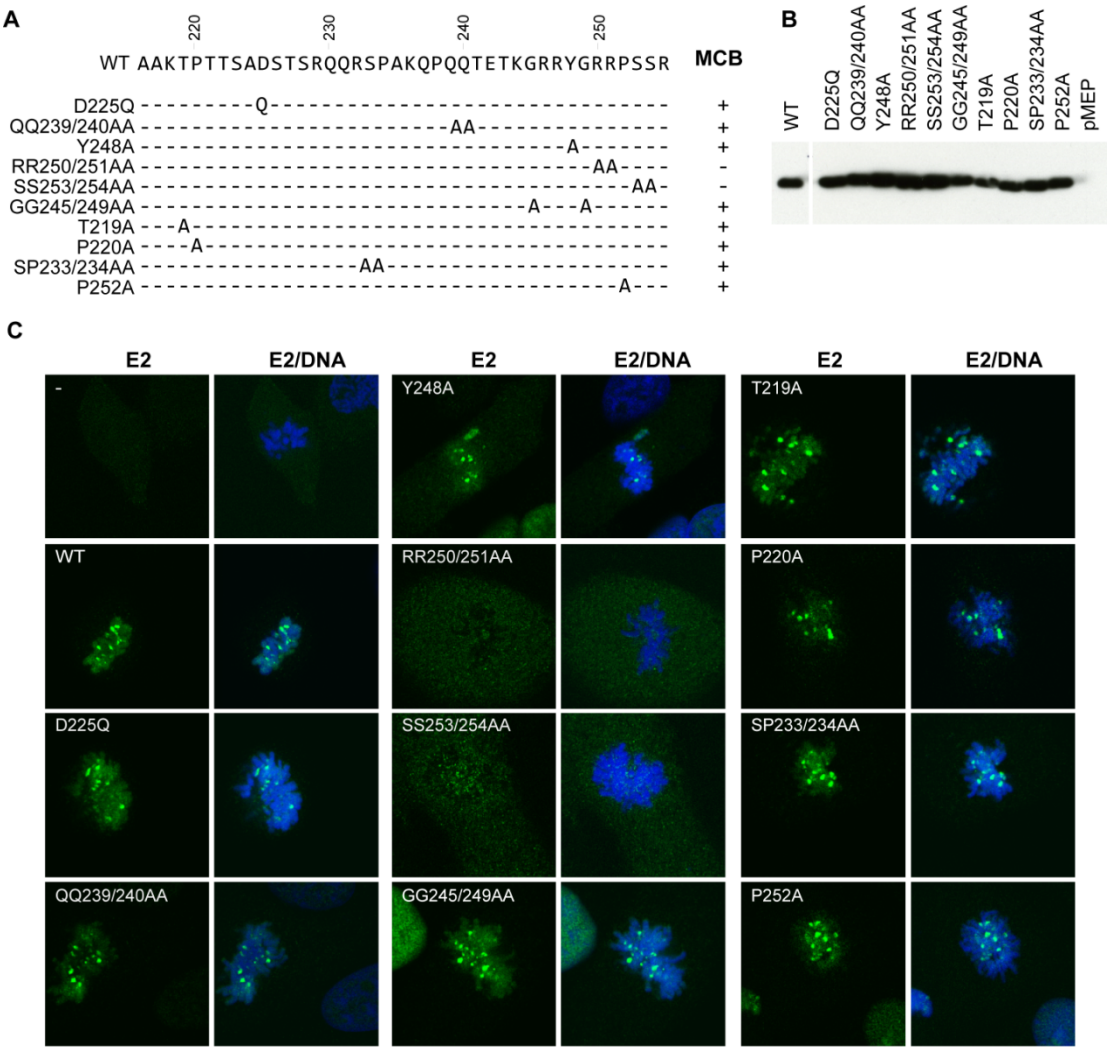


Figure 3.8
Mutational analysis and mitotic chromosome binding phenotype of 216-255-C E2 proteins

A. Amino acid substitutions in the background of E2 proteins with a 40 amino acid region from the hinge (216-255) fused to the CTD. MCB: mitotic chromosome binding phenotype.

B. Immunoblot analysis showing the expression levels of the mutated E2 proteins. E2 was detected using an anti-FLAG antibody.

C. Immunolocalization of the mutated 216-255-C E2 proteins in mitotic cells. Negative (-) represents cells transfected with empty vector. E2 proteins were detected using anti-FLAG antibody (green) and cellular DNA was stained with DAPI (blue). The percentage of E2 expressing mitotic cells and the percentage of positive E2 expressing mitotic cells showing the foci binding pattern are shown in Table 3.2.

Table 3.2 - Percentage of positive E2 expressing mitotic cells showing the foci binding pattern (Mutations in 216-255-CTD hinge background)

E2 Protein	N (# mitotic cells examined)	% cells expressing E2	% E2 positive cells with E2 foci on mitotic chromosomes
WT(216-255-C)	33	69.7	91.3
D225Q	49	81.6	85.0
QQ239/240AA	50	92.0	95.7
Y248A	46	50.0	87.0
RR250/251AA	53	30.2	0
SS253/254AA	48	31.3	0
GG245/249AA	52	63.5	84.8
T219A	50	68.0	79.4
P220A	54	70.4	89.5
SP233/243AA	51	60.8	90.3
P252A	49	59.2	89.7

on mitotic chromosomes [205], these mutated E2 proteins were also tested for their colocalization with UBF. All mutated E2 proteins, except RR250/251AA and SS253/254AA showed overlapping localization with UBF on mitotic chromosomes (data not shown). The percentage of positive E2 expressing mitotic cells showing the foci binding pattern is shown in table 3.2. Thus, the region between position 250 and 254 in the HPV8 E2 hinge region is important for HPV8 mitotic chromosomal binding.

Further examination of the RR250/251AA and SS253/254AA amino acid sequences determined that both mutations disrupted two overlapping 'RXXS' kinase motifs. This RXXS motif is a common consensus for several different kinases such as the aurora kinases, protein kinase A and C, calmodulin dependent kinases and the Rho-kinase (ROCK) [174,251,279]. Furthermore, these four residues map to the region of sixteen amino acids (240-255) identified in parallel by deletion analyses. To further analyze which of the two serine and arginine residues were actually contributing to the mitotic binding phenotype, we designed another set of mutations. In this case, they were generated in the background of the sixteen amino acid residues 240-255, fused to the C-terminal DNA binding domain (240-255-C). The residues arginine 250 and 251 and serine 253 and 254 were individually substituted with alanine residues. Each serine residue was also substituted with aspartic acid to mimic the effect of constitutive phosphorylation. To identify additional residues required for chromosomal association, the remaining E2 proteins in this set also contained substitutions of conserved residues that had not been previously mutated in this region (RR246/247, E242 and K244). Finally, a protein was generated in which all

five potential phosphorylation sites in the 240 to 255 region were substituted with alanine (A5). The sequences of these mutations and the expression level of the mutated proteins are shown in Figure 3.9A and 3.9B respectively.

Figure 3.9C shows the intracellular mitotic localization of the mutated 240-255-C proteins. This analysis revealed that arginine 250 and serine 253 were crucial for mitotic localization of the HPV8 E2 protein; R250A and S253A, as well as S253D, were deficient in mitotic binding function and did not form mitotic chromosomal foci (Figure 3.9C). These residues lie within the $R_{250}XXS_{253}$ motif. As mentioned above, this is a motif common to a number of protein kinases. However, the phosphor-mimetic S253D was not able to substitute for phosphorylated S253 as this protein was excluded from mitotic chromosomes. The overlapping $R_{251}XXS_{254}$ motif was not required as R251A and S254A E2 proteins could bind mitotic chromosomes. Chromosome binding was also abolished in the A5 protein, which had alanine substitutions in all potential phosphorylation sites within the 240-255 region. All other mutated E2 proteins exhibited a binding pattern similar to that of the wild-type protein. The E2 proteins that bound to mitotic chromosomes also colocalized with UBF (data not shown). The percentage of positive E2 expressing mitotic cells showing the foci binding pattern is shown in table 3.3. Therefore, the motif $R_{250}XXS_{253}$ in the hinge region is essential for the mitotic binding function. Notably, arginine 250 and serine 253 and the consensus motif 'RXXS' are absolutely conserved among all of the beta-viruses examined (Figure 3.7).

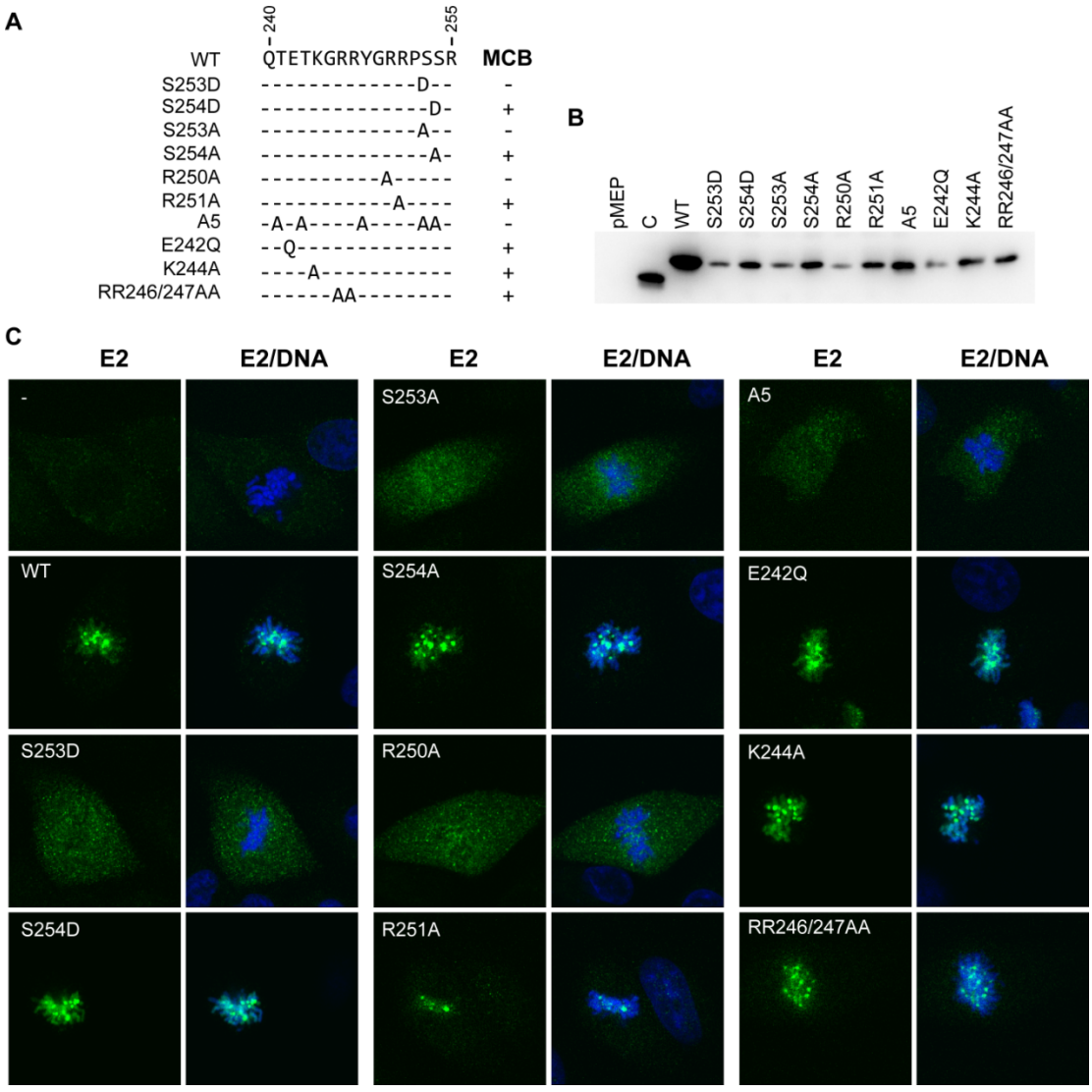


Figure 3.9
Arginine 250 and serine 253 are critical residues mediating chromosomal association function of HPV8 E2 protein
A. Amino acid substitutions in the background of E2 proteins with a 16 amino acid hinge region (240-255) fused to the CTD. MCB: mitotic chromosome binding phenotype.
B. Immunoblot analysis showing the expression levels of the mutated E2 proteins. E2 was detected using an anti-FLAG antibody.
C. Immunolocalization of the mutated 240-255-C E2 proteins on mitotic chromosomes. Negative (-) represents cells transfected with empty vector. E2 proteins were detected using anti-FLAG antibody (green) and cellular DNA was stained with DAPI (blue). The percentage of E2 expressing mitotic cells and the percentage of positive E2 expressing mitotic cells showing the foci binding pattern are shown in Table 3.3.

Table 3.3 - Percentage of positive E2 expressing mitotic cells showing the foci binding pattern (mutations in the 240-255-CTD background)

E2 Protein	N (# mitotic cells examined)	% cells expressing E2	% E2 positive cells with E2 foci on mitotic chromosomes
WT(240-255-C)	181	24.9	93.3
S253D	160	25	0
S254D	210	29	95.1
S253A	142	16.9	0
S254A	196	25	98.0
R250A	158	26.6	0
R251A	149	29.5	100
RR246/247AA	172	25.6	100
A5	180	23.9	0
E242Q	164	25.6	95.2
K244A	222	22.1	100

HPV8 E2 Serine 253 is a Phosphorylation Site

To investigate the phosphorylation status of the E2 proteins, we expressed the E2 proteins in CV-1 cells in the presence of [^{32}P]-orthophosphate or a combination of [^{35}S]-methionine and [^{35}S]-cysteine. E2 proteins were immunoprecipitated from equivalent counts of total protein and analyzed by gel electrophoresis, autoradiography and phosphor-imaging (see Figure 3.10). An initial study included the full length E2 protein, the HC protein (206-403-C) and the series of mutated proteins shown in Figure 3.8A in the 216-255-C background. Figure 3.10A shows that the wild-type, HC and 216-255-C were all labeled with ^{32}P, indicating that they were phosphorylated. Notably, the full-length E2 and HC proteins were highly phosphorylated, as determined by the ratio of ^{32}P to ^{35}S incorporation, compared to the smaller 216-255-C protein. However, all 216-255-C proteins containing the amino acid substitutions shown in Figure 3.8A were also phosphorylated. There was a slight reduction in ^{32}P incorporation in the RR251/252AA and SS253/254AA proteins, but it was difficult to conclude whether this was due to the elimination of phosphorylation in one of the overlapping RXXS motifs because of the potential background phosphorylation due to the additional 12 potential sites of phosphorylation in the 216 to 255 region (data not shown).

To further determine whether the $R_{250}XXS_{253}$ motif is phosphorylated, the experiment was repeated with the series of mutated proteins in the 240-255-C background. As shown in Figure 3.10B, the 240-255-C protein was phosphorylated and minimal, if any, phosphorylation was detected with C alone. Mutation of all five potential phosphorylation sites (A5) also eliminates detectable phosphorylation.

Furthermore, both S253A and S253D proteins have no detectable phosphorylation, confirming that residue 253, in the $R_{250}XXS_{253}$ motif, is a phosphorylation site. The 240-255-C proteins with substitutions in residue 254 (S254A and S254D) were phosphorylated and so S254, in the overlapping $R_{251}XXS_{254}$ motif, is not a major phosphorylation site. However, substitution of either R250 or R251 resulted in a substantial decrease in phosphorylation. Therefore, the optimal motif for phosphorylation is RRXS. However, although the R251A 240-255-C protein showed greatly reduced phosphorylation, it was able to bind to mitotic chromosomes in the characteristic HPV8 E2 binding foci. Therefore, even though R251 and S253 are essential for association with mitotic chromosomes, phosphorylation of the $R_{250}XXS_{253}$ motif may not be absolutely required. Alternatively, the minimal phosphorylation observed may be sufficient for the observed chromosome binding. The *in vivo* labeling experiments were carried out in asynchronous cells and, if the observed phosphorylation is cell cycle regulated, the residual phosphorylation might be present at higher levels in mitotic cells.

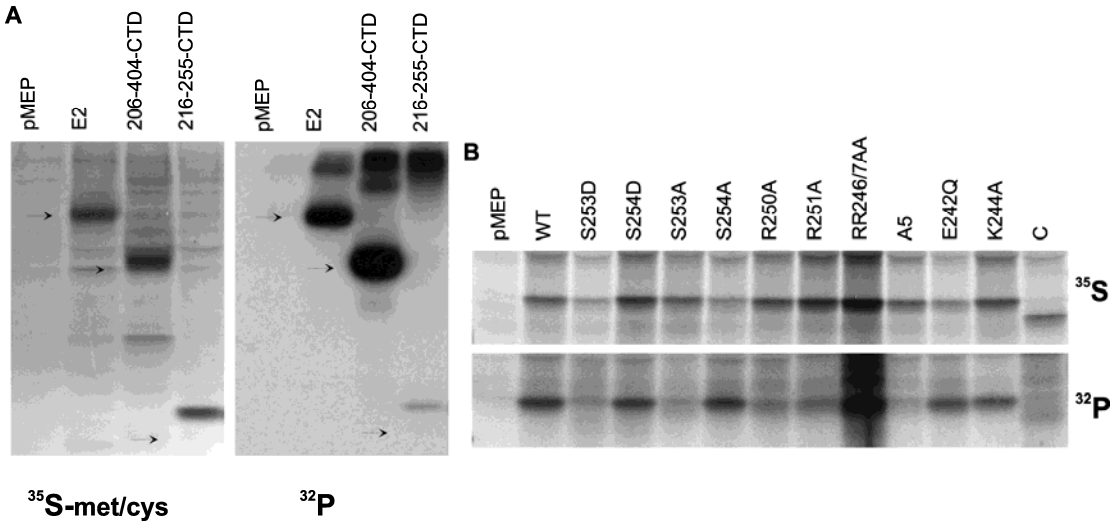


Figure 3.10
Phosphorylation analysis of the HPV8 E2 proteins
A. CV-1 cells expressing full-length, HC (206-404-CTD) and 216-255-CTD E2 proteins were metabolically labeled with either [^{32}P]-orthophosphate or [^{35}S]-methionine and cysteine. Equivalent amounts of total protein were immunoprecipitated using anti-FLAG beads and the samples were analyzed by 15% SDS-PAGE. Incorporation of labeled isotopes was determined using a Phosphorimager. E2 proteins are indicated by arrows.
B. CV-1 cells expressing the series of mutated proteins shown in Figure 7A were metabolically labeled with [^{32}P]-orthophosphate or [^{35}S]-methionine and cysteine, as described for A.

Discussion

Several studies have shown that the papillomavirus E2 proteins link the viral genome to the host chromosomes to support maintenance, stability and partitioning of the viral genome. For BPV1, both the N-terminal transactivation domain and C-terminal DNA binding domain of E2 are required for efficient mitotic chromosome interaction and genome partitioning [18,123,243]. The N-terminal domain is required for interaction with cellular factors bound to mitotic chromosomes, such as the Brd4 protein [19,281], and the C-terminal domain is required for binding to DNA sites in the viral genome [123,243]. Specific residues within the transactivation domain of E2 are required for interaction with Brd4 [19,124,236,281] and the C-terminal dimerization function of E2 augments the E2-Brd4-mitotic chromosome interaction [41]. We have previously shown that the domains required for chromosomal association of HPV8 E2 protein are different from those required for BPV1 E2 [205]. Unlike BPV-1 E2, the N-terminal domain of HPV8 E2 is not required for chromosomal association; instead, the C-terminal DNA binding and dimerization domain and the hinge region are essential. In this study we have identified a 16 amino acid chromosome binding region in the hinge that, when fused to CTD, is sufficient for E2 chromosomal association. Further analyses identified two specific residues within the mapped region, arginine 250 and serine 253, which are critical for the E2 mitotic chromosome binding function. Notably, the identified $R_{250}XXS_{253}$ motif, along with the arginine 250 and the serine 253 residues, is completely conserved among the 29 beta-papillomaviruses that have been sequenced to date. We have also demonstrated that the analogous chromosomal binding region in the HPV5 E2 protein

confers an identical chromosomal binding phenotype. Thus, evolutionary conservation of this region, in an otherwise divergent part of the E2 protein, highlights its functional significance in the viral life cycle.

The hinge region of the beta-papillomavirus E2 proteins is also responsible for a unique intracellular localization in interphase cells. The beta E2 proteins are localized in nuclear speckles; these speckles are enriched in splicing factors and are adjacent to sites of active transcription ([120] and this study). Lai et al. have shown that the HPV5 E2 protein can also associate with splicing factors and can augment splicing [141]. In HPV8 E2, the hinge region is essential for nuclear speckle localization but it is not sufficient; the C-terminal domain of E2 is also required for efficient nuclear speckle association. Progressive removal of SR dipeptide motifs in the hinge region results in loss of nuclear speckle localization. However, expression of the hinge region alone results in nucleolar localization. In addition, proteins containing truncated regions of the hinge region fused to the C-terminal domain, which no longer associate with nuclear speckles, also localize to the nucleolus. Thus, in the presence of the DNA binding domain, nuclear speckle localization is dominant but in the absence of this domain, E2 proteins derived from hinge and CTD are often observed in the nucleolus. It is not clear whether the nucleolar localization is of physiological significance. The hinge region contains many GR-rich regions that may function as RNA binding regions that could associate with the high amounts of RNA found in the nucleolus. We have shown that the hinge region is important for the colocalization of HPV8 E2 with UBF and the ribosomal DNA loci on mitotic chromosomes [205] and so localization to the nucleoli in interphase might not be

unexpected. However, the mitotic chromosome binding region of the hinge (240 to 255) does not localize E2 specifically to the nucleolus. Furthermore, E2 proteins that do localize to the nucleoli are usually found diffusely throughout the nucleolus and are not located specifically in the fibrillar centers or dense fibrillar components that contain the rDNA genes and UBF factor.

Within the 16 amino acid chromosome binding region of the E2 hinge we have identified two, highly conserved residues that are crucial for mitotic chromosome association. Arginine 250 and serine 253 lie within a consensus kinase motif, $R_{250}XXS_{253}$ and we have demonstrated that serine 253 is phosphorylated and that mutation of arginine 250 greatly decreases this modification. An overlapping $R_{251}XXS_{254}$ motif is not required for mitotic chromosomal foci and serine 254 is not a major phosphorylation site. However, mutation of arginine 251 also results in greatly reduced phosphorylation although the resulting protein is not defective for chromosome binding. Therefore, an RRXS motif is required for phosphorylation but only RXXS is required for localization to mitotic chromosomal foci. Thus, phosphorylation does not seem to be directly required for the chromosome binding of the 240-255-C E2 protein, unless it is cell cycle regulated and the residual phosphorylation observed is found primarily in mitotic cells. We propose that phosphorylation may instead regulate another property of the E2 protein that is related to but not directly required for mitotic chromosome association. For example, the BPV1 E2 proteins are phosphorylated in the hinge region by CK2 and this modification triggers a conformational switch that targets the E2 protein for proteolysis through the ubiquitin-proteosome pathway, which in turn indirectly

regulates genome copy number [166,200,201].

The RXXS motif is a consensus for many protein kinases including protein kinase A and C, calcium-calmodulin dependent kinase, aurora kinase B and the ROCK kinases. Notably, some of these, such as aurora kinase B, play an important role during mitosis in regulating host chromosomal segregation and cytokinesis. Phosphorylation of the E2 chromosome binding region requires more than the minimal $R_{250}XXS_{253}$ motif since R251A results in decreased phosphorylation and the S254 in the overlapping $R_{251}XXS_{254}$ motif is not phosphorylated. The requirements for S253 phosphorylation indicate that the kinase recognition motif might be RRXS, a protein kinase A site.

Notably, the chromosome binding regions of LANA and EBNA1 have been mapped to short peptide sequences that have sequence similarities to the chromosome binding region of HPV8 E2 that we have mapped in this study. As shown in Figure 3.11, the identified RXXS kinase motif is also present in the chromosome binding region of the LANA and EBNA1 tethering proteins. In EBNA1 several of the RXXS motifs overlap with AT hook regions that are required for chromosome binding and partitioning [232]. In LANA, substitution of residues overlapping the RXXS motif also disrupts episomal persistence, chromosome binding and interaction with histones H2A and H2B [14]. In addition, the chromosome binding regions all contain GR motifs. In EBNA1 these arginine residues are methylated [240] and the serine residues are phosphorylated and substitution of the serines disrupts the EBNA1 partitioning function [71,240]. EBNA1 is thought to associate with mitotic chromosomes by either interacting directly with chromosomal DNA through AT-

hook DNA binding regions [232] and\or or by interacting with a host nucleolar protein hEBP2 [239]. LANA interacts with a number of cellular chromosomal proteins and the N-terminal chromosome binding region of LANA interacts with the nucleosomal surface of histones H2A and B [14]. These observations suggest that, although these proteins may have different chromosomal targets, phosphorylation by certain cell cycle dependent kinases could be a common mechanism of regulation of chromosome binding function.

Future studies will address the molecular interactions of the chromosome binding region of the betapapillomavirus E2 proteins. Several functions and protein interactions have already been assigned to the PV E2 hinge region. For example, the HPV8 E2 hinge region can activate transcription by recruitment of Sp1 to cellular promoters [248]. The studies presented here show that the full-length HPV8 E2 protein interacts with several different complexes that direct its intracellular localization. One of the strongest associations is with nuclear speckles. As mentioned previously, the hinge of HPV5 E2 interacts with splicing factors and enhances splicing. The N-terminal domain of HPV8 E2 binds the Brd4 protein and this interaction strengthens the association of Brd4 with cellular chromatin [170]. However, Brd4 is not required for the prominent foci observed bound to the rDNA loci on mitotic chromosomes [205]. In the absence of the N-terminal domain and the RS regions of the hinge, the E2 protein was observed in the nucleolus. As discussed above, this localization might be due to putative RNA binding regions of the hinge region or might be due to interaction with nucleolar proteins.

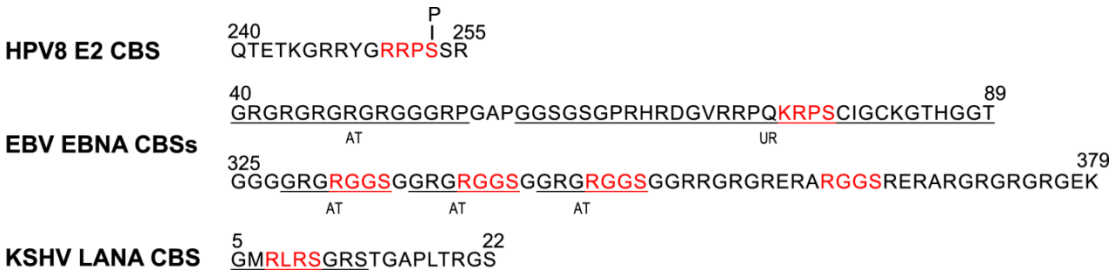


Figure 3.11
Similarities among the chromosome binding regions of HPV8 E2, EBNA1 and LANA. AT hook regions are underlined and RXXS motifs are shown in red. The E2 serine 253 residue, shown to be phosphorylated in this study, is indicated by a P. The EBNA1 “unique region”, important for transactivation, is also indicated.

The betapapillomavirus E2 proteins have distinct properties compared to the E2 proteins from other papillomavirus genera. To date, the interphase localization to nuclear speckles and mitotic association with rDNA loci has only been characterized for the beta PV E2 proteins. HPV8 E2 can give rise to tumors in transgenic mice [203] and it will be of interest to see if any of these unique properties are connected and relate to the unique pathogenesis of beta PV infection. The experiments described here have characterized sequences required for the interaction of the HPV8 E2 protein with mitotic chromosomes. By analogy with other papillomaviruses, we assume that this interaction is crucial for the virus to tether its genome to host chromosomes during persistent infection. Understanding the mechanism by which the E2 protein tethers the viral genome to host chromosomes during persistent infection will assist in the development of anti-viral therapies to inhibit E2 interaction with mitotic chromosomes and thus eliminate viral genomes from infected cells.

Following mapping of the chromosome binding elements in the HPV8 E2 protein, we next wanted to elucidate the mechanism of regulation of E2-chromosomal association. In the following chapter (Chapter 4), studies were conducted to understand the regulation of the HPV8 E2 chromosome binding function by investigating the role of S253 phosphorylation.

Chapter 4

The following chapter is based on a manuscript ready for submission.

Phosphorylation by PKA regulates the chromosome binding function of the HPV8 E2 protein-Sekhar,V and McBride A.A

Contributions: I wrote the manuscript and performed all the experiments presented in it. McBride A.A performed the prediction of PKA sites using the pkaPS predictor program.

Chapter 4: Phosphorylation regulates the chromosome binding function of the HPV8 E2 protein

Introduction

Papillomaviruses (PV) are small, ubiquitous, double stranded DNA viruses that infect either the mucosal or the cutaneous epithelia of their natural hosts. They are the etiological agents of a wide spectrum of diseases that range from mild asymptomatic infections to malignant carcinomas. HPV8 belongs to the betapapillomavirus genus which contains viruses that infect the cutaneous epithelium. In healthy individuals these viruses are associated with asymptomatic infections, but in individuals with immune disorders such as EV they cause lesions that can become cancerous after decades of infection [86]. Members of this genus have also been implicated in certain types of non-melanoma skin cancer [80].

One of the hallmarks of papillomavirus infection is the ability of the virus to establish persistent infection of the host. An essential feature of persistent infection is the ability of the viral E2 protein to tether the viral genomes to the host chromosomes during mitosis as a means to ensure their partitioning and nuclear retention at the end of cell division. The E2 protein consists of two conserved domains; the carboxy-terminal DNA binding and dimerization domain (CTD) that binds to a palindromic 12bp target sequence on the viral genome and the amino-terminal transactivation domain that along with the CTD is involved in viral replication and transcription. The two domains are separated by a highly flexible and non-conserved hinge region [165]. Although initially the hinge region was thought to function only as a flexible linker,

many diverse functions have now been mapped to hinges of different E2 proteins. Regions that regulate nuclear localization in HPV11 E2 [286], proteasomal degradation in BPV1 E2 [201] and transcriptional regulation and chromosome binding functions have been mapped to the HPV8 E2 hinge [235,248].

E2 proteins from different papillomaviruses have been shown to associate with distinct chromosomal targets [191]. BPV1 E2 binds as small speckles over the arms of all mitotic chromosomes in association with the cellular protein Brd4, but HPV8 E2 is observed as large pericentromeric foci on mitotic chromosomes [171,205]. BPV1 E2 interacts with host chromosomes through the amino-terminal transactivation domain but HPV8 E2 does not require either the N-terminal transactivation domain or the Brd4 interaction to bind to mitotic chromosomes [205]. The HPV8 E2 protein has been shown to associate with the repetitive rDNA loci on the short arms of human acrocentric chromosomes and colocalize with the rDNA transcription factor, upstream binding factor (UBF) on mitotic chromosomes [205]. We have previously identified a 16 amino acid region of the HPV8 E2 hinge that, when fused to the C-terminal DNA binding domain, is both essential and sufficient to bind pericentromeric foci on mitotic chromosomes. Furthermore, two residues within this hinge region, arginine 250 (R250) and serine 253 (S253), are critical for E2 mediated chromosomal interaction [235].

The regulatory mechanisms governing E2 mediated papillomavirus chromosome tethering and genome partitioning have not yet been completely elucidated. Many viruses have evolved to rely on post-translational modifications to regulate the functions of viral proteins and their interactions with cellular proteins.

For example, post-translational modifications have been implicated in regulating the chromatin binding function of the herpesvirus tethering protein EBNA1. The 325-376 GR-rich chromosome binding region of EBNA1 is both phosphorylated, likely by calmodulin-dependent kinase II, and methylated by protein arginine methyl transferases (PRMT), PRMT1 and PRMT5 [240]. Phosphorylation of multiple serine residues within the GR-rich region is important for the partitioning function of EBNA1 [240]. EBNA1 is a nuclear protein with small amounts of protein observed in the nucleolus. Inhibition of EBNA1 arginine methylation alters its localization within the nucleolus from a diffuse pattern to a peri-nucleolar ring. This suggests that methylation affects the EBNA1 interaction with either nucleolar proteins or RNA [240].

The PV E2 proteins are also subjected to such post-translational modifications that regulate their various functions and properties. In the case of BPV1 E2 protein, phosphorylation of serine residue 301 within the hinge region by casein kinase 2 (CK2) triggers a conformational switch that targets the E2 protein for proteosomal degradation [201]. Mutating the serine 301 residue in the BPV1 viral genome resulted in expression of a highly stable E2 protein and very high levels of viral DNA [166], indicating that E2 phosphorylation regulates viral genome copy number. The observation that when the half-life of E2 protein was increased, significantly higher number of viral genomes were found to be tethered to the mitotic chromosomes further supported the role of phosphorylation in regulating E2 function [243]. In the case of HPV16, phosphorylation of E2 results in increased stabilization of the E2 protein in the S-phase of the cell cycle [129], but

the functional significance of this E2 modification in the viral life cycle is not completely known. Conversely, recent work has also shown that nuclear receptor interaction protein (NRIP), a novel calmodulin binding protein, activates the phosphatase calcineurin that promotes dephosphorylation of HPV16 E2 protein, which in turn increases E2 stability and E2 mediated transcription [45]. In addition, studies have also reported a role for sumoylation in influencing E2 stability [274].

Similarly, the HPV8 E2 protein is highly phosphorylated [235]. The two residues crucial for chromosomal association, R250 and S253, lie within a common kinase motif, RXXS that is highly conserved in the E2 proteins of all members of the beta genus. We have also shown that S253 within this motif is phosphorylated [235]. The fact that the residues required for HPV8 E2 chromosomal association and phosphorylation are the same, raises the possibility that S253 phosphorylation plays a role in regulating the chromosome association function of the HPV8 E2 protein. Additionally, a comparison of the chromosome binding regions of the gamma herpesvirus proteins, EBNA1 and LANA with HPV8 E2 reveals that all three tethering proteins contain RXXS motifs within their respective chromosome binding regions [235]. This suggests that the chromosome binding function of these proteins might be regulated by a common mechanism. To gain further insight into regulation of the chromosome binding function of the HPV8 E2 protein, we investigated the role of phosphorylation of S253. Here, we show that phosphorylation of S253 by protein kinase A (PKA) regulates the interaction of the HPV8 E2 protein with host chromatin.

Results

Residues arginine 250 and serine 253 are essential for the chromosome binding function of the full length HPV8 E2 protein

We have previously shown that residues R250 and S253 within the hinge of the HPV8 E2 protein along with the C-terminal DNA binding and dimerization domain, are essential to bind to mitotic chromosomes [235]. In the current study, we wanted to ensure that mutating these residues in the background of the full-length protein abrogated the chromosome binding phenotype of the mutated proteins. We individually mutated each of these residues in the background of the full length HPV8 E2 protein and examined the localization of the E2 proteins on the mitotic chromosomes by indirect immunofluorescence. As shown in figure 4.1, alanine substitutions of residues R250 and S253 abrogated the chromosome binding function of the mutated E2 proteins. These residues lie within a common kinase motif, RXXS that is highly conserved among all betapapillomaviruses. We have previously reported that S253, within this motif, is phosphorylated [235].

Detection of HPV8 E2 protein phosphorylated at residue serine 253

To further investigate the role of S253 phosphorylation on the chromosome binding function of the E2 protein, we screened two phospho-specific antibodies that could detect proteins phosphorylated at the serine residue within the RXXS motif. One of these antibodies detects phosphorylated serine within an RRXS motif (Cell Signaling, 9624) while the other recognizes phosphorylated serine with an RXXS motif (Cell Signaling, 9621). To determine whether these antibodies could

specifically detect S253 phosphorylated E2 proteins, cell extracts prepared from CV-1 cell lines expressing inducible wild-type E2 protein and the S253 mutated E2 protein, were immunoprecipitated using the flag epitope tag and analyzed for phosphorylation by immunoblotting. Both phospho-RXXS and phospho-RRXS motif specific antibodies could detect the S253 phosphorylated E2 protein (Figure 4.2). Furthermore, phosphorylated E2 proteins were not detected in the S253A mutated E2 protein samples. For all further studies we used the phospho-RRXS motif specific antibody (Cell Signaling, 9624).

We also tested a number of other phospho substrate antibodies directed against phospho-MAPK/CDK substrate, phospho-Akt substrate and phospho-(Ser) PKC substrate, to determine whether they could detect S253 phosphorylation. None of these antibodies recognized the phosphorylated E2 proteins. Thus, the phospho-RXXS motif specific antibodies provide a valuable tool to detect HPV8 E2 proteins specifically phosphorylated at S253.

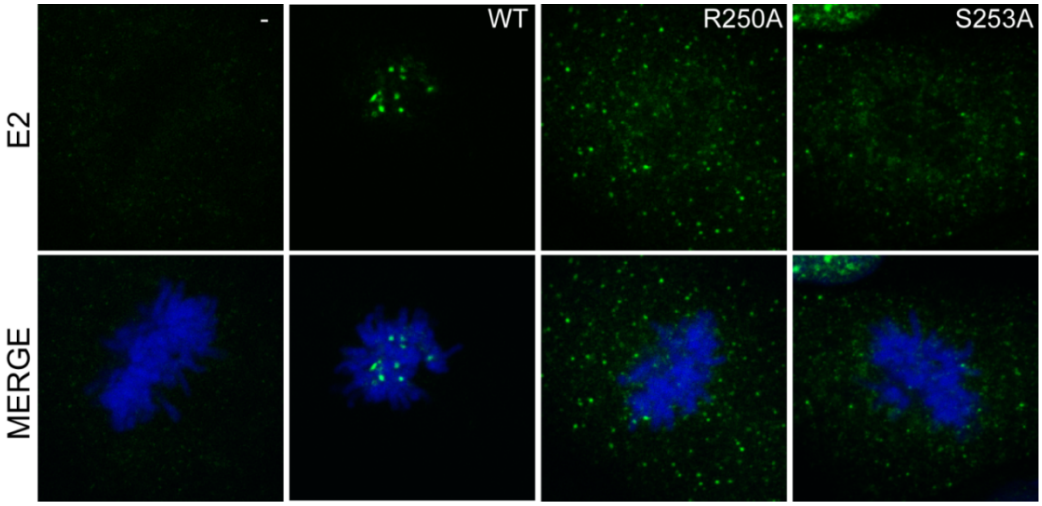


Figure 4.1
Residues arginine 250 and serine 253 are critical for the full length HPV8 E2 chromosomal association. CV-1 cell lines carrying control vector plasmid (-) or plasmids expressing wild-type HPV8 E2 protein (WT), R250 mutated E2 protein (R250A) or S253 mutated E2 (S253A) protein were visualized by indirect immunofluorescence to determine the location of E2 proteins on the mitotic chromosomes. E2 proteins were detected using anti-flag antibody (green) and cellular DNA was stained with DAPI (blue).

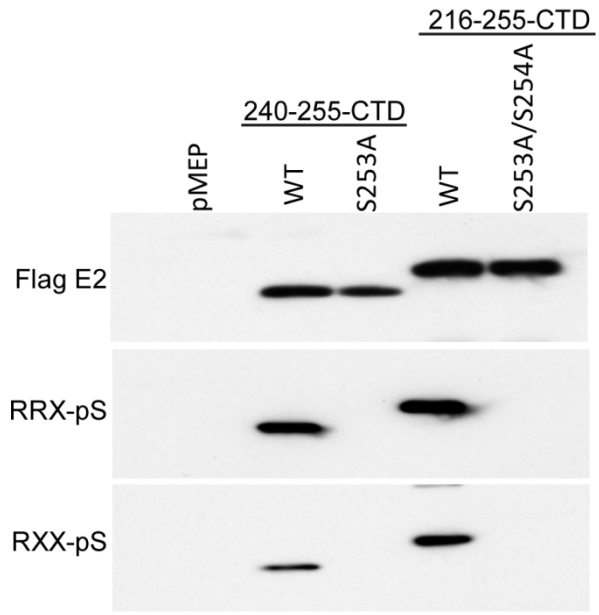


Figure 4.2

Phospho-RXXS motif specific antibodies can detect HPV8 E2 proteins phosphorylated at S253. Proteins extracted from CV-1 cell lines carrying control vector plasmid or plasmids expressing truncated E2 proteins were immunoprecipitated with M2 anti-flag antibody beads. Two versions of WT and mutated E2 proteins were used; one contained a 16 amino-acid region of the hinge fused to the C-terminal domain (240-255-CTD) and the other a 40 amino-acid region of the hinge fused to the CTD (216-255-CTD). The mutated E2 protein in the 240-255-CTD background has an alanine substitution at residue 253 (S253A). The mutated E2 protein in the 216-255-CTD background has alanine substitutions at residues S253 and S254 (S253A/S254A). Phosphorylated E2 proteins were detected by phospho-RXXS and phospho-RRXS motif specific antibodies. Total E2 protein was detected using rabbit anti-flag antibody.

HPV8 E2 protein is phosphorylated at S253 residue by Protein Kinase A (PKA)

The RXXS motif is a common kinase motif that is shared by a number of different cellular kinases including protein kinase A and aurora kinases. To understand the role of S253 phosphorylation in regulating the chromosome binding function of the HPV8 E2 protein, we proceeded to first identify the cellular kinase responsible for S253 phosphorylation. We used inhibitors against various cellular kinases such protein kinase G, CaM kinase II and Rho-kinase and examined their effects on E2 phosphorylation (Figure 4.3A). Treatment with the inhibitors did not affect the levels of S253 phosphorylation. In addition, we also treated cells with different concentrations of inhibitors against PKA and aurora kinases (Figure 4.3B). Treatment with PKA inhibitor H89 resulted in reduction in S253 phosphorylation levels whereas; treatment with aurora kinase inhibitor ZM447439 did not affect the levels of S253 phosphorylation (Figure 4.3B). The decrease in the levels of E2 phosphorylation observed with 2μM ZM447439 was not reproducible. Moreover, treatment with a PKA enhancer forskolin resulted in enhanced E2 phosphorylation (Figure 4.3B). Additionally, using a program called pkaPS predictor that predicts the PKA phosphorylation sites based on a simplified kinase substrate binding model [184], S253 was predicted to be a strong PKA substrate site (Figure 4.4). Thus, the above findings indicated that PKA might be responsible for S253 phosphorylation. PKA is a cAMP dependent protein kinase that is involved in phosphorylation of many different cellular proteins involved in regulating signal transduction pathways, immune regulation and gene expression [219].

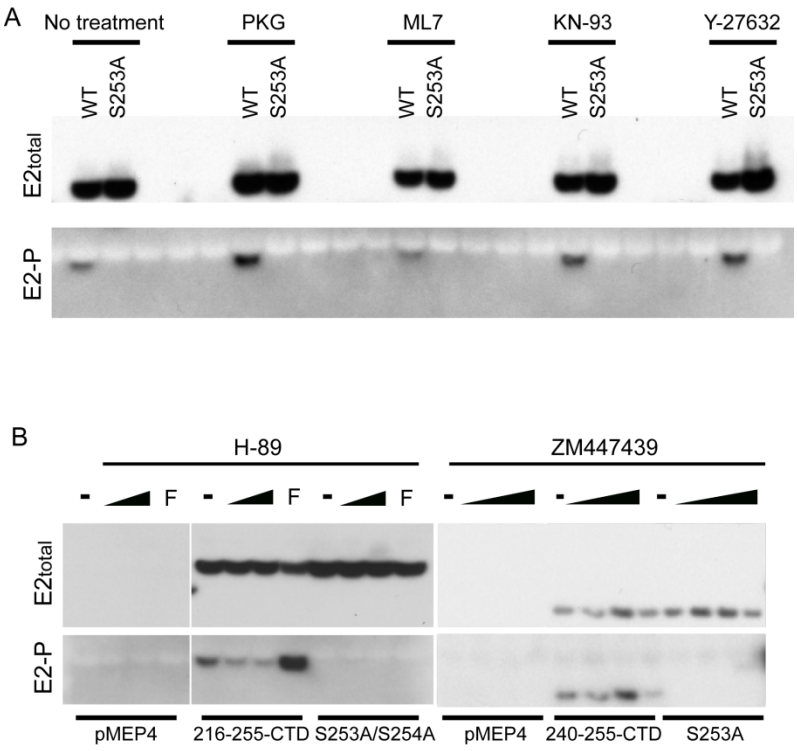


Figure 4.3

Effect of kinase inhibitors on S253 phosphorylation. (A) CV-1 cell lines carrying plasmids expressing 216-255-CTD E2 (WT) or mutated E2 (S253A) were either untreated or pretreated for 2h with PKG inhibitor (1μM), CaM kinase II inhibitor, KN-93 (5μM) or Rho-kinase inhibitor, Y-27632 (10μM). Following pretreatment, E2 was induced for an additional 3h. RIPA cell extracts were prepared and equivalent amounts of total proteins were immunoprecipitated with M2 anti-flag antibody beads. Phosphorylated E2 protein was detected using phospho-RRXS motif specific antibody, followed by re-blotting for total E2 protein using rabbit anti-flag antibody.
(B) CV-1 cell lines carrying pMEP plasmid, plasmid expressing wild-type (216-255-CTD or 240-255-CTD) or mutated E2 (S253A/S254A or S253A) were either untreated (-) or pretreated for 2h with different concentrations of PKA inhibitor, H-89 (10μM, 20μM) or PKA enhancer 10μM forskolin (F) or aurora kinase inhibitor, ZM447439 (100nM, 1μM, 2μM). Following pretreatment, E2 was induced for an additional 3h and E2 proteins were detected as described in panel (A).

To further confirm the role of cellular PKA in S253 phosphorylation, we used different PKA modulators to either activate or inhibit PKA activity and examined their effects on the phosphorylation status of the E2 protein. CV-1 cells expressing 240-255-CTD E2 and S253A mutated E2 were pretreated for 2h with the PKA inhibitors H89 and KT- 5720 or PKA enhancers, forskolin and cholera toxin (CTX). Treatment was followed by induction of E2 expression for 3h in the presence of the PKA modulators. H89 and KT-5720 are competitive antagonists that bind to the ATP site on the catalytic subunit of PKA and prevent phosphorylation of target proteins. On the contrary, the PKA enhancers, forskolin and CTX act by increasing the levels of cAMP in the cells, which results in increased cellular PKA activity. Treatment of E2 expressing CV-1 cells with 10μM H89 resulted in reduced levels of S253 phosphorylation compared to untreated cells (Figure 4.5). Treatment with KT-5720 twice resulted in lower lewels of E2 phosphorylation, whereas the third time no reduction was observed. There is some residual amount of phosphorylated E2 protein observed even after the inhibitor treatment. This could either be due to incomplete inhibition of PKA at the concentrations used or due to redundancy with a secondary protein kinase that can phosphorylate E2 in the absence of PKA. Reciprocally, treatment of E2 expressing cells with enhancers of PKA activity (10μM forskolin or 100ng/ml CTX) greatly increased the levels of S253 phosphorylation compared to that in untreated cells (Figure 4.5). Thus, modulation of cellular PKA activity alters the level of HPV8 E2 phosphorylation at S253 confirming that PKA can phosphorylate residue S253.

Results

Description	Position	Score	Sequence
HPV8 E2	151	0.35	MWKHIYYTDADDKWHKTTSGVNQTGIYYMQGSFRHYYVVFAD
	221	0.19	PPGSPPGQADTDTAAKTPTTSADSTSRQQRSPAKQPQQTETK
>	253	2.32	AKQPQQTETKGRRYGRRPSSRTRPQKEQRRSRSRHRTRSRSR
	254	0.37	KQPQQTETKGRRYGRRPSSRTRPQKEQRRSRSRHRTRSRSRS
	265	1.01	RYGRRPSSRTRPQKEQRRSRSRHRTRSRSRSLSRVRAVGSTT
	267	1.53	GRRPSSRTRPQKEQRRSRSRHRTRSRSRSLSRVRAVGSTTVS
	271	1.37	SSRTRPQKEQRRSRSRHRTRSRSRSLSRVRAVGSTTVSRSRS
	273	2.03	RTRPQKEQRRSRSRHRTRSRSRSLSRVRAVGSTTVSRSRSSS
	275	1.73	RPQKEQRRSRSRHRTRSRSRSLSRVRAVGSTTVSRSRSSSLT
	277	1.74	QKEQRRSRSRHRTRSRSRSLSRVRAVGSTTVSRSRSSSLTKA
	279	1.67	EQRRSRSRHRTRSRSRSLSRVRAVGSTTVSRSRSSSLTKAVR
	294	0.85	RSLSRVRAVGSTTVSRSRSSSLTKAVRPRSRSRSRGRATATS
	295	1.25	SLSRVRAVGSTTVSRSRSSSLTKAVRPRSRSRSRGRATATSR
	296	1.74	LSRVRAVGSTTVSRSRSSSLTKAVRPRSRSRSRGRATATSRR
	305	0.75	TTVSRSRSSSLTKAVRPRSRSRSRGRATATSRRRAGRGSPRR
	307	1.66	VSRSRSSSLTKAVRPRSRSRSRGRATATSRRRAGRGSPRRRR
	309	2.10	RSRSSSLTKAVRPRSRSRSRGRATATSRRRAGRGSPRRRRST
	314	1.13	SLTKAVRPRSRSRSRGRATATSRRRAGRGSPRRRRSTSRSPS

Figure 4.4
Prediction of the PKA phosphorylation sites within the HPV8 E2 protein using the pkaPS predictor program (174). Residue S253 within the HPV8 E2 hinge has a high score of 2.32 that is highlighted in red.

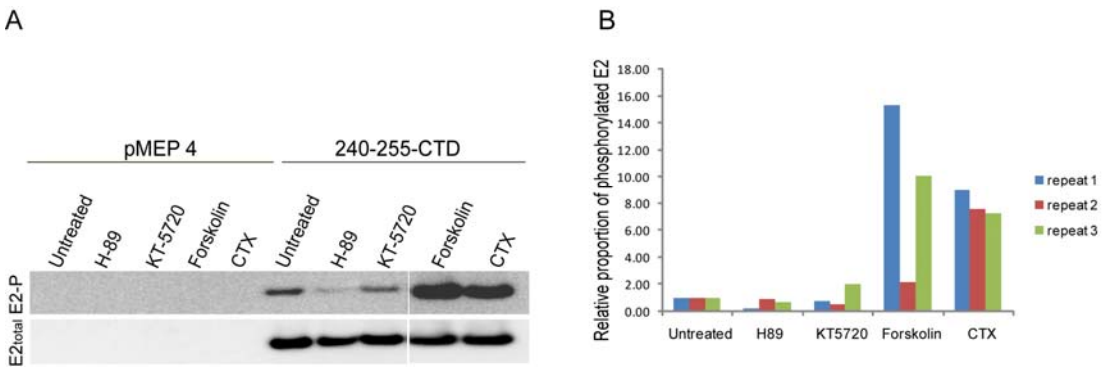


Figure 4.5

PKA phosphorylates HPV8 E2 protein at serine 253 residue. CV-1 cell lines carrying control vector plasmid or plasmid expressing 240-255-CTD E2 protein were either untreated or pretreated for 2 h with PKA inhibitors, 10μM H-89 and 6μM KT-5720 or PKA enhancers, 10μM forskolin or 100ng/ml cholera toxin (CTX) followed by E2 induction for 3h. (A) RIPA cell extracts were prepared and equivalent amounts of total proteins were immunoprecipitated with M2 anti-flag antibody beads. Phosphorylated E2 protein was detected using phospho-RRXS motif specific antibody, followed by re-blotting for total E2 protein using rabbit anti-flag antibody. (B) Quantitative representation of the proportion of phosphorylated E2 protein at indicated times relative to phosphorylated E2 in asynchronous cells observed by immunoblotting in different experimental repeats. The repeat 1 (blue bar) for treatment with KT5720 was performed at 2μM concentration.

Modulation of cellular PKA activity affects the mitotic localization of the HPV8 E2 protein

Since the two residues, R250 and S253, which are critical for the chromosomal association function of the HPV8 E2 protein are also important for E2 phosphorylation [235], we wanted to examine whether modulating the activity of cellular PKA affected mitotic localization of the E2 protein. CV-1 cell lines expressing 240-255-CTD E2 proteins were treated with the different PKA modulators as described above and E2 localization in mitotic cells were examined by indirect immunofluorescence. Cells were also stained for UBF to detect the ribosomal DNA loci on mitotic chromosomes. Following treatment with the PKA inhibitor H89, there was a reduction observed in the number of mitotic cells with E2 foci on mitotic chromosomes compared to the untreated cells (Figure 4.6A and 4.6B). The cells without E2 foci on mitotic chromosomes had undetectable levels of E2 expression and there were almost no mitotic cells observed with E2 protein excluded from the chromosomes. These observations raise the possibility that E2 protein that is not bound to chromosomes is unstable and undetectable by immunofluorescence. The percentage of cells expressing E2 in untreated and treated interphase cells was similar, ranging from 54% of E2 expressing cells in untreated cells to 46% in treated cells. Also no change in the level of total E2 protein was observed following treatment with the different PKA modulators, as seen in the immunoblot in figure 4.5A. In contrast, treatment with PKA enhancers, forskolin and CTX, resulted in an increase in the number of mitotic cells with visible E2 foci,

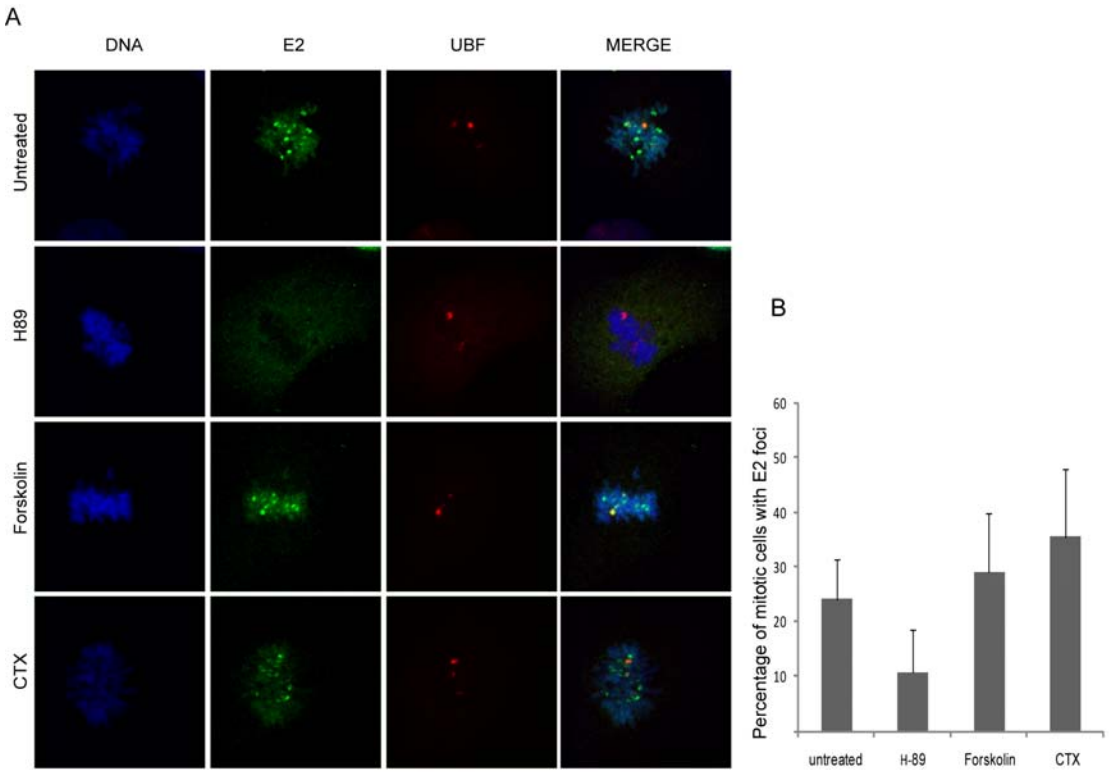


Figure 4.6
Mitotic localization of the HPV8 E2 protein is affected by modulators of cellular PKA activity. (A) CV-1 cell lines carrying control vector plasmid or plasmid expressing 240-255-CTD E2 protein were either untreated or pretreated for 2h with 10μM H-89 or 10μM forskolin or 100ng/ml CTX followed by E2 induction for 3h. Cells were visualized by indirect immunofluorescence to determine the location of E2 proteins on the mitotic chromosomes. E2 proteins were detected using anti-flag antibody (green), UBF with anti-UBF antibody (red) and cellular DNA was stained with DAPI (blue). (B) Quantitative representation of the percentage of mitotic cells with E2 foci on the mitotic chromosomes in the untreated, H-89, forskolin or CTX treated samples. Error bars represent standard deviation from two experimental repeats.

compared to the untreated cells (Figure 4.6). However, there was no significant difference observed in the number of E2 foci per mitotic cell. Thus, modulating the activity of cellular PKA affects the mitotic localization of the HPV8 E2 protein, indicating that cellular PKA regulates the chromosomal association function of the HPV8 E2 protein.

We have previously shown that both the full length HPV8 E2 protein and the truncated 240-255-CTD E2 protein localize as distinct pericentromeric E2 foci on the mitotic chromosomes of both African green monkey CV-1 cells and human C33A cells [205,235]. In case of the C33A cells, these pericentromeric E2 foci localize to the rDNA loci on the acrocentric chromosomes and colocalize completely with the RNA polymerase I transcription factor UBF [205]. However, in CV-1 cells there are two populations of E2 associated pericentromeric foci, one is UBF positive and one is UBF negative (Figure 4.6). Thus in CV-1 cells, there are additional UBF negative E2 binding sites located adjacent to the centromere. Notably, PKA affects the association of E2 with both UBF positive and UBF negative E2 regions.

Enhanced E2 phosphorylation results in an increase in the number of interphase cells with E2 associated pericentromeric foci

In CV-1 cells during interphase, the 240-255-CTD E2 protein appears in a diffuse nuclear pattern with some nucleolar staining. However, in a subset of cells 240-255-CTD E2 also localizes in distinct, bright foci that are adjacent to the centromeres, stained with an anti-centromere antibody (ACA) (Figure 4.7). Notably,

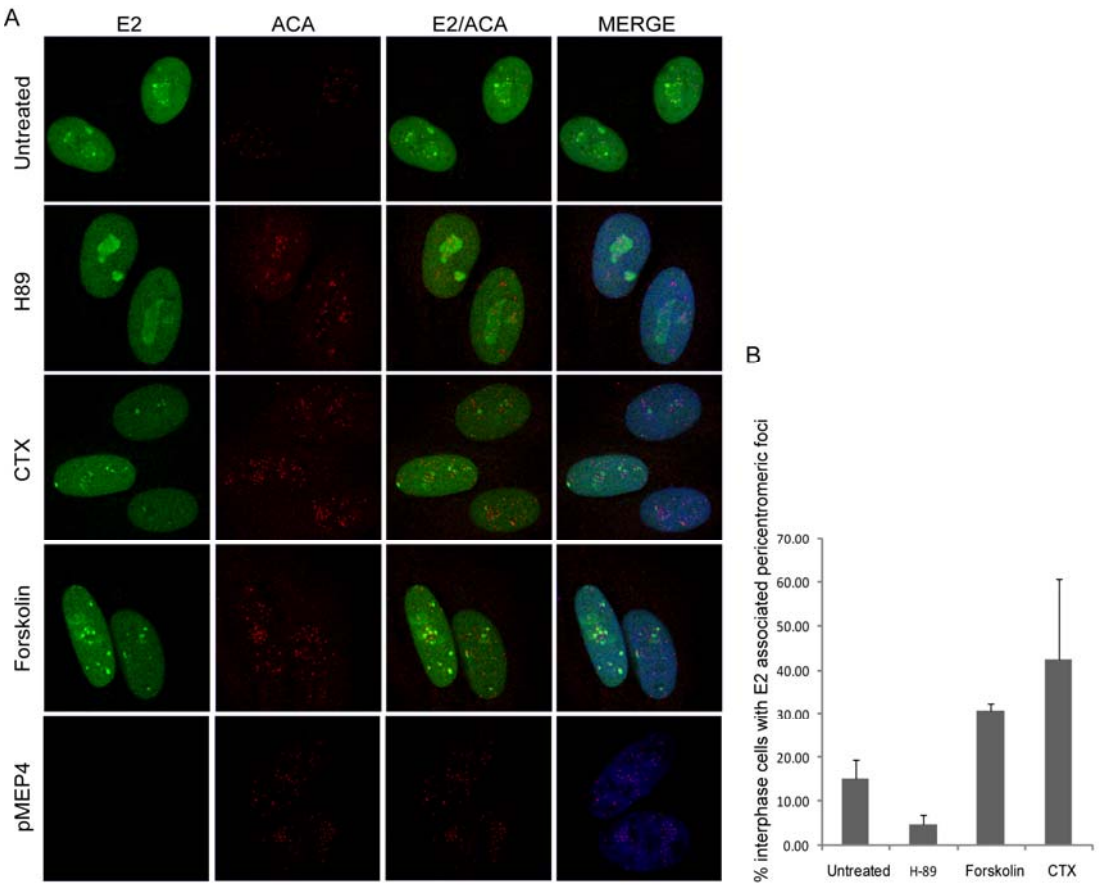


Figure 4.7
Modulation of PKA activity alters the association of E2 with pericentromeric foci in interphase cells. (A) CV-1 cell lines carrying control vector plasmid (pMEP) or plasmid expressing E2 protein 240-255-CTD were either untreated or pretreated for 2h with PKA inhibitor 10μM H-89 or PKA enhancers, 10μM forskolin or 100ng/ml CTX followed by E2 induction for 3h. Cells were visualized by indirect immunofluorescence to determine the location of E2 proteins in interphase cells. E2 proteins were detected using anti-flag antibody (green), centromere with anti-ACA antibody (red) and cellular DNA was stained with DAPI (blue). (B) Quantitative representation of the percentage of E2 expressing interphase cells with E2 associated pericentromeric foci in untreated, H-89, forskolin and CTX treated samples. Error bars represent standard deviation from two experimental repeats.

these pericentromeric interphase foci are completely absent in C33A cells, and in cells expressing the S253A mutated 240-255 CTD E2 protein. We believe that these foci represent the UBF negative foci observed in mitotic CV-1 cells. To examine whether the appearance of the E2 associated pericentromeric foci in interphase cells correlated with the phosphorylation status of the E2 protein, their localization was examined following treatment with the different PKA modulators. As shown in figure 4.7A, treatment of wild-type 240-255-CTD E2 protein expressing cells with H89 almost completely abrogated the appearance of E2 bound to pericentromeric foci in interphase cells. Most interphase cells showed only a diffuse nuclear E2 staining pattern, with some minor nucleolar localization. However, there was a dramatic increase in the number of interphase cells with E2 associated pericentromeric foci in cells treated with the PKA enhancers (Figure 4.7A and 4.7B). These results indicate that the localization of E2 adjacent to the centromere in CV-1 interphase cells is linked to the phosphorylation status of the E2 protein and these foci most likely represent the UBF negative pericentromeric E2 foci in CV-1 cells. Thus, enhanced levels of E2 phosphorylation result in an increase in the number of interphase cells with pericentromeric E2 foci, whereas in the presence of the PKA inhibitor, there is nearly a complete loss of these foci (Figure 4.7B).

S253 phosphorylationa of HPV8 E2 increases during S–phase and mitosis

To further understand the dynamics of regulation of the chromosomal association function of the HPV8 E2 protein, we examined the levels of E2 phosphorylation during the different stages of the cell cycle. CV-1 cells carrying a

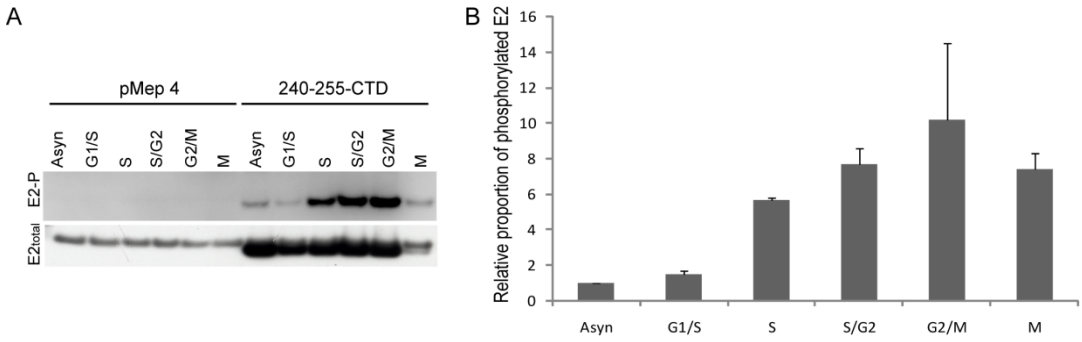


Figure 4.8

HPV8 E2 phosphorylation at S253 increases in S-phase and remains high until mitosis. (A) CV-1 cell lines carrying control vector plasmid or plasmid expressing 240-255-CTD E2 protein were synchronized with thymidine and proteins extracted at 0h (G1/S), 3h (S), 6h (S/G2) and 9h (G2/M) post thymidine release in RIPA extraction buffer following E2 induction for 3h. Mitotic cells (M) were collected by mitotic shake off at 9h post thymidine release. Equivalent amounts of total protein were immunoprecipitated with M2 anti-flag antibody beads. Phosphorylated E2 protein was detected using phospho-RRXS motif specific antibody followed by re-blotting for total E2 protein using rabbit anti-flag antibody. (B) Quantitative representation of the proportion of phosphorylated E2 protein at indicated times relative to phosphorylated E2 in asynchronous cells observed by immunoblotting in panel (A). The error bar shown is a representation of standard deviation of two experimental repeats.

plasmid expressing the E2 protein or the control vector pMEP, were synchronized using 2mM thymidine for 16h, which arrests cells at G1/S boundary and in the S-phase of the cell cycle. After releasing the E2 expressing cells from thymidine block, the cells were allowed to progress through the cell cycle. E2 expressing mitotic cells were collected by mitotic shake off at 9h post thymidine release. E2 proteins were extracted in modified RIPA buffer at the indicated times and analyzed for E2 phosphorylated and total E2 levels by immunoprecipitation and immunoblotting. As shown in figure 4.8A, there is a significant increase in the level of E2 phosphorylation during S-phase, compared to the G1-phase or in an asynchronous population of cells. The levels of phosphorylated E2 remain high during the late stages of S-phase, G2/M and mitosis. Thus, as shown in the quantitative data in 4.8B, during mitosis the level of E2 phosphorylation is high, further indicating that phosphorylated E2 is bound to host chromatin, since only chromosome bound E2 can be detected at this stage.

Increase in phosphorylation of the E2 protein in S-phase correlates with an increase in association of E2 protein with pericentromeric foci

Since, an increase in the number of interphase cells with E2 associated pericentromeric foci corresponds with an increase in E2 phosphorylation and the level of E2 phosphorylation increases in S-phase; we wanted to determine whether the interphase foci were S-phase specific. 240-255-CTD E2 protein expressing cells were synchronized with thymidine. Cells were released from thymidine block and allowed to progress through the cell cycle. The cells were then fixed with 4% paraformaldehyde at 0h and 3h post release, in G1/S phase and S-phase respectively.

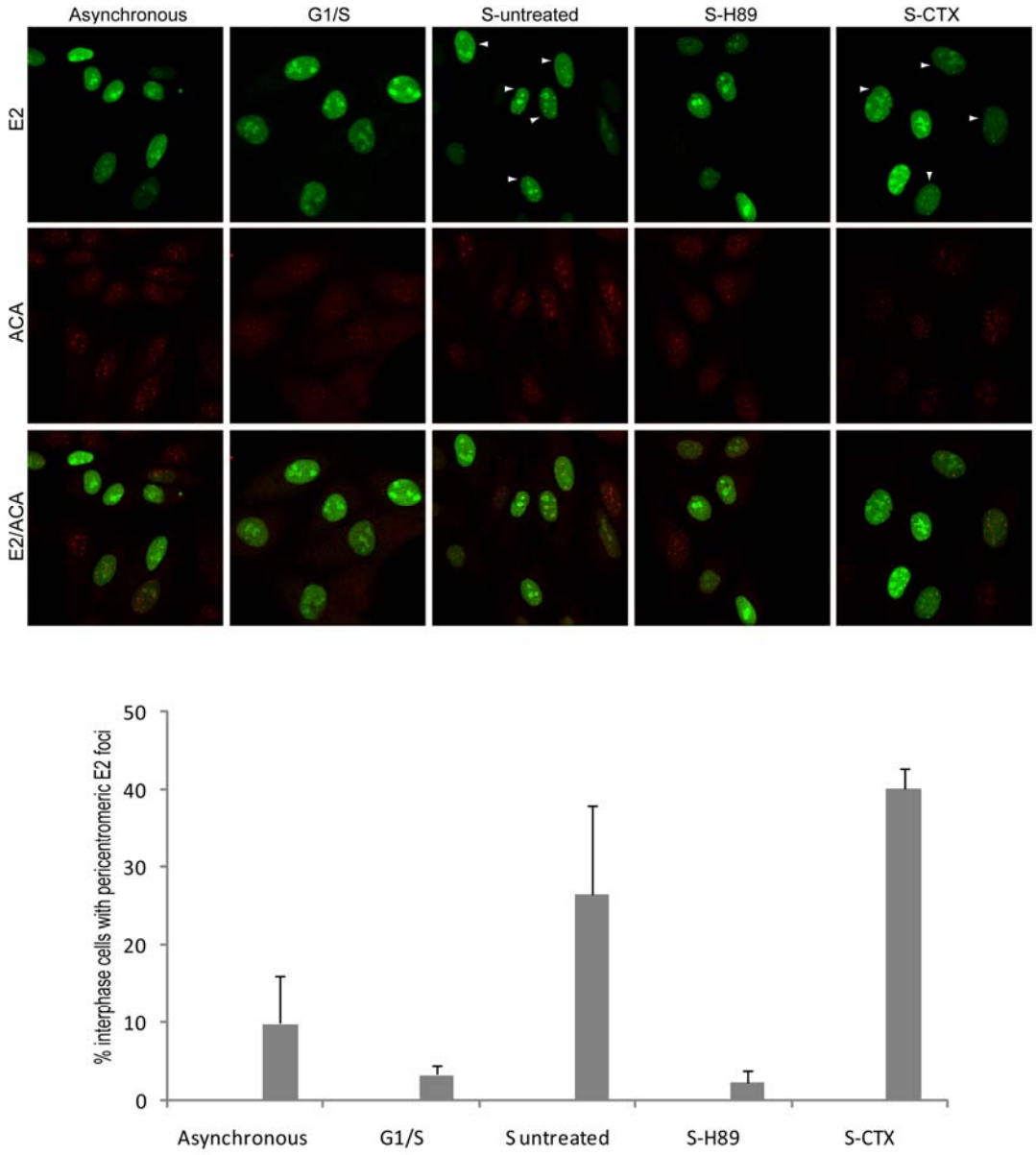


Figure 4.9
E2 association with pericentromeric regions increases in S-phase. (A) CV-1 cells expressing 240-255-CTD E2 protein were synchronized with thymidine and fixed at 0h (G1/S) and 3h (S) post thymidine release. In addition, S-phase samples were also treated with H89 (10μM) or CTX (100ng/ml) for 2h followed by E2 induction for 3h prior to fixing. Asynchronous cells were also fixed following E2 induction for 3h. Cells were visualized by indirect immunofluorescence to determine the localization of E2 proteins. E2 protein was detected using M2 anti-flag antibody (green) and centromeres with anti-ACA (red). (B) Quantitative representation of the percentage of E2 expressing interphase cells with E2 associated with pericentromeric foci in different stages of the cell cycle. The experiments were repeated two times.

Asynchronous cells were also fixed with 4%paraformaldehyde. As shown in figure 4.9A, there is an increase in the number of interphase cells with pericentromeric E2 foci in the cells enriched for S-phase compared to the asynchronous population and the G1/S phase enriched cells. Notably, there is a marked reduction in the number of foci seen in G1/S phase cells. Modulation of PKA activity in S-phase cells resulted either in a modest increase in the number of cells with foci in the presence of CTX or reduction in the presence of H89 (Figure 4.9A and 4.9B). Thus, there is an increase in E2-pericentromeric foci in S-phase and this correlates with enhanced E2 phosphorylation.

HPV8 E2 proteins phosphorylated at S253 have a longer half-life

While examining the mitotic localization of E2 protein following H89 inhibitor treatment, we observed that there were almost no mitotic cells with E2 protein excluding the chromosomes. It appeared that in mitotic cells without chromosome bound E2 the levels of E2 protein were undetectable. These observations lead us to hypothesize that E2 protein that is not bound to chromosomes is unstable and hence, is not observed by immunofluorescence. To investigate this possibility further, we carried out a pulse-chase experiment and compared the half-life of the 240-255-CTD E2 with that of the S253A mutated E2 protein. As shown in figure 4.10A and 4.10B, 240-255-CTD E2 protein had a longer half-life than phosphorylation defective S253A E2 protein. However, with wild-type E2, there was a dramatic decrease in the levels of E2 protein in the first 20 minutes following inhibition of protein synthesis; however, from 40 minutes to 3h the levels of wild-

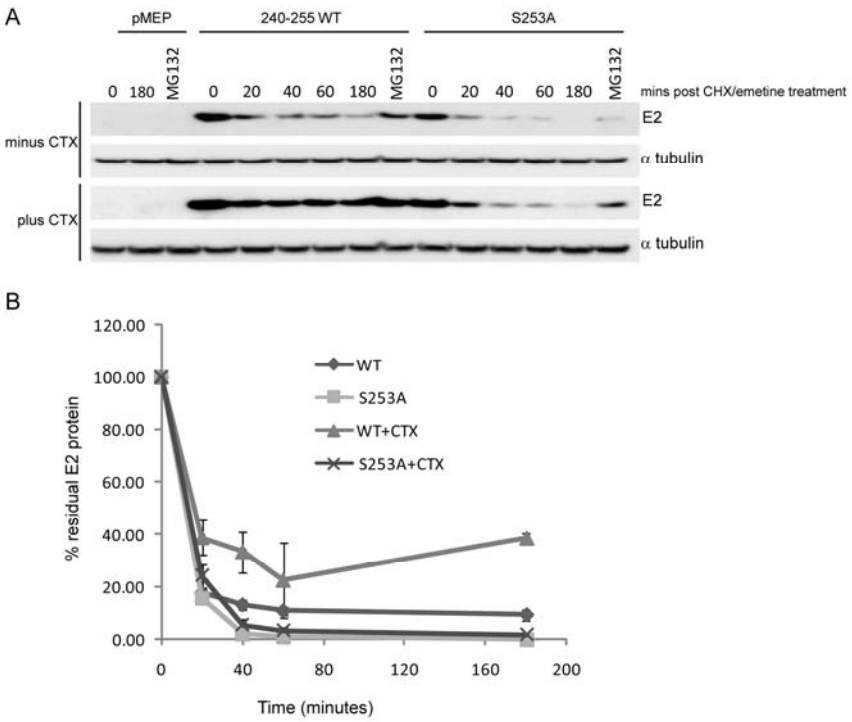


Figure 4.10

Phosphorylated E2 protein has a longer half-life than phosphorylation defective E2 protein. (A) CV-1 cell lines carrying control vector plasmid or plasmids expressing 240-255-CTD E2 protein or S253 mutated E2 protein (S253A) were either untreated or treated with 100ng/ml CTX for 2h followed by E2 induction for 2h with 4μM $CdSO_4$. Thereafter, cells were treated with protein synthesis inhibitors 25μM cycloheximide (CHX) and emetine (25μM) for the indicated time periods. Cells were also treated with MG132 (20μM) for 3h post E2 induction as indicated. Cell extracts were prepared in SDS extraction buffer at the indicated time points and E2 protein was detected using M2 anti-flag antibody and α tubulin was detected using anti-α tubulin. (B) Quantitative representation of the blot in (A) showing the percentage of E2 protein remaining plotted against time in minutes.

type protein remained stable. Thus, E2 decay was biphasic. In contrast, the S253A mutated E2 protein undergoes much more rapid degradation and by 40 minutes has almost disappeared. The proteosome inhibitor MG132 was also added to the cells for 3h, following inhibition of protein synthesis to see if it could rescue degradation of the E2 proteins. MG132 was only able to rescue the levels of wild-type E2 to about 20% of the initial protein amount and in the case of the S253A mutated E2 protein; there was minimal effect on protein recovery (Figure 4.10A). This suggests that the proteasome is not primarily responsible for the degradation of the labile population of E2. To determine whether enhancement of PKA phosphorylation would result in enhanced stability of the wild-type protein, E2 expressing cells were pretreated with CTX for 2h followed by induction of E2 expression, as described above. Following CTX treatment, the amount of wild-type E2 protein was higher at the zero time point compared to untreated E2. Pulse-chase analysis showed that the wild-type 240-255 E2 protein had an increased half-life in CTX treated cells and 40% of E2 protein remained stable for at least 3h, while the stability of the S253A protein was unchanged (Figure 4.10A and 4.10B). Thus, PKA phosphorylation increased the half-life of the wild-type E2 protein.

Next, we wanted to investigate whether the observed increase in E2 phosphorylation and stability were related to chromatin binding. To this end, we compared the half-life of the E2 protein in chromatin bound and unbound fractions. The unbound fraction was extracted using a standard lysis buffer (0.5% Triton, 0.1M NaCl, 2mM EDTA, 0.1M Tris pH 8) and the chromatin bound fraction was extracted in 2% sodium dodecyl sulfate (SDS), 50mM Tris-HCl (pH 6.8), 10% glycerol from

the insoluble pellet of the unbound fraction. As can be observed in figure 4.11, chromatin-bound wild-type 240-255 E2 protein is quite stable through the different time points following inhibition of protein synthesis irrespective of MG132 treatment. This indicates that the chromatin bound wild-type protein is resistant to proteosomal degradation. It is possible that the wild-type E2 population that we observe in figure 4.10A following MG132 treatment is the chromatin bound stable E2 that is resistant to degradation. On the contrary, almost no S253A mutated protein could be detected in the bound fraction. This further supports the finding that S253A is defective in chromatin binding. Furthermore, both E2 proteins were observed to be unstable in the unbound fraction, supporting our hypothesis that there is a correlation between chromatin binding and protein stability. Notably, MG132 treatment did not rescue either the wild-type or the S253A mutated E2 protein in the unbound fraction.

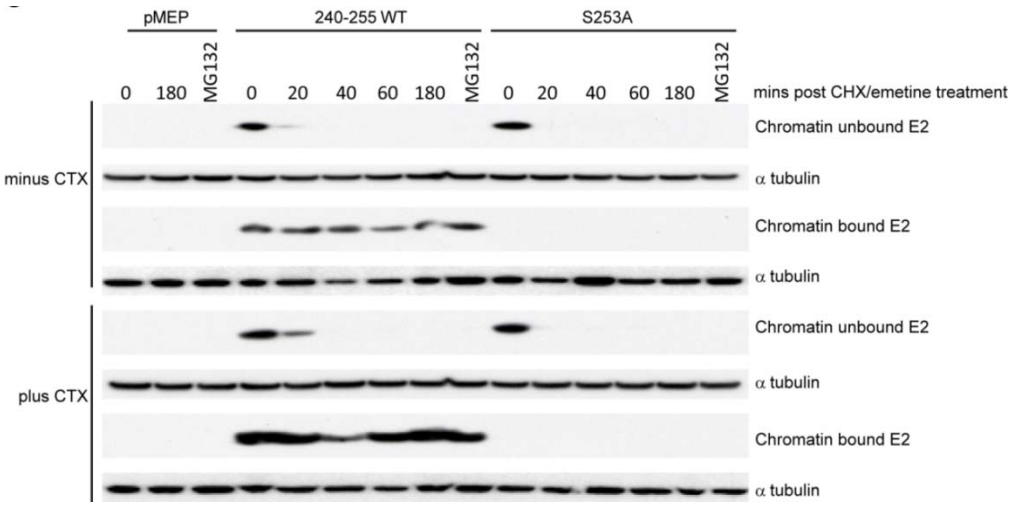
We also analyzed the difference in chromatin binding of E2 protein extracted from cells treated with CTX to enhance E2 phosphorylation. Increasing PKA phosphorylation with CTX resulted in a dramatic increase in the amount of wild-type E2 protein in the chromatin bound fraction whereas there is no effect on the distribution of the S253A E2 protein (Figure 4.11). Therefore, the increase in E2 protein half-life correlates perfectly with the increase in chromatin binding that we observe following enhanced E2 phosphorylation in mitotic cells. The E2 proteins in the non-chromatin bound fraction are unstable and do not show much difference in half-life following CTX treatment. Taken together, these observations indicate that enhanced E2 phosphorylation correlates with increased binding to host chromatin and increased half-life of the HPV8 E2 protein.

Discussion

We recently reported that residues R250 and S253, located within the chromosome binding region of the HPV8 E2 protein, are required for E2's chromosome binding function. Furthermore, S253 located within the highly conserved RXXS motif is phosphorylated [235]. In the current study, we have shown that phosphorylation of S253 regulates the chromosome binding function of the HPV8 E2 protein. RXXS is a common kinase motif shared by various cellular kinases, including protein kinase A (PKA). Using modulators of cellular PKA activity we have demonstrated that S253 is most likely phosphorylated by PKA.

PKA is a cAMP dependent protein kinase that regulates many biological processes [254]. PKA is involved in multiple functions during the different stages of the cell cycle, ranging from regulation of S-phase replication to mitotic progression [278]. We report here that the level of E2 phosphorylation at residue S253 increases in S-phase and remains high through mitosis. This finding is in line with previous studies that have shown that PKA is required for chromosomal DNA replication, and so is active during the S-phase of the cell cycle [53]. PKA is ubiquitous and hence, is under strict spatial and temporal regulation. The A kinase anchoring proteins (AKAPs) play an important role in directing PKA to specific cellular sites so that they are in close proximity to their substrates [244]. One such protein is AKAP95, which recruits the regulatory subunit of PKA to chromosomes during mitosis and plays a key role in chromatin condensation at mitosis [50,142].

A



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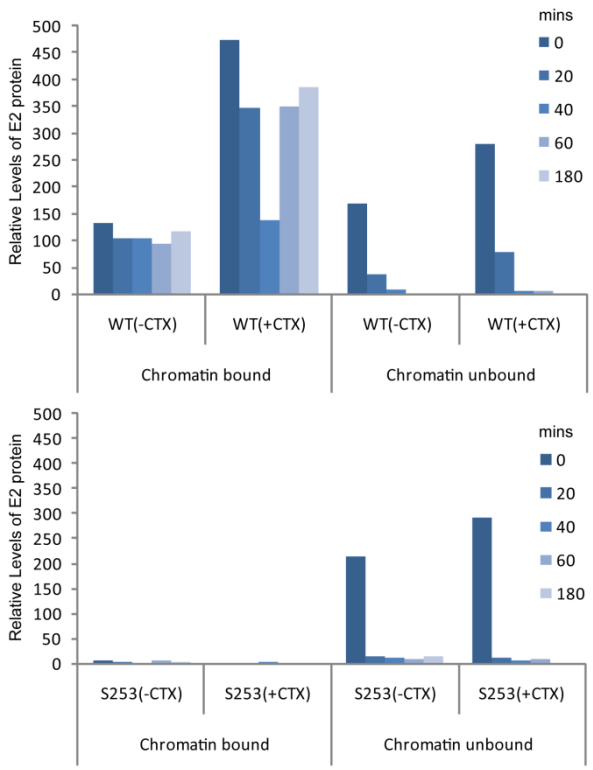


Figure 4.11
Chromatin bound E2 protein has a longer half-life than unbound E2. (A) Chromatin bound and chromatin unbound fractions were prepared from CV-1 cell lines carrying control vector plasmid or plasmids expressing 240-255-CTD E2 protein or S253 mutated E2 protein (S253A) that were either untreated or treated as described in Figure 4.10A. E2 protein was detected using M2 anti-flag antibody and α tubulin was detected using anti-α tubulin. (B) Quantitative representation of the relative levels of E2 protein in the chromatin bound and unbound fractions.

Since E2 also binds host chromatin, it is possible that PKA anchored to chromatin by AKAP95 is readily available to phosphorylate E2 protein.

Previous studies have also implicated PKA in the HPV life cycle. PKA has been shown to phosphorylate the Dlg/PDZ binding motif of high-risk HPV E6 [138] and regulates the E6 dependent degradation of the human discs large protein (Dlg). PKA also plays a role in HPV16 E7 mediated transformation by contributing to cellular alkalinization, a common characteristic of transformed cells [42]. More recently, the HPV16 E5 protein has been reported to utilize the PKA pathway to inhibit apoptosis of transformed cervical epithelial cells by stimulating proteosomal mediated degradation of the Bax protein [189]. Although the functional significance is not known, the HPV11 E1^E4 protein has also been reported to be phosphorylated in vitro by PKA and MAPK [37]. These findings, along with our study, highlight the importance of PKA in the HPV life cycle.

The HPV8 E2 protein binds to mitotic chromosomes as large distinct pericentromeric foci in human cells. These foci colocalize with UBF and overlap with the rDNA loci found on the short arm of acrocentric chromosomes [205]. However, in CV-1 cells, two populations of E2 associated pericentromeric foci are observed on mitotic chromosomes. One population is the UBF positive E2 foci that colocalize with UBF at the rDNA loci. The other consists of UBF negative foci that are localized adjacent to the centromeres of non-acrocentric chromosomes [235]. We believe that the latter regions consist of pericentromeric satellite DNA. Similar heterochromatic satellite regions are observed in mouse cells [102] and the gamma herpesvirus

tethering protein LANA has been shown to bind to these murine chromocenters [162]. The E2-associated UBF-negative pericentromeric foci are not observed in human cells and it is possible that in human cells similar repetitive regions are dispersed throughout the chromosomes and hence, are difficult to detect. Although, the HPV8 E2 protein has been shown to colocalize with the rDNA genes in human cells, the exact target has not been identified. It is possible that E2 is interacting with repetitive DNA sequences that are interspersed among the copies of rDNA genes and that these regions are similar to the monkey pericentromeric satellite DNA.

While the HPV8 E2 protein binds to different mitotic chromosomal locations in cell types from different species, the binding is regulated in a very similar manner. Mutation of the S253 phosphorylation site in E2 eliminates chromatin binding of both populations in human and monkey cells, as does the inhibition of PKA activity. There is no colocalization of E2 with UBF-positive foci in either monkey or human interphase cells, most likely because the rDNA loci are sequestered into the nucleoli. However, one advantage of monkey cells is that the 240-255-CTD E2 protein can be observed binding to the UBF-negative pericentromeric regions in interphase as well as in mitosis. This interphase localization provides a useful tool to elucidate the regulation of E2-chromatin association throughout the cell cycle. Our analysis shows that E2 is bound to the UBF-negative foci during S-phase and that this interaction is increased by PKA phosphorylation of E2.

There is a strong correlation between E2 phosphorylation, protein half-life and chromatin binding. PKA phosphorylation of HPV8 E2 enhances both chromatin binding and protein half-life. However, it is not clear whether E2 phosphorylation

directly regulates protein half-life thus leaving stable, phosphorylated E2 to bind to host chromatin or whether phosphorylation regulates chromatin binding and chromatin bound E2 has a much longer half-life than unbound protein. A dramatic decrease in the amount of E2 protein is observed by both immunofluorescence and western blotting as cells enter mitosis and only chromosome bound E2 can be detected in mitosis. Presumably, the labile population of unbound E2 protein rapidly disappears as cells cease transcription and translation at the onset of mitosis. It has also been shown that HPV16 E2 has both increased phosphorylation and extended half-life in S-phase [129].

Our findings are in accordance with a recent study that showed that the chromosome binding Gly-Arg repeat regions of the EBV tethering protein, EBNA1, were shown to inhibit its proteasomal degradation and stabilize the protein [52]. These chromosomal tethering regions are thought to resemble A-T hook motifs that bind with high avidity to A-T rich sequences on cellular DNA [232]. Another study demonstrated that EBV replicons replicate while bound to host chromatin [181]. Furthermore, the daughter molecules remain paired at the site of DNA synthesis until they are partitioned in mitosis. HPV8 replication could follow a similar scenario in which viral genomes are tethered to host chromatin by the E2 protein in S-phase and the daughter molecules remain bound throughout mitosis. Additionally, the presence of RXXS motifs within the chromosome binding regions of EBNA1 and LANA further underscore the possibility of a common mechanism regulating the chromosome binding function of all three tethering proteins.

In summary, we have shown that PKA phosphorylates the HPV8 E2 protein at S253. E2 phosphorylated at S253 can bind to specific, UBF-negative regions of host chromatin in interphase and has a much greater half-life than unphosphorylated E2. We propose that E2 is phosphorylated primarily in S-phase and that phosphorylated, chromatin bound E2 remains stabilized at these sites through mitosis. However as cells transition from interphase to mitosis, additional phosphorylated E2 protein can also associate with UBF-positive regions of chromosomes that become available when the nucleolus disassembles. Both types of binding could be used to tether viral DNA to host chromosomes for viral genome partitioning. On the contrary, the population of E2 protein that is unphosphorylated, and not bound to chromatin, has a short half-life and disappears quickly as transcription and translation ceases at the onset of mitosis. Finally, when infected cells exit from mitosis and re-enter interphase, the E2 protein is likely dephosphorylated and degraded. Newly synthesized E2 protein would not be highly phosphorylated on serine 253 until the next S-phase.

The CTD of HPV8 E2 is required for mitotic chromosome association and localization to nuclear speckles. Primary functions of this domain include mediating dimer formation [165] and specifically binding to a DNA motif that is found at multiple positions in the viral genome [10]. In Chapter 5, studies were performed to understand the role of HPV8 E2 CTD in the chromosome binding function of the E2 protein.

Chapter 5: Role of the C-terminal DNA binding and dimerization domain in the chromosomal association function of the HPV8 E2 protein

Introduction

As described in earlier chapters, our mapping studies in the HPV8 E2 protein have shown that in addition to the elements in the hinge region, the CTD is also essential for the chromosomal association function of the HPV8 E2 protein [235]. The CTD of the PV E2 proteins belong to a unique structural class of proteins that form a dimeric beta-barrel structure, with each half of the barrel consisting of four beta strands and two alpha helices [110]. One of the alpha helices, termed the 'recognition helix', recognizes and binds a specific 12bp palindromic DNA sequence on the viral genome. The 12bp consensus E2 binding sites on the viral genome consist of two conserved four base pair sequences separated by a variable spacer region. The sequence specific interaction between the recognition helix of the E2 protein and the viral DNA is mediated by both the backbone and the side chains of the alpha helix [110]. The E2 protein directly binds to the sequences by inserting the recognition helix into the major groove of the DNA while the variable spacer regions that lie in the minor groove are not contacted. Although the spacer sequences are variable, their conformation affects the relative affinity with which the E2 proteins bind to the E2 binding sites [28,114]. For instance, HPV16 E2 has been observed to have a stronger preference for an 'AATT' spacer than a 'TTAA' spacer [114]. The conformational flexibility within the AT rich spacers allows for bending towards the major groove of the DNA [36]. The percent amino acid identity among the E2 CTDs ranges from 80%

among closely related types to approximately 30% among distant viral types [27]. The electrostatic interactions of the CTDs among the different papillomaviruses are conserved, which suggests that specific interactions of the E2 protein with DNA are also conserved [27]. E2 proteins are expressed as dimers in cells [165,175]. Over the years, several crystal structures have been solved such as those of high-risk HPV16 and HPV31 and low-risk HPV6 and BPV1 CTDs [39,61,109,110] . Several NMR solution structures have also been solved and these include the CTDs from HPV16, HPV31 and BPV1 [151,180,263].

Notably, the EBNA1 tethering protein of EBV also forms a dimeric beta-barrel structure similar to the E2 protein [29]. Although, the structure of EBNA1 CTD also consists of two alpha helices positioned similar to those of the E2 protein, it carries an additional flanking domain consisting of an alpha helix and an extended chain that seems to interact with the viral DNA in the crystal structure. Additionally, the CTD of the tethering protein LANA of KSHV has been predicted to form a dimeric beta-barrel structure similar to EBNA1 [101,105]. This prediction is further supported by studies wherein identification of residues in the LANA protein important for DNA binding and chromosome association functions were found to correlate with analogous EBNA1 residues [133,136]. These observations thus, point to a partial functional homology between these proteins.

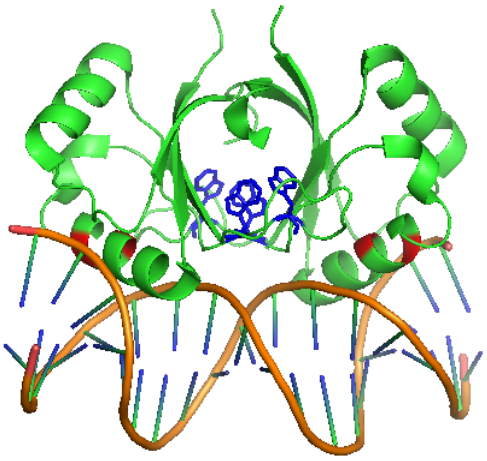
As mentioned earlier, the CTD of E2 proteins is important for both dimerization and binding to specific DNA motifs on the viral genome. The critical amino acid residues of the E2 protein that mediate dimerization and DNA contact have been clearly defined and mutations of these residues have been shown to

abrogate dimerization and DNA binding, respectively [206]. The residues that make crucial contact at the dimer interface for the HPV8 E2 proteins are residues W449 and W451 (Figure 5.1A). Similarly, residues R431 and R433 are important for binding to sequences on the viral DNA (Figure 5.1A). The dimerization function of the CTD is essential for efficient BPV1 E2 mediated mitotic chromosome binding [41]. Substitution of the BPV1 E2 CTD with the dimeric DNA binding domain of Gal4 from *Saccharomyces cerevisiae* or EBNA1 could rescue both the mitotic chromosome binding phenotype and Brd4 binding function of the E2 protein [41]. However, substitution with a monomeric red fluorescence protein did not rescue either function of the BPV1 E2 protein [41].

Preliminary studies conducted in our laboratory showed that, in addition to the HPV8 E2 dimerization defective protein (W449A/W451A) expressed in the full length background, a DNA binding defective protein (R431K/R433K) was also deficient in chromosome binding. We examined the mitotic localization of both the dimerization and DNA binding defective proteins in the background of 240-255-CTD (Figure 5.1B). In all the W449A/W451A and R431K/R433K E2 expressing mitotic cells, E2 proteins were excluded from the mitotic chromosomes and did not show any colocalization with UBF.

At first glance, these observations point towards a requirement for both dimerization and DNA binding functions of the CTD, for the chromosomal association function of E2. However in our tethering model, the DNA binding function of E2 is required to bind to motifs in the viral genome and would not be predicted to affect the E2 localization on host chromosomes. Hence, before.

A



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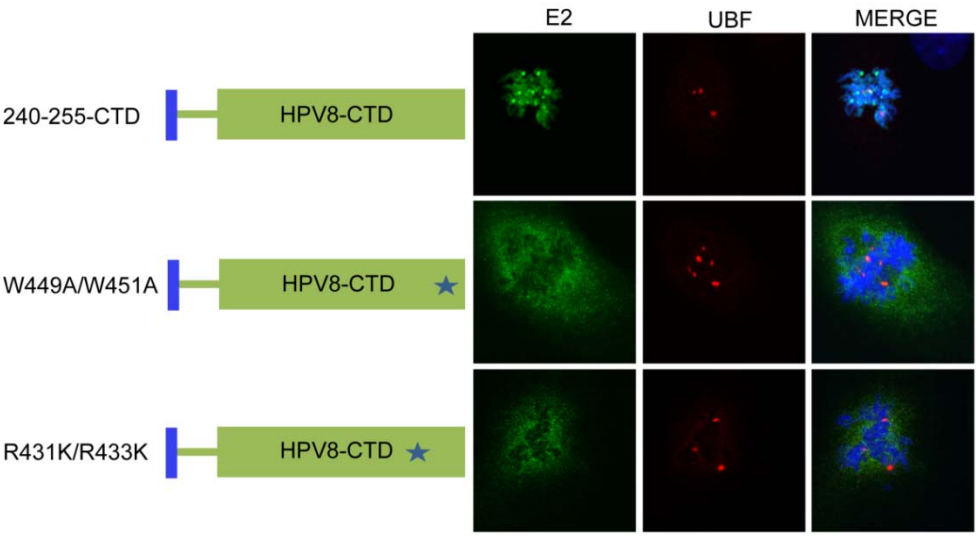

Figure 5.1
Dimerization defective and DNA binding defective HPV8 E2 proteins are unable to bind mitotic chromosomes. (A) Structure of HPV6a E2 CTD with the residues required for DNA binding highlighted in red and dimerization residues highlighted in blue reconstructed using PyMol (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC) (2AYG (HPV6a CTD) Hooley, Fairweather et al., 2006) (B) CV-1 cell lines expressing the 240-255-CTD wild-type protein, dimerization defective (W449A/W451A) and DNA binding defective (R431K/R433K) proteins were visualized by indirect immunofluorescence to determine the localization of the E2 proteins and UBF on mitotic chromosomes. E2 proteins were detected by anti-flag antibody (green), UBF with anti-UBF antibody (red) and cellular DNA was stained with DAPI (blue).

concluding that the DNA binding function is required for E2 host chromosomal association, we wanted to assess whether other functions such as dimerizaton of the CTD was affected by the R431K/R433K mutation. In this chapter, we have designed experiments to further elucidate the role of the CTD in the chromosome binding function of the HPV8 E2 protein

Results

The DNA binding defective E2 protein R431K/R433K is capable of dimerization

Firstly, it was important to rule out the possibility that the R431K/R433K E2 protein's inability to bind to chromosomes was due to a defect in dimerization. It is possible that the lysine substitutions disrupted the structure of the E2 protein such that it is unable to dimerize. An *in vitro* dimerization assay was performed to check for the ability of the R431K/R433K E2 protein to self-associate. The putative dimerization defective W449A/W451A HPV8 E2 protein was included as a negative control and the wild-type E2 protein as a positive control. We expressed an N-terminal flag tagged version of the wild-type 240-255-CTD E2 protein along with the W449A/W451A and R431K/R433K proteins, also in the 240-255-CTD background (Figure 5.2A). In addition, we also expressed the untagged versions of all the proteins in the full length HPV8 E2 background (Figure 5.2A). The flag tagged and untagged versions of the proteins were generated by *in vitro* translation in rabbit reticulocyte lysates. For the dimerization assay, both untagged and flag tagged proteins were either in vitro translated independently and mixed together or co-translated. For E2

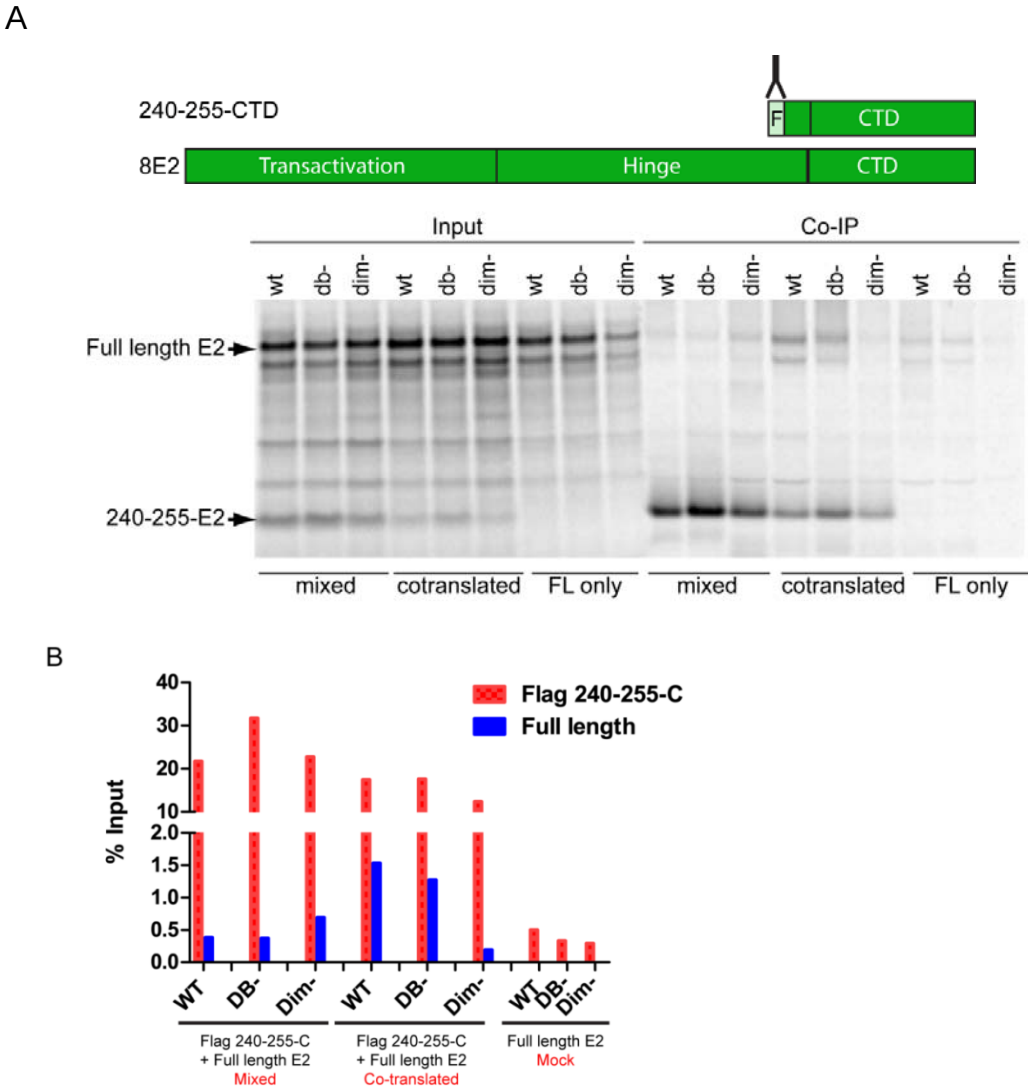

Figure 5.2
DNA binding defective E2 protein R431K/R433K can form dimers. (A) Untagged version of the full length (FL) HPV8 E2 WT (represented in the top panel as 8E2), DNA binding defective E2 protein R431K/R433K and dimerization defective W449A/W451A were generated by *in vitro* translation. Similarly, the flag tagged versions of the wild-type (represented in the top panel as 240-255-CTD with an N-terminal flag tag shown in blue and an anti-flag antibody), R431K/R433K and W449A/W451A proteins, in the 240-255-CTD background were also translated *in vitro*. For the dimerization assay, both untagged and flag tagged proteins were generated by either *in vitro* translation independently and mixed together or co-translated together. E2 proteins were immunoprecipitated using anti-flag antibody and co-immunoprecipitation of untagged proteins were detected by western blot analysis. (B) Graphical representation of the immunoprecipitation of the untagged proteins (blue bar) relative to the % input.

proteins to dimerize efficiently it has been shown that they need to be co-translated together [165]. E2 proteins were then immunoprecipitated with an anti-flag antibody and the co-immunoprecipitation of the untagged version was detected by western blot analysis. The presence of the untagged R431K/R433K E2 protein in the immunoprecipitated complex confirmed its ability to dimerize. As can be observed in figure 5.2, E2 proteins only dimerize when they are co-translated.

Similarly, the untagged wild-type full length E2 protein was also immunoprecipitated with the tagged version. However, as expected the dimerization defective W449A/W451A HPV8 E2 protein did not immunoprecipitate with the flag tagged version, further confirming that it is clearly dimerization defective (Figure 5.2). Thus, the *in vitro* dimerization assay indicates that the DNA binding defective R431K/R433K protein is capable of dimerization showing that its inability to bind to mitotic chromosomes is not related to a defect in dimerization.

Substitution of the HPV8 E2 CTD with CTDs from other PVs

The *in vitro* dimerization experiment indicates that the inability of the R431K/R433K protein to bind to chromosomes was unlikely to be due to a defect in dimerization. Therefore, the possibility that the DNA binding function is required for E2-host chromosomal association could still not be dismissed. To test this hypothesis further, we decided to substitute the HPV8 E2 CTD with E2 CTDs from PVs belonging to different genera. As mentioned above, the E2 CTDs of PVs belonging to different genera are conserved and capable of both dimerization and DNA binding. Our rationale behind the domain swap approach was that if the HPV8 E2 CTD's contribution to its chromosome binding function was limited to dimerization and

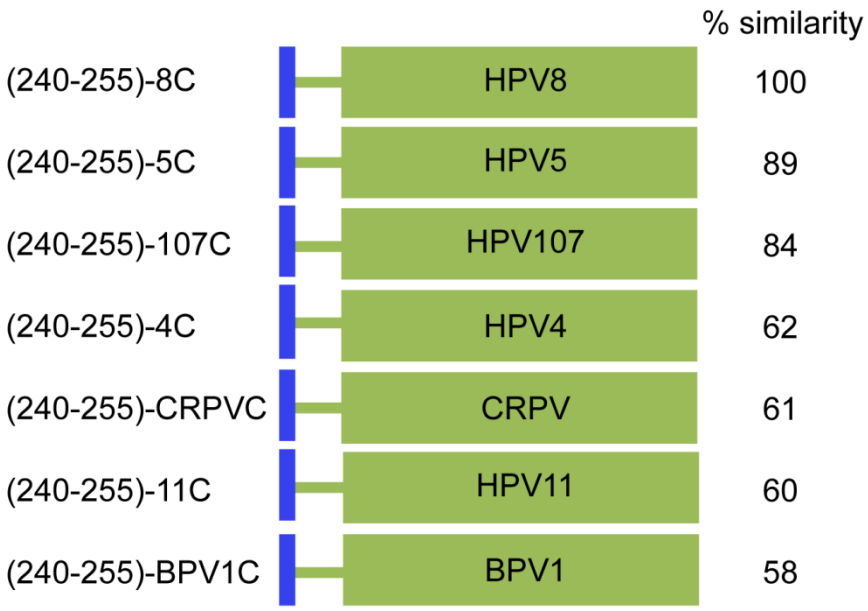

Figure 5.3
Substitution of the HPV8 E2 CTD with CTDs from other PVs. Fusion proteins were generated by substituting the CTD of the HPV8 E2 with CTDs from different PVs fused to the 240-255 hinge peptide. These included the E2-CTDs from species 1 of beta PV (HPV5), species 2 of beta PV (HPV107), gamma PV (HPV4), kappa PV (SfPV1), alpha PV (HPV11) and delta PV (BPV1). The percent amino acid similarity (% similarity) of the CTDs were calculated relative to HPV8 E2 CTD using the BioEdit program.

DNA binding, then substitution of the HPV8 E2 CTD with CTDs from other PVs should be able to restore the chromosome binding function of fusion proteins. Various fusion proteins were generated by substituting the HPV8 E2 CTD, fused to the 240-255 hinge peptide. The HPV8 E2 hinge peptide was fused to the CTDs of HPV11 from alpha, HPV5 from beta species 1, HPV107 from beta species 2, HPV4 from gamma, delta BPV1 and kappa SfPV1 (Figure 5.3). The percent amino acid similarity between the CTD of the E2 proteins from different genera relative to the HPV8 E2 CTD is shown in figure 5.3. The members belonging t beta species 2 are phylogenetically closest to HPV8, a member of the species 1 of beta papillomaviruses, followed by gamma HPV4, kappa SfPV1, alpha HPV11 and delta BPV1. We also included the DNA binding domain of a non-PV protein, LANA. As mentioned above, the predicted structure of the LANA CTD is similar to that of the E2 CTD [134]. Moreover, the LANA CTD has been shown to bind to pericentromeric and telomeric regions of mitotic chromosomes similar to E2 protein [132]. Additionally, Kelley-Clarke *et. al* have shown that a mutated LANA-CTD (LANA-SHP) protein, although capable of dimerization and binding to viral DNA, is defective in the chromosome binding function. The LANA-SHP carries alanine substitutions at serine 1125, histidine 1126 and proline 1127 of the CTD. We generated a fusion protein by fusing the E2 hinge peptide to LANA-SHP. We wanted to examine whether fusing the 240-255 peptide from the HPV8 E2 hinge to the LANA-SHP could rescue the chromosome binding phenotype of this protein. We also generated E2 proteins lacking the 240-255 hinge peptide and consisting of only the flag-tagged CTDs. All of the flag-tagged proteins were expressed in CV-1 cells and

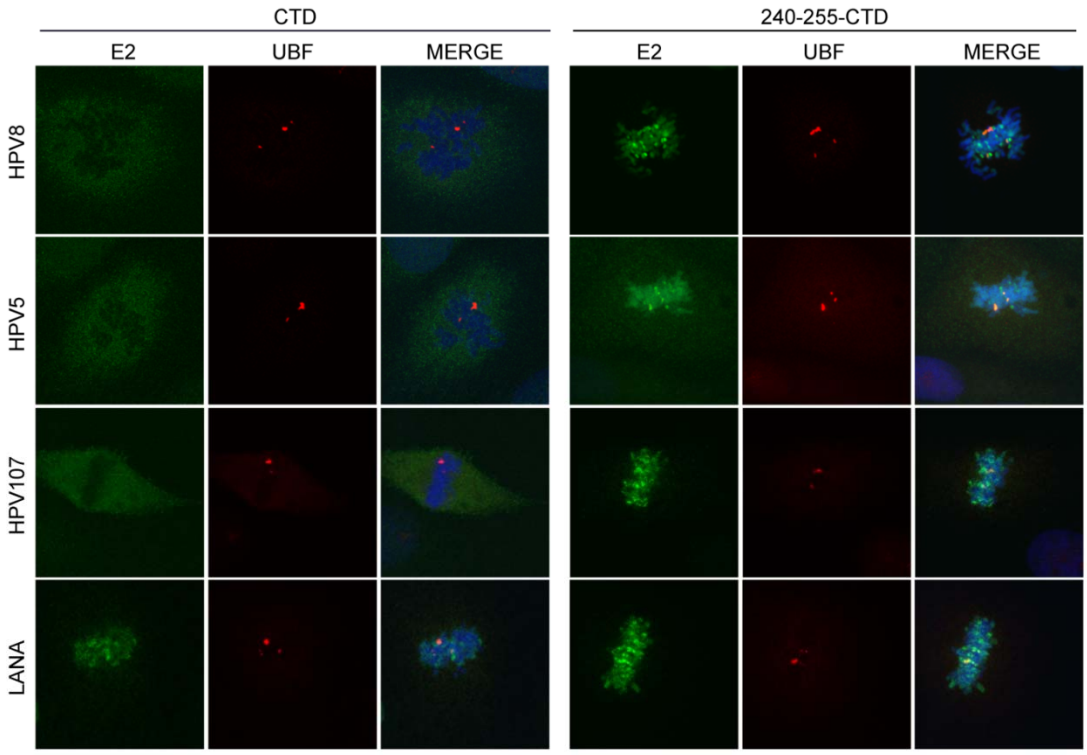

Figure 5.4
E2 CTDs from species 1 and 2 of beta papillomaviruses can functionally substitute the HPV8 E2 CTD when fused to the 240-255 hinge peptide. The LANA CTD can bind mitotic chromosomes in the presence and absence of 240-255 hinge peptide. CV-1 cell lines expressing either PV CTDs alone or fusion proteins consisting of the 240-255 hinge fused to the CTDs from species 1 and 2 of beta PVs (HPV8, HPV5, HPV107) or to the LANA CTD were visualized by indirect immunofluorescence to examine the location of E2 proteins and UBF on mitotic chromosomes. E2 was detected using anti-flag antibody (green) and UBF with anti-flag antibody (red). Cellular DNA was stained with DAPI (blue).

their localization on mitotic chromosomes was examined using indirect immunofluorescence. We also examined if the CTDs or the fusion proteins colocalized with UBF, as seen in the case of the HPV8 E2 protein. From our confocal microscopic analysis, it is clear that none of the E2 proteins carrying the CTD alone but lacking the hinge peptide could bind to mitotic chromosomes (Figure 5.4 and 5.5). The only exception was the LANA-CTD, which has already been shown to bind to mitotic chromosomes as pericentromeric and telomeric foci (Figure 5.4). However in the case of the fusion proteins, the CTDs from beta species 1 and 2 could substitute for the chromosomal association function of HPV8 E2 when fused to the 240-255 hinge peptide (Figure 5.4). These fusion proteins bound to mitotic chromosomes in a focal pattern reminiscent of the wild-type HPV8 E2 protein. Similar to the wild-type protein, they also showed partial colocalization with UBF. Thus, both the HPV5 and HPV107 CTDs could functionally compensate for the CTD of HPV8 E2 protein.

On the contrary, fusing the 240-255 peptide to the CTDs of BPV1, HPV11, HPV4 and SfPV1 did not result in mitotic chromosome binding (Figure 5.5). Each of these fusion proteins were excluded from the mitotic chromosomes. The fusion protein with the LANA CTD showed similar pattern of mitotic binding to that of the LANA CTD alone. There was no obvious difference in mitotic localization in the presence and absence of the peptide (Figure 5.4). Furthermore, fusing the 240-255 peptide to the LANA-SHP did not rescue the chromosome binding phenotype. The resulting fusion protein was distributed around the mitotic chromosomes (Figure 5.5). Thus, from the fusion studies it is clear that substituting the CTD of HPV8 E2 protein

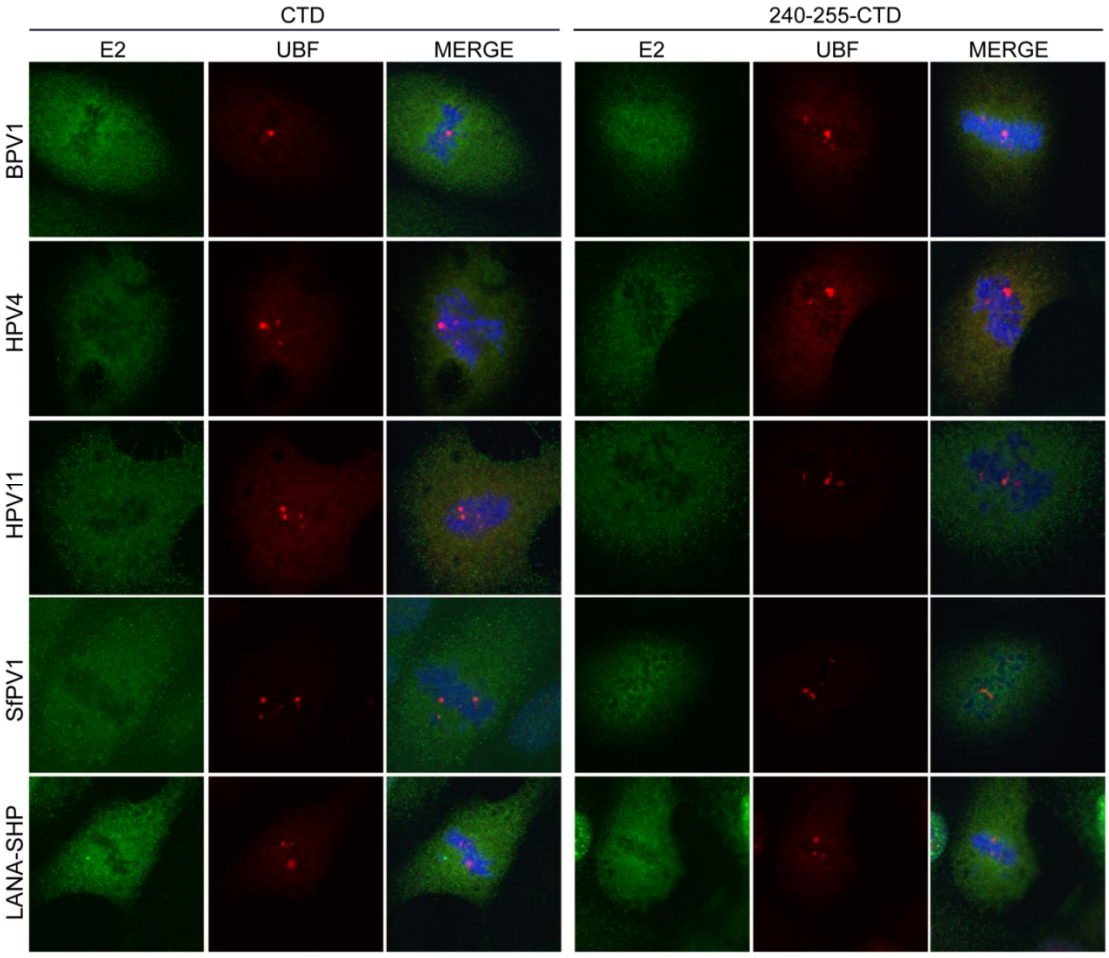

Figure 5.5
E2 CTDs from alpha, gamma, kappa or delta PVs cannot functionally substitute for the HPV8 E2 CTD when fused to the 240-255 hinge peptide. The mutated LANA-SHP is also deficient in chromosome binding when fused to hinge peptide. CV-1 cell lines expressing either PV CTDs alone or fusion proteins consisting of the 240-255 hinge fused to the CTDs from alpha PV (HPV11), gamma (HPV4), kappa (SfPV1), delta (BPV1) or to the mutated LANA-SHP CTD were visualized by indirect immunofluorescence to examine the location of E2 proteins and UBF on mitotic chromosomes. E2 was detected using anti-flag antibody (green) and UBF with anti-flag antibody (red). Cellular DNA was stained with DAPI (blue).

with the CTDs from the beta species 1 and 2, which are phylogenetically very close to one another, results in retention of the chromosome binding function of the HPV8 E2 protein. However, CTDs from distantly related types are unable to compensate for HPV8 E2 chromosomal binding.

Notably in the interphase cells, the CTDs from the species 1 of beta genera were localized to the cytoplasm. But when the hinge peptide was fused to the CTD, the resulting fusion protein almost exclusively localized to the nucleus (Figure 5.6). In the case of HPV4 E2 protein, both the CTD alone and the fusion proteins were observed to be mainly cytoplasmic (Figure 5.6). Fusing the hinge peptide to the HPV4 CTD did not alter its cytoplasmic localization. However, the interphase localizations of all the other fusion proteins (BPV1, HPV11, HPV107, SfPV1 and LANA) were strictly nuclear (data not shown).

There is still the possibility that the differences in the DNA binding specificities of the different CTDs, could contribute to the inability of the CTDs from different PVs to functionally replace the HPV8 E2 CTD. To gain a clearer understanding of this possible scenario, we compared the consensus E2 binding sites within the URR of the different PV genus, using the Multiple EM Motif Elicitation (MEME) software. Comparison of the consensus E2 binding sites of the beta PVs with those of the gamma, alpha and delta PVs, indicates that they have different DNA binding specificities (Figure 5.7). The members of the beta PV genus have a stronger preference for a ‘TTAA’ spacer sequence compared to the alpha PVs, which show a preference for the ‘AATT’ rich spacer instead. In contrast, the members of the gamma and delta genera do not have any specific requirement for spacer sequences

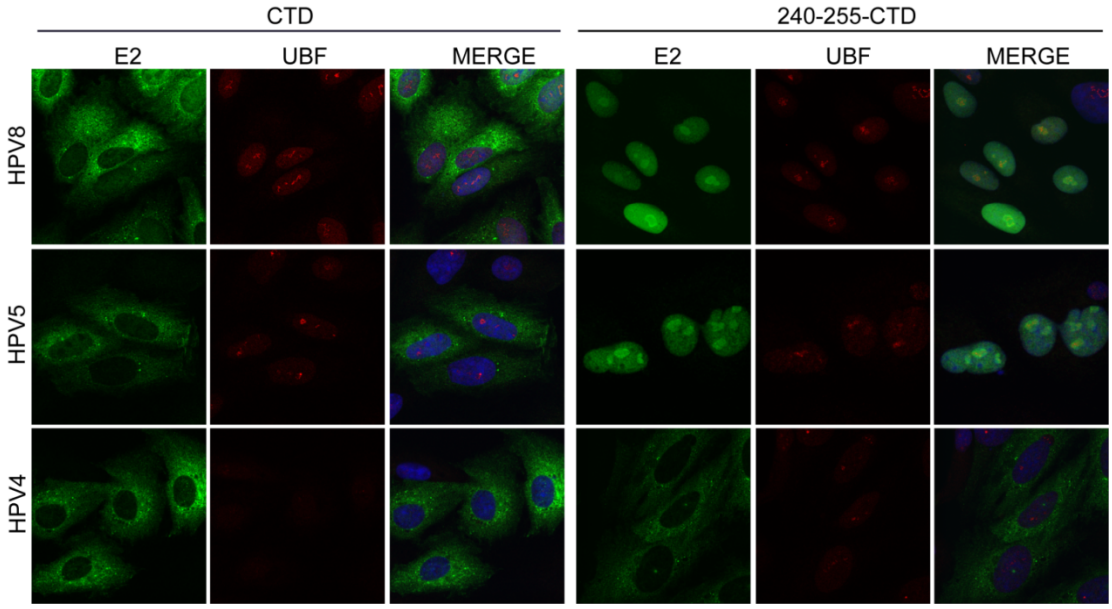

Figure 5.6
Interphase localization of HPV5, HPV8 and HPV4 CTDs and their respective fusion proteins. CTDs of HPV5, HPV8 and HPV4 localize to the cytoplasm in the absence of the peptide. Fusion of 240-255 peptide to HPV5 and HPV8 CTDs results in nuclear localization whereas fusion of 240-255 peptide to HPV4 CTD does not localizes the protein to the nucleus. CV-1 cell lines expressing either PV CTDs alone or fusion proteins consisting of the 240-255 hinge fused to the CTDs from HPV5, HPV4 and HPV8 were visualized by indirect immunofluorescence to examine the location of E2 proteins and UBF on mitotic chromosomes. E2 was detected using anti-flag antibody (green) and UBF with anti-flag antibody (red). Cellular DNA was stained with DAPI (blue).

when binding to DNA (Figure 5.7). This would suggest that the CTDs of the gamma and delta PVs because of their less stringent consensus sequence are more likely to bind to cellular DNA sequences. However in the domain swapping experiments, the fusion proteins with gamma and delta CTDs are excluded from the mitotic chromosomes. Thus, the CTD swapping experiments suggest that the beta CTDs have a specific role in chromosome binding that cannot be compensated by other PVs.

Discussion

Two known functions attributed to the CTD of PV E2 proteins are dimerization and DNA binding. The latter is mediated through its ability to bind to specific sequences on the viral genome. Additionally, we have shown that the CTD is required for the chromosomal association function of the HPV8 E2 protein [235]. Thus, this study was aimed towards understanding the role of the CTD in the chromosome binding function of the HPV8 E2 protein.

Although earlier work with BPV1 E2 has shown that dimerization is essential for mitotic chromosome binding [41], the observation that the DNA binding defective R431K/R433K HPV8 E2 protein was also defective in chromosome binding was unexpected. In accordance with our tethering model, the DNA binding function of the E2 protein is required to bind to sequences in the viral genome. Mutating residues involved in contacting the viral DNA would not be predicted to affect the localization of E2 on host chromosomes. In fact in the case of the BPV1 E2 protein, mutations of

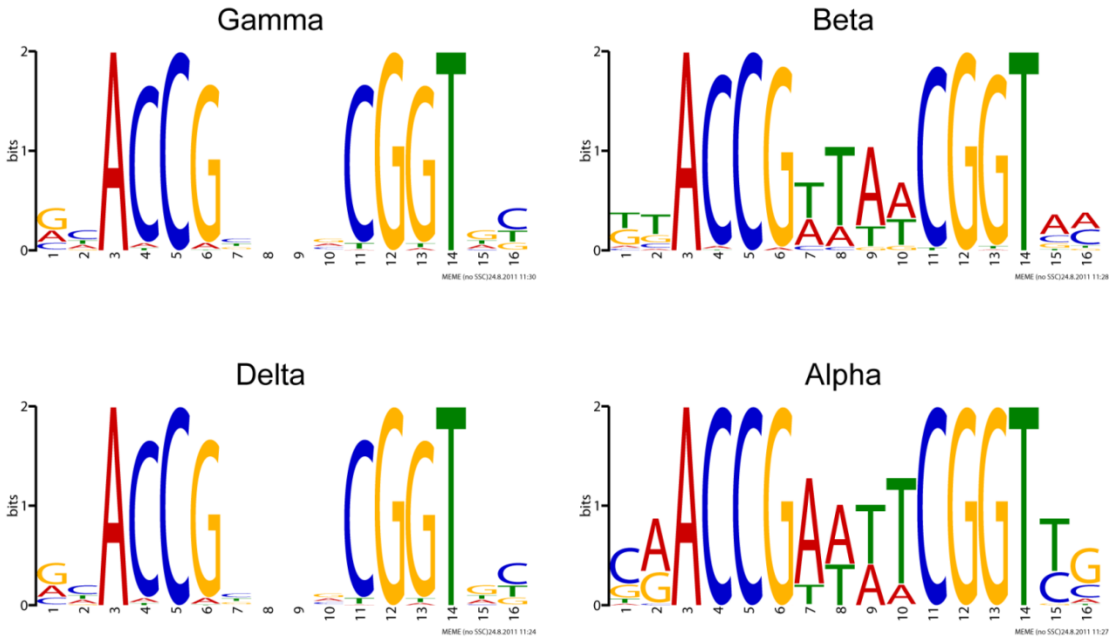

Figure 5.7
Consensus E2 binding sites varies among each genus of PVs. Sequence logo indicating the consensus E2 binding site motif for each of the PV genus obtained after comparing the URR sequences of all the members of the genera alpha (75 types), beta (44 types), gamma (25 types) and delta PVs (10 types) using the Multiple EM Motif Elicitation (MEME) software.

the DNA binding residues did not affect the chromosome binding function of the E2 protein [243]. Additionally, the *in vitro* dimerization assay ruled out the possibility that the DNA binding defective E2 protein's inability to associate with mitotic chromosomes was due to a dimerization defect. Instead, these observations indicated that the DNA binding function of the CTD might be required to mediate E2 chromosomal association. The CTDs from different PVs are capable of dimerization and DNA binding functions. We hypothesized that if the role of the CTD in the chromosome binding function of the HPV8 E2 protein was limited to dimerization and DNA binding, then substitution of the HPV8 E2 CTD with CTDs from other PVs should be able to restore the chromosome binding function of the resulting fusion protein. However, domain swapping experiments indicate that only CTDs belonging to the beta genera, of which HPV8 is a member, are capable of functionally compensating for the ability of CTD of the HPV8 E2 protein to associate with mitotic chromosomes. CTDs from different species of the beta genera such as the HPV5 E2 from species 1 and the HPV107 E2 from species 2 could associate with mitotic chromosomes when fused to the peptide. In contrast, the CTDs from the E2 proteins of alpha (HPV11), gamma (HPV4) or kappa (SfPV1) genus could not functionally substitute for the HPV8 E2 CTD and restore the chromosome binding phenotype. However, the fact that those different PVs have slightly different DNA binding specificities make it difficult to determine if this could contribute to the lack of functional replacement of the HPV8 E2 CTD with CTDs from other PVs.

Nonetheless, it is possible that the role of the beta CTDs in the chromosome binding function is not due to dimerization and DNA binding, rather, they have a

specific role that cannot be substituted by the CTDs from others PVs. It is possible that the CTDs from the beta-papillomavirus genus are involved in specific protein-protein interactions that are regulated by phosphorylation of the hinge peptide. PVs are highly species and tissue specific. It would not be surprising if different PVs show highly specific interactions that are essential for establishing infections in their respective niche tissues and hence, are not shared by members belonging to other PV genus.

We observed that the HPV8 E2, HPV5 E2 and HPV4 E2 CTDs by themselves are localized to the cytoplasm compared to the CTDs from other PVs. The CTDs of all the other PVs were nuclear. Nonetheless, fusing the 240-255 peptide from the hinge region to the CTDs of the HPV5 and HPV8 E2 proteins changes the sub-cellular localization of these proteins from the cytoplasm to the nucleus. This suggests that the peptide might carry a nuclear localization signals (NLS) that results in nuclear retention of these fusion proteins. There is precedence for the presence of NLS in the hinge region, as has been previously reported for the HPV11 E2 protein [286]. The NLS in the hinge region was shown to be important for targeting the E2 protein to the nuclear matrix. In conducting the mapping studies described in chapter 3, we observed that a number of truncated HPV8 E2 proteins lacking the 240-255 peptide region of the hinge, although, deficient in chromosome binding were localized to the nucleus. It is possible that multiple NLSs are present within the HPV8 E2 hinge region. An alternative explanation for the nuclear localization of the HPV5 and HPV8 fusion proteins could be that the phosphorylation of the hinge peptide results in unmasking of a putative NLS in the CTD by altering its conformation. In

the case of BPV1 E2, an NLS has been reported in the CTD [242]. Likewise, an NLS has also been detected in the HPV16 E2 CTD [135]. The NLS overlaps with the alpha recognition helix in the CTD [135]. Nevertheless, as described above, different E2 proteins use different NLS to efficiently localize the protein to the nucleus. The fact that fusing the 240-255 peptide to the HPV4 CTD did not alter the localization of the fusion protein of HPV4 from the cytoplasm to the nucleus, further underscores this point.

Using the 'Muscle' algorithm as interpreted in Geneious software [73]; we aligned and compared the CTD sequences from the different PVs to examine unique characteristics within the HPV4 E2 CTD (Figure 5.8). As can be observed from the Geneious alignment of the CTDs, HPV4 CTD has some unique sequence features compared to CTDs of other PVs. For example, HPV4 has a valine in place of a conserved threonine at residue 43 in a stretch of conserved residues spanning residues 41 to 47 in the alignment. It is possible that the conserved threonine undergoes a post-translational modification that is lacking in HPV4 E2 CTD and thus, can be attributed to differences in its CTD properties. Likewise, conserved basic arginine/lysine residues at position 54 and glycine at position 56 are replaced by a serine and aspargine respectively, in HPV4. The replacement of a hydrophobic residue, glycine, with hydrophilic aspargine could also affect the structure of HPV4 CTD. Additionally, a conserved bulky histidine/tyrosine at residue 33 is replaced by aspargine in HPV4 (Figure 5.8). In order to better understand how these differences in the HPV4 E2 CTD sequences affect its function and cellular localization, detailed mutagenesis studies targeting above described residues are required. Similarly, the

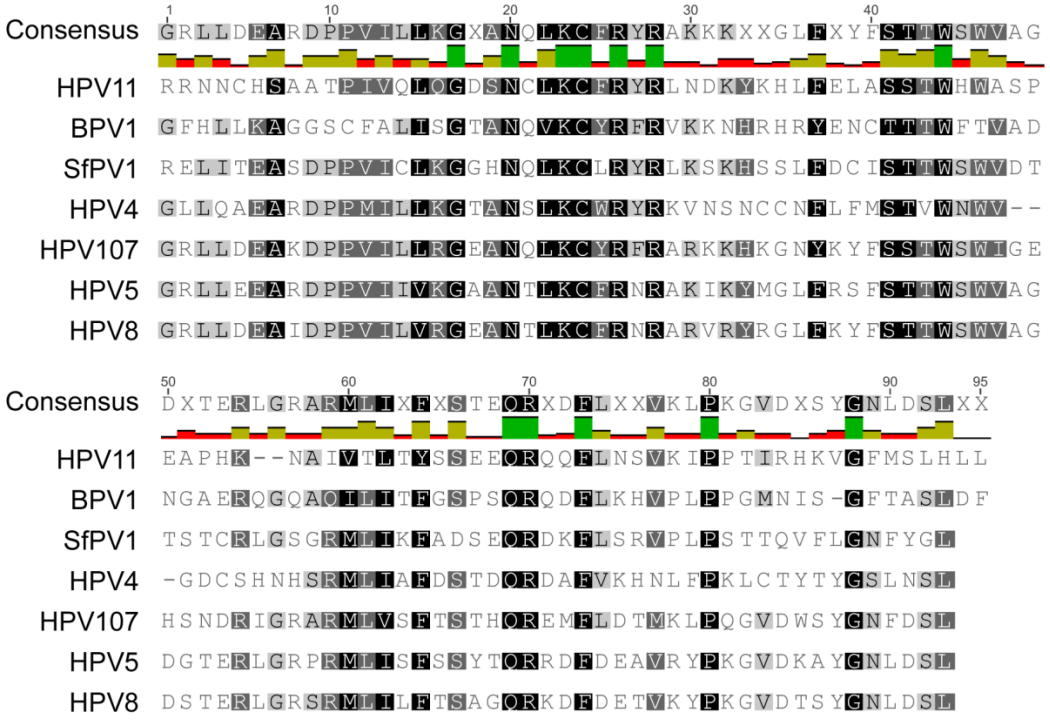

Figure 5.8
Alignment of the CTDs of HPV11, BPV1, SfPV1, HPV4, HPV107, HPV5 and HPV8 using Muscle as interpreted in Geneious. Black bars represent 100% amino acid similarity, grey bars represent 90% and light grey bars represent 80% amino acid similarity. Green bars represent 100% amino acid identity, yellow bars represent 80-90% identity and red represents < 50% identity.

alignment comparison of the CTD sequences of the beta PVs with other PVs indicates that there are conserved residues in the beta CTDs that are absent in others. For example, beta CTDs have a negatively charged residue at position 5 that is absent in other PVs. Likewise, position 35, position 40 and position 57 have conserved glycine, phenylalanine and arginine, respectively, in beta PVs. Comparison of the sequences further downstream indicates that a basic residue occupies position 78 whereas positions 84 and 91 are occupied by negatively charged acidic residues in beta CTDs. Whether the basic residues are important for binding DNA or the acidic residues aid in protein interactions need to be examined in future studies. Mutagenesis targeting these conserved residues in beta PVs will be useful in understanding the unique characteristics of beta CTDs.

The CTD swapping experiments indicate that CTDs from the closely related species of beta genera can functionally replace the HPV8 E2 CTD. However, it is still not clear why the DNA binding defective R431K/R433K protein is unable to bind chromosomes. One possibility is that the R431K/R433K mutation affects another function of the CTD that is unrelated to DNA binding. Similar observations have been made with the LANA CTD, whereby mutations in the DNA binding domain can affect both DNA binding and chromosome binding function of the LANA protein [133]. However, these functions can be separated, indicating that the CTD has multiple roles in chromosome binding. As mentioned above, this might indicate that the CTD could be involved in certain protein-protein interactions to mediate chromosome binding. Due to the similarity between the CTD structures of EBNA1

and E2 and the predicted structure of LANA, using homology between EBNA1 and E2, we can develop a computer model of the LANA DNA binding domain

Future work would involve using this model to identify the position of residues shown to be important for chromosome binding in the LANA protein and correlate this with residues in the analogous position in the HPV8 E2 protein. Further, we could examine whether these residues are specifically conserved in the E2 proteins of beta-papillomaviruses and then test, using a mutagenesis approach, if these mapped residues contribute to the chromosomal association function of the E2 protein. If we are able to identify regions of the CTD that are required for chromosomal interaction but not dimerization or DNA binding, this would indicate that they are required for other functions such as interaction with various cellular proteins that might mediate binding to mitotic chromosomes. This approach will further allow us to identify potential interacting cellular partners of the E2 protein.

It is not clear whether the chromosomal association function of the HPV8 E2 protein is mediated through direct DNA binding, RNA binding or protein-protein interactions. In order to address this question, we performed the studies that have been described in the following Chapter-6

Chapter 6

Contributions: Protein complex purification was performed by Moon Kyoo Jang. I performed the Western blot analysis and all the other experiments presented in this chapter.

Chapter 6: Role of protein-protein and protein-RNA interactions in the chromosomal association function of the HPV8 E2 protein

Introduction

In previous chapters we have described mapping of HPV8 E2 domains that are essential for its chromosomal association function. However, it is not yet clear whether the chromosomal association function of the HPV8 E2 protein is mediated through protein-protein, protein-RNA or protein-DNA interactions. The possibility of E2-chromosomal interaction through direct DNA binding was discussed in Chapter 5. However, it is possible that protein-protein or protein-RNA interactions could be involved in mediating HPV8 E2-chromosomal association. E2 proteins from different PVs show variable patterns of mitotic binding on chromosomes [171,205,262], suggesting that they interact with distinct chromosomal targets. One of the best characterized E2 binding partners is the cellular double bromodomain protein, Brd4 that binds to acetylated lysine residues on histone H3 and H4 [64]. The N-terminal residues R37 and I73 of the BPV1 E2 protein interact with the C-terminal domain of Brd4 [281]. Notably in the presence of BPV1 E2, both Brd4 and E2 colocalize in punctate dots all over the mitotic chromosomes [243]. In addition, E2 protein stabilizes the interaction of Brd4 on chromosomes in interphase and mitosis [171]. Although E2 proteins of all PVs interact with Brd4 for E2 mediated transcriptional regulation, it is not a partner for chromosome binding in all PVs [124,170,273]. This is exemplified in the case of the beta and alpha PVs, where R37A and I73A mutations that abrogate E2-Brd4 interactions do not affect the chromosomal binding function of

the E2 proteins from either genus [170,205]. Unlike the pattern of small E2 speckles observed in association with Brd4, all over the chromosomes with BPV1 E2, the HPV8 E2 protein binds as large distinct foci to the rDNA loci [205]. However, the chromosome tethering partner for HPV8 E2 still remains elusive. Additionally, various other cellular proteins such as TopBP1, MKlp2 and ChlR1 have been reported to interact with different E2s [66,196,284].

Similarly, several studies have reported protein interactions for the gammaherpesvirus tethering proteins such as LANA and EBNA1. LANA associates with methyl CpG-binding protein 2 (MeCP2) and DEK to bind to mouse chromosomes [137]. LANA also binds directly to histone proteins H2A and H2B [14], in addition to interacting with cellular Brd4 and Brd2/Ring3 proteins [163,194,204,264,283]. More recently, interaction of the C-terminus of LANA with the nuclear mitotic apparatus protein (NuMA) has been reported to contribute to KSHV genome maintenance and partitioning [241]. Another recent study also demonstrates that LANA binds the centromeric protein CENP-F and the kinetochore associated protein Bub1, on host chromosomes of KSHV infected cells [275]. Thus, all of the studies so far seem to indicate that the mechanism of LANA mediated chromosome tethering function is complex and involves interactions among multiple protein partners. Similarly in the case of EBNA1, EBNA1 binding protein 2 (EBP2) was initially identified as an interacting partner [239]. This interaction was shown to be essential for EBNA1 mediated partitioning of terminal repeat containing plasmids using a reconstituted yeast segregation assay [131].

In addition to protein-protein interactions, protein-RNA interactions can also be implicated in mediating chromosomal tethering functions. Recently EBNA1 was shown to bind to G-rich RNA that formed G-quadruplex structures, through its chromosome binding domains [187]. Moreover, treatment of EBNA1 expressing cells with compounds like BRACO-19 that interacts with G-quadruplex structures interfered with the EBNA1 dependent viral replication and chromosome tethering functions [187]. These observations suggested that EBNA1 binds to RNA structures and thus, interaction with RNA plays an important role in regulating EBNA1 functions [187]. As described previously, the chromosome binding domains of EBNA1, rich in GR and RS repeats that are the RNA binding motifs share sequence similarity with the chromosome binding regions of HPV8 E2 protein. Hence, it is possible that similar RNA interactions could mediate HPV8 E2 chromosome binding. Additionally, the structural fold of the RNA recognition motif (RRMs) of the heterogeneous nuclear ribonucleoprotein A1 is very similar to the dimeric beta barrel structure of the PV E2 CTD [57]. Crystallographic studies have shown that the RRMs form a compact alpha/beta structure in which the four beta strands form the major RNA binding surface [265]. Thus, the similarities in the structural fold of the RRMs and the E2-CTD, further, raise the possibility of the E2 protein interacting with RNA during chromosomal tethering.

All of the above described observations propelled us to examine whether the HPV8 E2-chromosomal interaction was mediated through either protein-protein or protein-RNA interactions. One of the means to obtain a comprehensive list of cellular proteins interacting with E2 is to use a proteomics approach. Thus, to identify

potential cellular factors that mediate E2 chromosomal association, our laboratory had previously isolated protein complexes in association with various E2 proteins using tandem affinity purification (TAP) and mass spectrometry (Moon Kyoo Jang, Eric Anderson and Alison McBride unpublished data). A number of proteins including nuclear matrix proteins, splicing factors, chromatin remodeling proteins and enzymes involved in post-translational modifications were identified in the HPV8 E2 immunoprecipitated complexes (Moon Kyoo Jang, personal communication). Notably, a large number of HPV8 E2 protein complexes that were immunoprecipitated using TAP were RNA binding proteins, further highlighting a potential interaction of the E2 protein with RNA. One of the aims of the study described in this chapter was to determine if the chromosomal association of the truncated 240-255-CTD E2 protein correlated with interaction with the identified interacting partners of HPV8 E2 protein isolated from the TAP immunoprecipitated complexes. The objective of the study was to validate the interaction of the HPV8 E2 protein with some of the candidate proteins identified in the TAP immunoprecipitated complex. Additionally, since the HPV8 E2 protein colocalizes with the RNA polymerase I transcription factor, upstream binding factor (UBF), we also examined if the full length and the truncated HPV8 E2 proteins interact with UBF in vivo. Hence, this study is useful in determining the role of cellular proteins in the molecular interaction of the HPV8 E2 protein with mitotic chromosomes. The second aim of the study described in this chapter was to analyze the role of RNA interactions in mediating the chromosome binding functions of the HPV8 E2 protein. To this end,

HPV8 E2 expressing CV-1 cells were treated with RNAse A and the mitotic localization of the E2 protein was examined by indirect immunoflourescence.

Results

Validation of interactions of the HPV8 E2 protein with candidate proteins identified from the TAP/mass spectrometry study

For this study, we generated C33A cell lines expressing different flag tagged HPV8 E2 proteins. We included the full length HPV8 E2 protein along with a truncated version called 8E2ΔHC that lacks both the N-terminal domain and the C-terminal half of the hinge region from residues 312-402. We also expressed the wild-type 240-255-CTD protein along with mutated E2 proteins S253A, R250A, R251A and the DNA binding defective protein (DB-) in the truncated 240-255-CTD background (Figure 6.1). Nuclear extracts prepared from E2 expressing cells were used to immunoprecipitate E2 protein using M2 flag beads. The protein complexes were eluted twice from the beads; once with a flag peptide and the second time with 2% sarkosyl. The eluates were mixed in a 1:1 ratio and separated on 4-12% gradient NuPage polyacrylamide gels with 5µl of input (10% protein). Using western blot analyses, we analyzed the complexes for some of the candidate proteins previously identified from the proteomics study as shown in figure 6.2. Cellular proteins such as SAF-A, matrin3, SRPK1 and WSTF were detected in the protein complexes immunoprecipitated with the full length HPV8 E2 protein but failed to show any interactions with the truncated 240-255-CTD protein. However, the following candidate proteins were selected for screening from the TAP/mass spectrometric studies for the reasons described below.

SAF-A- Scaffold attachment factor A (SAF-A) or heterogeneous nuclear ribonucleoprotein-U (hnRNP-U) is a nuclear scaffold protein that interacts with the DNA sequences of nuclear matrix attachment regions (MARs). SAF-A interacts with the MAR sequences in the nuclear matrix via its SAF-box domain [139] and binds to RNA through its RGG-box domain [139]. The nuclear matrix is important for the structural organization of the nucleus and for maintaining the integrity of the nucleus. SAF-A plays an important role in chromatin organization and transcriptional regulation by interacting with proteins such as heterochromatic HP1 [8]. SAF-A has been recently reported to localize to mitotic spindles during mitosis and is an essential component of the mitotic apparatus [157]. RNAi mediated repression of SAF-A expression results in mitotic delay and abnormal alignment of chromosomes [157]. A recent genome-wide RNAi screening also identified SAF-A as one of the proteins required for chromosome segregation [121]. Moreover, SAF-A has also been observed on mitotic chromosomes in mouse cells where it binds to pericentromeric satellite regions [139]. It has been hypothesized that, to stably maintain viral genomes during persistent infection, PV E2 proteins might require additional cis-elements on the viral genome in addition to E2 binding sites. One such candidate is the (MARs) that could be potentially bound by the SAF-A protein. MAR sequences have been mapped to the PV genomes in vitro [253]. There are studies that show that the interaction of MAR elements with SAF -A allows for stable maintenance of a MAR element containing plasmid by attachment to cellular mitotic chromosomes [128]. Since the above described evidence implicated SAF-A in genome maintenance and chromosome binding, we wanted to determine if SAF-A interacted with E2 and

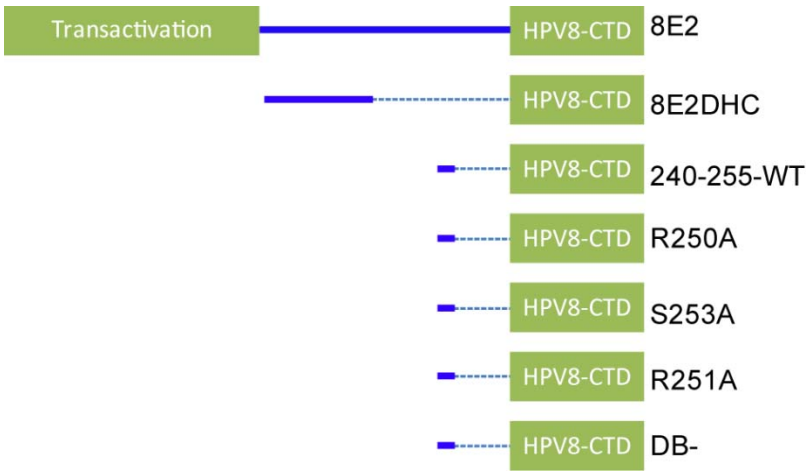

Figure 6.1
Schematic representation of the different HPV8 E2 proteins used for the immunoprecipitation assay. These included the full length HPV8 E2 protein (8E2), a truncated version that lacks both the N-terminal domain and the C-terminal half of the hinge region from residues 312-402 (8E2ΔHC), the 240-255-CTD wild-type protein, mutated E2 proteins S253A, R250A, R251A and the DNA binding defective protein (DB-) all in the 240-255-CTD background.

played any role in the chromosome tethering function of the HPV8 E2 protein. In the western blot analysis, interaction of SAF-A could be observed with the full length HPV8 E2 protein and the truncated 8E2ΔHC protein. This indicates that SAF-A interacts with the full length HPV8 E2 protein and likely plays a role in E2 functions. But no SAF-A was immunoprecipitated with the wild-type 240-255-CTD protein compared to the control samples.

U5 116 – U5 is a small nuclear ribonucleoprotein (snRNP) of 116 kDa molecular weight and is a component of the spliceosome complex. It is a GTP binding protein involved in splicing [153]. This snRNP protein undergoes dephosphorylation by the PP1/PP2A phosphatases during the splicing process [238]. As described previously, HPV8 and HPV5 E2 protein interact with components of the spliceosome complex and hence, we examined if U5 116 protein was immunoprecipitated with the different HPV8 E2 proteins. In our immunoprecipitation assay, we could not demonstrate any specific interaction between U5 116 and HPV8 E2 proteins.

SRPK1- Serine-arginine protein kinase 1 (SRPK1) is a kinase that specifically phosphorylates serine residues within the serine-arginine (SR) or arginine-serine (RS) dipeptide motifs. This kinase phosphorylates SR splicing proteins in a cell cycle regulated manner, which is important for the assembly of the spliceosome complex and for the splicing process to form mature mRNAs [95]. Moreover, different SRPKs also play a role during viral infection. Viral proteins such as ICP27 of herpes simplex virus-1 and the E1^E4 protein of HPV1 interact with SRPK1 [21,230]. Notably,

interaction of ICP27 with SRPK results in lower host splicing activity (Bell, Martin et al., 2007). The E1^E4 protein is phosphorylated by SRPK [21,230]. In addition, SRPKs interact with nuclear matrix protein scaffold attachment factor (SAF-B) proteins, which negatively regulate SRPK activities [259]. The hinge regions of E2 proteins from the betapapillomaviruses such as HPV8 and HPV5 are rich in arginine-serine/serine-arginine repeats which make them ideal candidate substrates for SRPK activities. Additionally, HPV5 and HPV8 E2 proteins have been shown to colocalize with the spliceosome factor SC-35 [141,235]. Furthermore, many splicing factor proteins are a part of the TAP immunoprecipitated HPV8 E2 protein complexes. Given the possibility of a functional interaction between the HPV8 E2 protein and SRPK1, we wanted to determine if SRPK1 could be immunoprecipitated with HPV8 E2 proteins. It is clear that both the full length HPV8 E2 protein and the 8E2ΔHC can specifically immunoprecipitate SRPK1. These interactions are potentially mediated through the previously described RS/SR rich domains of the hinge region of E2 proteins. However, the 240-255-CTD wild-type protein does not interact with SRPK1. The 240-255-CTD protein carries only 16 residues of the hinge fused to the CTD and thus, lacks most or all of the RS/SR dipeptide motifs required for interaction with splicing factors and possibly SRPK1. Hence, it appears that SRPK1 interacts with the full length E2 protein and might be involved in playing a role in the HPV8 lifecycle but is most likely not involved in chromosome binding.

WSTF- Williams syndrome transcription factor (WSTF) is a subunit of the ATP-dependent chromatin remodeling complex that includes members such as WSTF

including the nucleosome assembly complex (WINAC), WSTF-ISWI chromatin remodeling complex (WICH) and B-WICH. It is involved in a number of diverse functions such as chromatin assembly, histone modifications, RNA polymerase I and III transcription and DNA repair [17]. Members of the B-WICH family have been found to play a role in nucleosome remodeling to allow RNA polymerase I and III gene transcription. Although the mechanism is unclear, WSTF, along with other chromatin remodelers, has also been reported to regulate ribosomal gene expression [202]. Additionally WSTF accumulates at pericentromeric heterochromatin in mouse cells and stably binds to mitotic chromosomes [33]. Since, the HPV8 E2 protein also targets the pericentromeric regions on mitotic chromosomes and binds to ribosomal DNA, we wanted to examine whether there is any in vivo interaction between WSTF and the E2 protein. From the complex purification assay, it appears that the full length HPV8 E2 protein and the truncated 8E2ΔHC protein do show some interaction with WSTF compared to the pMEP control. However, WSTF was not a part of the immune-complex pulled down with the truncated 240-255-CTD wild-type protein. Thus, interaction of WSTF with the HPV8 E2 protein indicates it is likely involved in mediating some function of E2 protein, however, it is most likely not involved in the HPV8 E2 tethering function.

PRMT5- Protein arginine methyltransferase 5 (PRMT5) are members of the methyltransferase family that methylate proteins at the arginine residues within the RGG, GRG or RXR motifs. They have been implicated in a number of cellular processes such as signal transduction, RNA processing, histone modifications and

protein transport [195,272]. Interestingly, PRMT5 has been also shown to methylate the chromosome binding regions of EBNA1 tethering protein of EBV [240]. Although most of the EBNA1 protein is expressed in the nucleus, inhibition of PRMT5 methylation altered the localization of the EBNA1 protein resulting in a peri-nucleolar ring suggesting that, it might be involved in regulating interaction of EBNA1 with RNA or RNA binding proteins [240]. Since the chromosome binding region of the HPV8 E2 protein contains GR dipeptides similar to the EBNA1 chromosome binding region, we wanted to investigate whether PRMT5 played a role in the tethering function of HPV8 E2. In the complex purification assay, we could not observe any specific interactions between PRMT5 and the HPV8 E2 proteins.

UBF- UBF is an RNA polymerase I transcription factor that plays an important role in maintaining the euchromatic state of rDNA chromatin. It is one of the few transcription factors that remain bound to chromosomes during mitosis when rDNA transcription is shut down [92]. UBF binds not only to high affinity sites on the promoter region but also binds to the transcribed region of the rDNA genes [217]. Since the HPV8 E2 protein localizes to the rDNA loci and colocalizes with RNA polymerase I UBF, we wanted to examine if HPV8 E2 protein interacts in vivo with UBF. The immunoprecipitation assay revealed no interaction between the different HPV8 proteins and UBF. However, UBF might weakly interact with the HPV8 E2 protein, such that, complex isolation using high salt results in loss of weakly interacting partners or conversely, it might be tightly bound to chromosomes such that it is not extracted.

TRIM28- Tripartite motif-containing protein 28 (TRIM28) or KAP-1(Krab-associated protein-1) is a cofactor of Kruppel-associated box zinc finger proteins (KRAB-ZFPs) that act as transcriptional repressors. It interacts with the multimolecular repressor complex containing histone methyl transferases (SETDB1) and histone deacetylases (HDACs) to repress gene expression [7]. It also interacts and colocalizes with the heterochromatin protein (HP1) and results in heterochromatin mediated gene silencing [267]. Recently, a truncated E2 protein of HPV31 known as E8^E2C protein was reported to interact with cellular repressor molecules such as HDACs, SETDB1 and the TRIM28 protein [9]. The E8^E2C protein lacks the N-terminal domain of E2, which is instead replaced with the 12 residues long E8 domain fused to the CTD of the E2 protein. This truncated E2 protein acts as a repressor of E2 mediated replication and transcription functions [250]. Interaction of the E8 domain with TRIM28 is thought to be one of the mechanisms of E8^E2 mediated transcriptional repression [9]. In our immunoprecipitation assay, no specific interaction was observed with the different HPV8 E2 proteins.

Nucleophosmin/B23- B23 protein has been shown to associate with rRNA to stimulate rRNA transcription. But during mitosis inactivation of its RNA binding activity by cdc2 kinase-mediated phosphorylation causes B23 to be released from chromatin. From the complex purification studies, it is interesting to note that B23 expression is almost completely lost in the cells expressing the HPV8 full length E2 and DNA binding defective E2 proteins as observed in the input samples. Whether the loss of B23 protein expression has any implications on the functions of the HPV8

E2 protein needs to be further investigated. Some B23 is immunoprecipitated with the 8E2ΔHC protein suggesting, that the 8E2ΔHC can interact with B23. However, the truncated 240-255-CTD protein does not interact with B23.

WDR5- WD repeat containing protein 5 **(**WDR5) is a member of the methyltransferase family mixed-lineage leukemia (MLL) complex that methylates lysine 4 residue of histone H3. This interaction is also important for rDNA transcription [82]. Additionally, WDR5 also associates with chromatin remodeling proteins such as chromodomain helicase DNA binding protein 8 (CHD8) [256]. It has been recently shown to be involved in induction of type I IFN response triggered during viral infection [156]. Although in the immunoprecipitation assay no specific interaction was observed between WDR5 and the different HPV8 E2 proteins, the level of WDR5 protein appeared to be reduced in cells expressing the full length HPV8 E2 protein. It would be interesting to investigate the effect of WDR5 down regulation on the papillomaviral life cycle.

Matrin 3- Matrin 3 is another abundant protein present in the nuclear matrix that binds DNA through MAR sequences. It is involved in many nuclear processes including retention of hyper-edited RNA within the nucleus [285] and is mainly observed in the euchromatic regions of the nucleus [159]. Additionally, matrin 3 has been demonstrated to colocalize and co-immunoprecipitate with SAF-A and other nuclear proteins such as SAF-B and hnRNP-L [159], suggesting that it is also

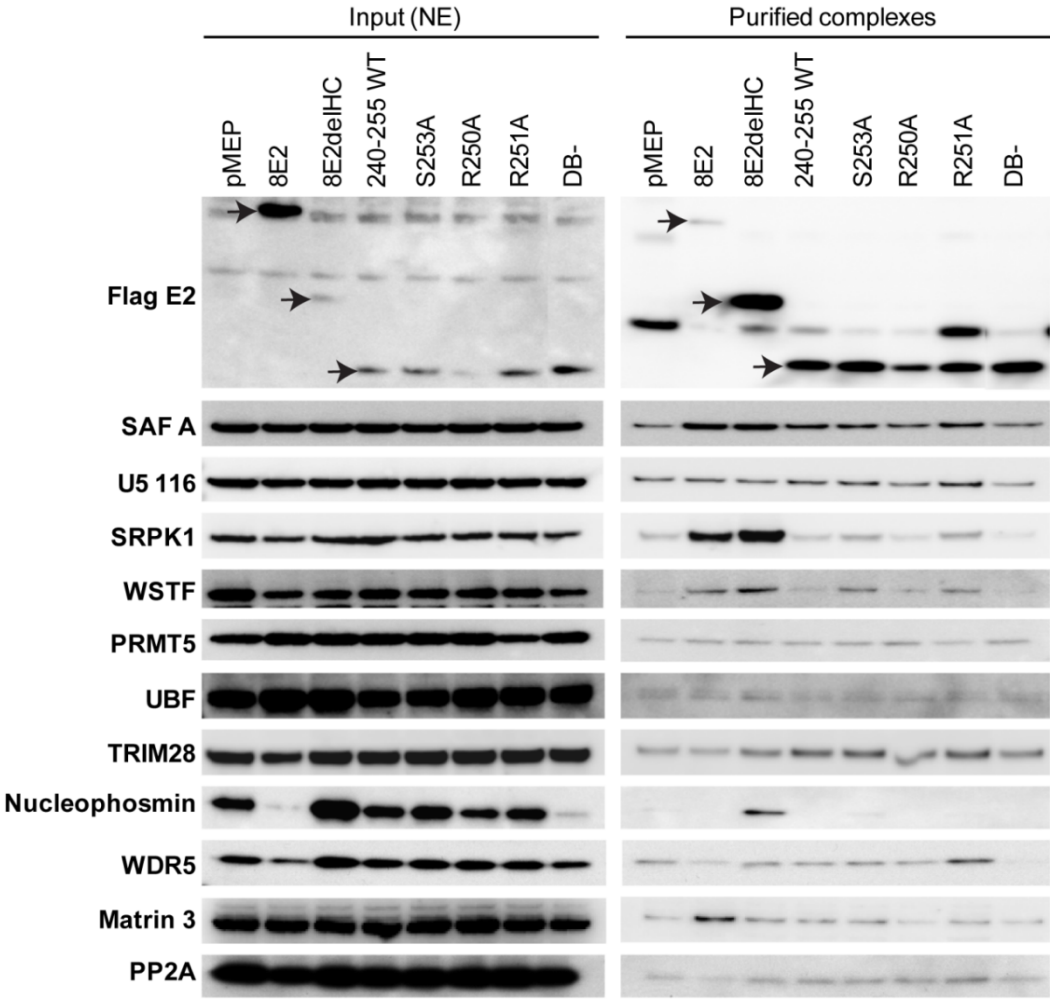

Figure 6.2
Validation of the protein interactions in the immunoprecipitated HPV8 E2 protein complexes. C33A cell lines expressing full length HPV8 E2, truncated 8E2ΔHC, 240-255-CTD WT, mutated E2 proteins S253A, R250A, R251A and the DNA binding defective protein also in the truncated 240-255-CTD background were used to prepare nuclear extracts (NE). E2 proteins were immunoprecipitated with M2 anti-flag antibody and the immunoprecipitated complexes were analyzed by western blotting. E2 was detected using rabbit anti-flag antibody.

involved in RNA processing and chromatin structural organization. Furthermore, viral kinases such as alphaherpesvirus US3/ORF66 protein kinase phosphorylate matrin 3 and affect its nuclear localization [77]. However in the immunoprecipitation assay, matrin 3 co-immunoprecipitated with only full-length HPV8 E2 protein but did not interact with any other truncated HPV8 E2 proteins. Further investigations are required to determine whether matrin 3 is involved in regulating the functions of HPV8 E2 along with the SAF-A protein.

PP2A- Protein phosphatase 2A (PP2A) belongs to the family of protein serine/threonine phosphatases that are important regulators of mitosis. PP2A plays a crucial role in regulating sister chromatid cohesion during mitosis. It has also been implicated in preventing premature mitotic exit by dephosphorylating many mitotic proteins [258]. PP2A acts as a tumor suppressor and hence is targeted by DNA tumor viruses. In the case of small DNA tumor virus (SV40), small t antigen inhibits PP2A activity. This inhibition contributes to SV40 mediated transformation [12]. Additionally, PP2A has been shown to a play a role in activation of the LCR region of HPV16 through its interaction with Sp1 [246]. However, in our immunoprecipitation assay PP2A was not immunoprecipitated in complex with any of the HPV8 E2 proteins, suggesting that there is no interaction between PP2A and the HPV8 E2 protein under our assay conditions.

RNAse treatment of E2 expressing cells did not affect the chromosome binding function of the HPV8 E2 protein

From the immunoprecipitation assays we could not identify a protein binding partner for HPV8 E2 and so we wanted to investigate the role of RNA binding in E2-chromosomal association. To examine the role of RNA interactions in mediating the chromosome binding functions of the HPV8 E2 protein, E2 expressing CV-1 cells were seeded onto glass slides and grown for two days. E2 expression was induced for 4h with cadmium sulfate. Following E2 induction, the cells were permeabilized with PBS containing 0.1% Triton X 100 and 0.1% Tween-20 for 15 minutes at room temperature. The permeabilized cells were treated with 1mg/ml RNAse A for 10 minutes at room temperature. Following RNAse treatment, the cells were washed twice with 1X PBS and fixed in 4% PFA for 20 minutes.

Fibrillarin staining is often used as a marker for nucleolar RNA staining. It was used as a marker to detect efficiency of the RNAse A digestion. Fibrillarin is a component of the snRNP particle and is involved in ribosomal RNA maturation. In normal cells, fibrillarin staining gives a globular pattern within the nucleolus. However, following RNAse A treatment, the globular appearance is lost and staining is uniform and dispersed (Figure 6.3). After RNAse treatment, the cells were stained for immunofluorescence with anti-flag and anti-fibrillarin antibodies and observed by confocal microscopy (Figure 6.3). From the microscopic analysis, it appears that treatment of E2 expressing CV-1 cells with RNAse A does not affect the chromosome binding phenotype of the HPV8 E2 protein. However following RNAse treatment, the staining pattern of fibrillarin is altered from a globular to a diffuse

pattern, but the HPV8 E2 protein is still bound to mitotic chromosomes as distinct foci.

To confirm these findings, the E2 expressing cells were stained with SYTO RNASelect green, a fluorescent cell stain that selectively stains RNA (Figure 6.4). This stain was used to further confirm the efficiency of RNAse A treatment. It exhibits bright green fluorescence when bound to RNA and weak fluorescence when bound to DNA. Thus, under normal conditions, maximal fluorescence is seen in the nucleoli with weak staining in the rest of the nucleus. However, following RNAse treatment, the nucleolar staining pattern is completely lost and the intensity of nuclear staining is reduced significantly. However, the SYTO RNASelect stain is sensitive to formaldehyde fixation and hence, the samples had to be fixed instead using methanol-acetone. E2 expressing cells were permeabilized with 0.1% Triton X 100, treated with RNAse A for 10 minutes at room temperature followed by fixation with methanol and acetone at -20^{O}C. Although co-staining with E2 weakens RNASelect RNA staining, it was clear that the SYTO RNASelect stained the nucleolus brighter than the nuclear staining in the absence of RNAse A treatment. But following RNAse treatment, the nucleolar staining pattern was lost. On the contrary, the HPV8 E2 protein was observed to bind mitotic chromosomes as large foci irrespective of RNAse treatment (Figure 6.4).

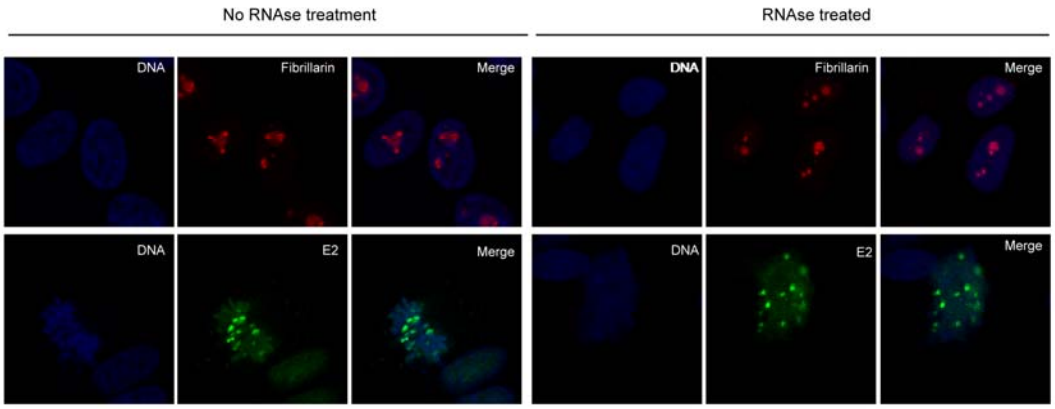

Figure 6.3
RNAse A treatment of 240-255-CTD expressing CV-1 cells does not affect HPV8 E2 chromosome binding. CV-1 cells expressing HPV8 E2 protein were either untreated or treated with RNAse A and examined for E2 and fibrillarin localization in interphase cells or on mitotic chromosomes using indirect immunofluorescence. E2 was detected using anti-flag antibody (green), fibrillarin using anti-fibrillarin antibody (red) and cellular DNA using DAPI (blue).

Thus, in the above experiments the association of the HPV8 E2 protein with mitotic chromosomes was not affected by RNAse treatment.

Discussion

Though one of the objectives of this study was to validate cellular factors interacting with the HPV8 E2 protein that might be involved in mediating the E2-chromosomal association function, we were unable to conclusively identify a chromosome tethering partner of the HPV8 E2 protein. For the immunoprecipitation assay, we used HPV8 E2 wild-type and mutated proteins. These included the full length HPV8 E2 protein, truncated 240-255-CTD wild-type protein and the S253A mutated E2 protein. Using indirect immunofluorescence, we have shown that both the full length HPV8 E2 and the truncated 240-255-CTD E2 proteins bind mitotic chromosomes as large distinct pericentromeric foci. Moreover, the wild-type protein can bind chromosomes whereas the S253A mutated E2 protein is defective in chromosome binding. Thus, one would expect that the proteins involved in chromosome binding will not be immunoprecipitated with S253 mutated protein. Our rationale behind using these proteins for the complex purification assay was that, we could possibly identify an interacting partner for HPV8 E2 protein by comparing the complexes immunoprecipitated by these proteins.

Some cellular proteins such as SAF-A, matrin3, SRPK1 and WSTF interact with the full length HPV8 E2 protein but failed to show any interactions with the truncated 240-255-CTD protein. Notably, since proteins like SAF-A, matrin3 and WSTF are involved in RNA processing and RNA binding, it is possible that the

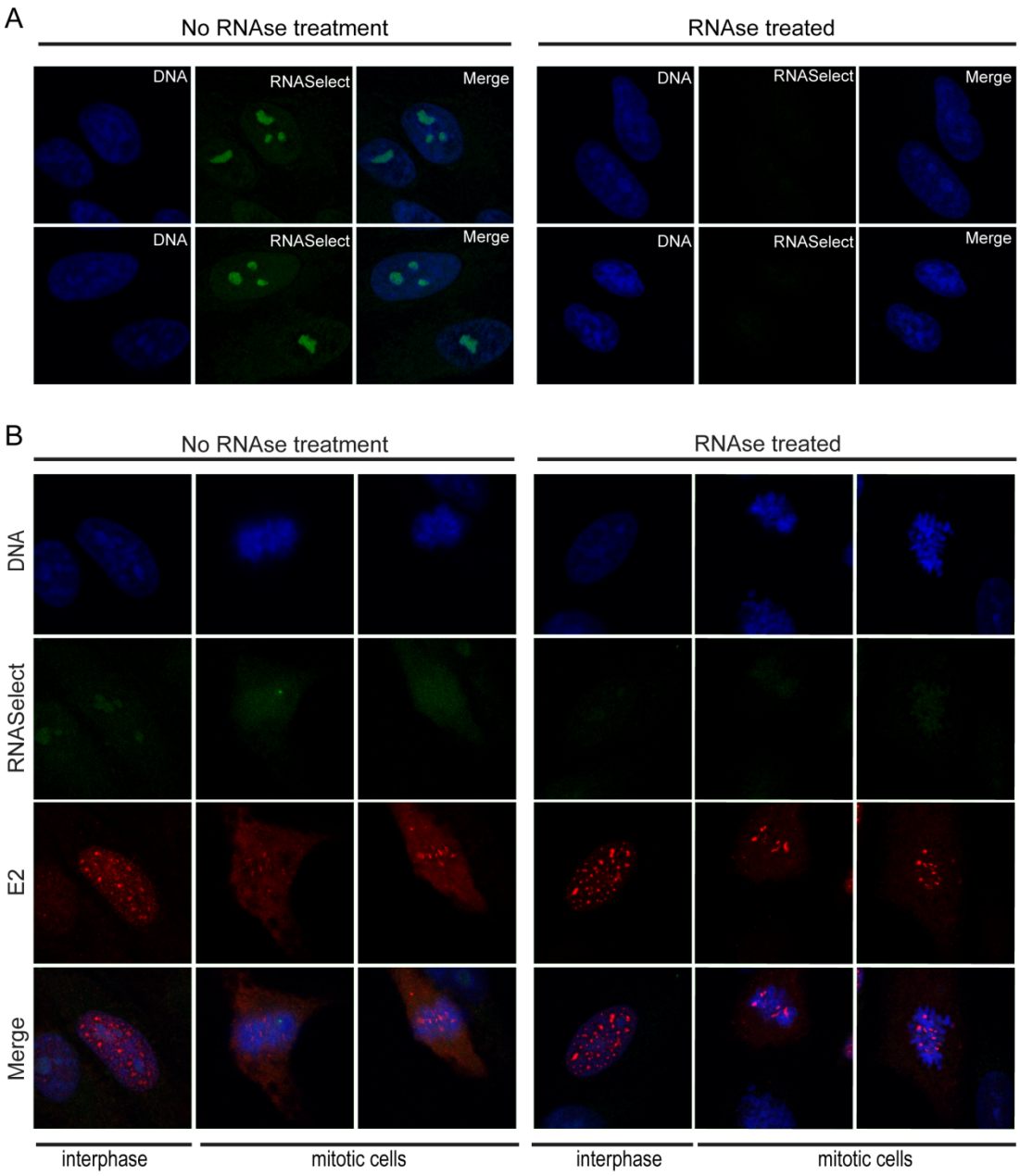

Figure 6.4
Full length HPV8 E2 chromosomal association is not mediated through RNA binding. CV-1 cells expressing HPV8 E2 protein were either untreated or treated with RNAse A and examined for E2 localization in interphase cells or on mitotic chromosomes using indirect immunofluorescence. (A) Efficiency of RNAse treatment was detected using the RNASelect stain in 2 examples of untreated and RNAse treated samples. (B) Co-staining of E2 and RNA using anti-flag antibody (red), RNASelect stain (green), respectively. Cellular DNA was stained using DAPI (blue). In B, the first panel under each treatment is interphase cell and the second and third panels are mitotic cells.

interaction of these proteins with HPV8 E2 could be mediated through binding to RNA rather than actual protein-protein interactions. This is not unexpected since, many RNA binding proteins were identified in the protein complexes immunoprecipitated in the original TAP/mass spectrometry study (Moon Kyoo Jang, personal communication). Moreover as described previously, the HPV8 E2 hinge region is rich in RG repeats that might have RNA binding properties and the CTD shows similarity to the structure fold of the RRMs. Thus, one of the caveats of our immunoprecipitation assay was that we did not treat protein complexes with RNAse before immunoprecipitating the complexes. Hence, we cannot conclusively state whether interaction with these proteins is mediated through direct protein-protein binding or through binding to RNA.

In the case of the SRPK1 protein, interaction with the HPV8 E2 protein is not surprising considering the fact that the hinge region of the E2 protein carries a number of SR/RS dipeptide motifs that are potential consensus sites for the SRPK1. Additionally, the HPV8 E2 proteins have been shown to colocalize with splicing factor SC35 suggesting their involvement in mRNA transcript splicing. Thus, further detailed studies could shed light on the role and the mechanism of the interaction of SRPK1 with E2 protein in the HPV8 life cycle. Notably, the expression levels of the proteins nucleophosmin/B23 and WDR5 were found to be low in the full length HPV8 E2 expressing cells. Likewise the levels of B23 have been reported to be low in cells undergoing apoptosis and differentiation [198][119]. Moreover, high-risk HPV16 and HPV18 E2 proteins have been shown to induce apoptosis in different cell types [26] [63]. Thus, it would be interesting to explore the effects of down regulation

of nucleophosmin/B23 and WDR5 proteins on the functions of the full length HPV8 E2 protein in future studies. Notably, there was no interaction observed between these proteins and the HPV8 E2 proteins in the immunoprecipitation assay.

None of the candidate proteins tested interacted with the truncated 240-255-CTD protein. One of the reasons for the differences in the interactions of the full length HPV8 E2 protein and the truncated 240-255-CTD protein with different cellular proteins could be attributed to the fact that the truncated 240-255-CTD protein lacks many regions of interaction present in the full length protein. Another explanation for the lack of interactions we observed in our validation study could be the use of high salt concentration in our extraction protocol that resulted in proteins with weaker interactions to be lost during purification. Conversely, it is also possible that proteins that are tightly bound to chromatin are not extracted at the salt concentration used for purification. Although many different candidate proteins that seemed relevant to HPV8 E2 functions were screened in the study, the chromosome tethering partner of the HPV8 E2 protein still remains elusive.

The second objective of the study was to examine if the interaction of the HPV8 E2 protein with mitotic chromosomes was mediated through RNA binding. Although we did not see any effect on E2-chromosomal association following RNAse treatment, it is possible that E2 masks the interaction of RNA with mitotic chromosomes and hence, the E2-RNA interaction is not inhibited by RNAse treatment. Notably, the markers fibrillarin and RNASelect, used to detect efficiency of the RNAse A digestion showed an effect following RNAse A treatment,

confirming the efficiency of RNAse treatment. Hence, in our assay RNAse treatment did not abrogate the E2-chromosomal association.

As described previously, the E2 protein is multi-functional and is involved in viral DNA replication and transcription in addition to genome partitioning. In the following chapter (Chapter 7), we examined whether alanine substitution of residues R250 and S253 affected the other functions of E2.

Chapter 7: The effect of the chromosome binding motif on the transactivation and replication functions of the HPV8 E2 protein

Introduction

In addition to its role in viral genome maintenance, the E2 protein functions in transcriptional regulation and viral genome replication. As described previously, the major determinants of the transactivation function of the E2 proteins have been mapped to the N-terminal domain with residues R37 and I73 identified as being important for Brd4 mediated transcriptional regulation [35,167]. Transcriptional activation by E2 involves interaction with many different transcription factors such as CBP/p300 and Sp1 [22,34,104,145,150,199]. There are also reports that E2 proteins are capable of stimulating transcription in the absence of the E2 binding sites [107,111]. In the case of the HPV8 E2 protein, the hinge region has also been shown to be capable of transactivation [248]. The hinge region of the HPV8 E2 protein was observed to interact with the transcription factor Sp1 and activate transcription of the human promoter $p21^{WAF1/CIP1}$ [248]. Thus, one of the objectives of this study was to determine if alanine substitutions of residues R250 or S253 within the chromosome binding region affected its transcriptional activation function. To this end, we used the luciferase assay system to determine the transactivation function of the mutated HPV8 E2 proteins.

PV genome replication is dependent on the host cellular replication machinery and is tightly linked to the differentiation state of the host keratinocytes [288]. The PV replication cycle can be divided into three different stages: the first stage involves limited amplification of the viral genome following entry of the viral genome;

second, maintenance replication that involves partitioning of the viral genome to daughter cells at the end of mitosis; third, vegetative amplification of the viral genome that provides genomes for packaging into newly synthesized viral particles. The PV genome undergoes bidirectional (theta-type) replication that requires the viral replication proteins E1 and E2 in addition to the viral origin of replication [211,261].

PVs are highly species and tissue specific, which limits the availability of the cell types that can support viral replication. This has been particularly challenging in the case of betapapillomaviruses that infect the cutaneous epithelia of the hosts. Until recently, no cell culture based system had been reported that could be used to study their genome replication. However, in contrast replication of PV origin containing plasmids has been achieved relatively easily in various cell types, by co-transfecting, expression vectors encoding viral proteins E1 and E2 along with the plasmid containing the origin [48,211]. However, recently the human osteosarcoma cell line U2OS was reported to support replication of different papillomaviral genomes including high-risk, low-risk and betapapillomaviruses [93]. Thus, in our study to examine the effect of R250 and S253 mutations on the replication function of the HPV8 E2 protein, performed HPV8 replication assays in U2OS cells.

Results

Alanine substitutions of residues R250 and S253 within the HPV8 E2 hinge region had no effect on the transactivation function of the HPV8 E2 protein

For the transactivation assay, we used pMEP-E2 expression plasmids expressing either full length HPV8 E2 protein or HPV8 E2 proteins carrying mutations R250A, S253A or the double mutation R250A/S253A in the full length background. BPV1 E2 was included as a positive control and pMEP4 as a negative control. The luciferase gene was encoded by the plasmid pBS1073 that consisted of four alpha HPV E2 binding sites upstream of the thymidine kinase promoter. CV-1 cells were co-transfected in triplicate with different concentrations of pMEP-E2 expression plasmids ranging from 25ng, 100ng, 250ng, 500ng to 1000ng and 1μg of the pBS1073 plasmid using the Fugene 6 transfection reagent. Cells were harvested 40 h post transfection in cell culture lysis reagent. Luciferase activity was measured as relative light units per second or RLU/sec using the Zylux Femtomaster FB12 luminometer. In the luciferase assay, there is a dose dependent increase in the transactivation of the luciferase gene by the different E2 proteins (Figure 7.1). As E2 expression increases from lower levels to higher levels with respect to increasing concentration of E2 expression plasmids, there is a concomitant increase in the levels of luciferase activity. However, the alanine substitutions of R250 or S253 do not significantly affect the transactivation function of the HPV8 E2 protein. The overall levels of transactivation appear to be similar at different concentrations of the wild-type E2 protein and the alanine substituted E2 proteins (Figure 7.1). The HPV8 and BPV1 E2 proteins transactivate to similar levels, although, at the highest plasmid concentration, the levels of luciferase activity induced by BPV1 E2 drops likely due to high expression of the E2 protein. At high concentrations of the E2 protein, the freely available transcription factors are bound by E2 and hence, are not available for

transcriptional activation in a process known as squelching [2]. Thus from the luciferase transactivation assay, we observe that the alanine substitution of residues R250 and S253 does not affect the transactivation function of the HPV8 E2 protein.

Alanine substitutions of residues R250 and S253 within the HPV8 E2 hinge region have no effect on the replication function of the HPV8 E2 protein in U2OS cells

To examine the replication of the HPV8 genome in U2OS cells, we transfected them with either recircularized HPV8 wild-type genome or HPV8 genomes carrying either R250A or S253A mutations in the E2 gene. Low-molecular weight DNA was isolated at three days post-transfection using the Hirt DNA extraction protocol to determine and compare the levels of transient HPV replication in U2OS cells. The isolated DNA was cleaved with BamHI to linearize the HPV genome and with DpnI. Digestion with the DpnI enzyme cleaves the methylated input DNA. Following Southern blot analysis using HPV8 wild-type genome as probe, we observed that all HPV8 genomes replicated in U2OS cells irrespective of the presence of mutations within the E2 gene (data not shown). Meanwhile, a transient replication assay conducted in the laboratory with an HPV8 genome carrying a translation

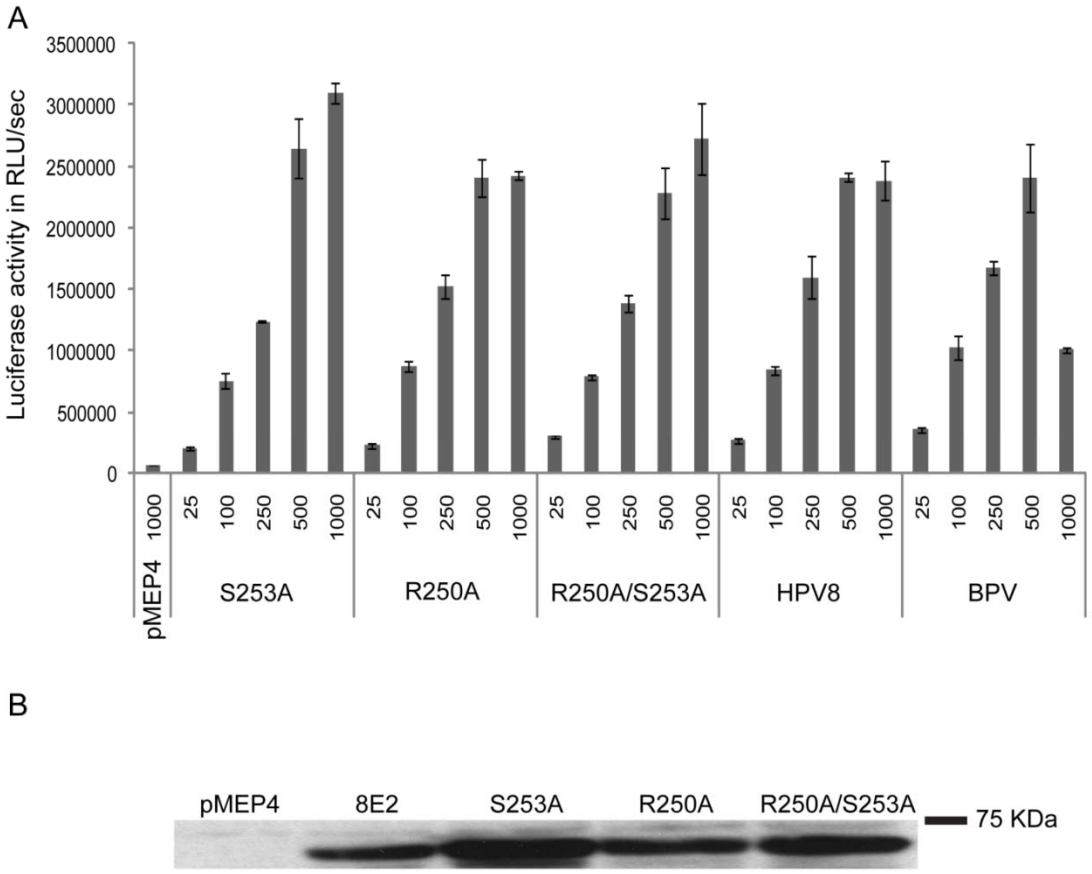

Figure 7.1
R250A and S253A mutations within the HPV8 E2 hinge do not affect the transactivation function of the HPV8 E2 protein. CV-1 cells were co-transfected in triplicate with different concentrations of pMEP-E2 expression plasmids ranging from 25ng, 100ng, 250ng, 500ng to 1000ng and 1μg of the pBS1073 plasmid. pMEP-E2 expression plasmids encoding HPV8 E2 full length protein, HPV8 E2 proteins carrying mutations R250A, S253A or the double mutation R250A/S253A in the full length background, BPV1 E2 and pMEP4 were used for the assay. (A) Graphical representation of the levels of luciferase activity recorded as relative light units per second (RLU/second) for the different E2 plasmids that were transfected into CV-1 cells. The error bars represent the standard deviation of the experiments done in triplicate. (B) Immunoblot showing expression levels of the wild-type and mutated HPV8 E2 proteins in E2 expressing CV-1 stable cell lines detected using anti-flag antibody.

termination linker inserted into the E2 ORF indicated that HPV8 genome was capable of replicating in the absence of E2 protein in U2OS cells (Raymond Fernalld, personal communication). Hence, for our studies aimed at examining the role of wild-type and mutated E2 proteins on the HPV8 genome replication, U2OS cells did not prove to be a useful replication assay system. However, we employed the U2OS replication assay to examine the replication of the plasmid containing the HPV8 origin in the presence of plasmids expressing the wild-type HPV8 E1 protein and wild-type or mutated HPV8 E2 proteins (Figure 7.2). In our assay, we included plasmids that expressed either the R250A mutated or S253A mutated HPV8 E2 proteins in addition to plasmids expressing the wild-type E1 or E2 proteins. We also included control plasmids that did not express either HPV8 E1 or E2 proteins. Moreover, we also used a plasmid that encoded a mutated HPV8 E2 protein that was defective in binding to E2 binding sites. Following day after transfection, E1 and E2 protein expression was induced at low levels with 0.1μM cadmium sulfate. Low molecular weight DNA was isolated from U2OS cells three days post transfection and digested with EcoRI and DpnI. Southern blot analysis with the HPV8 ori plasmid as probe revealed that, the HPV8 ori plasmids could replicate transiently in the presence of the R250A and S253A mutated E2 proteins. However, no replication was observed in the absence of the wild-type E1 or E2 protein. Notably in the presence of the mutated HPV8 E2 protein carrying the DNA binding mutation, no replication of the ori plasmid was observed (Figure 7.2). These observations indicate that the replication of the HPV8 ori plasmid in U2OS cells is dependent on both E1 and E2 proteins.

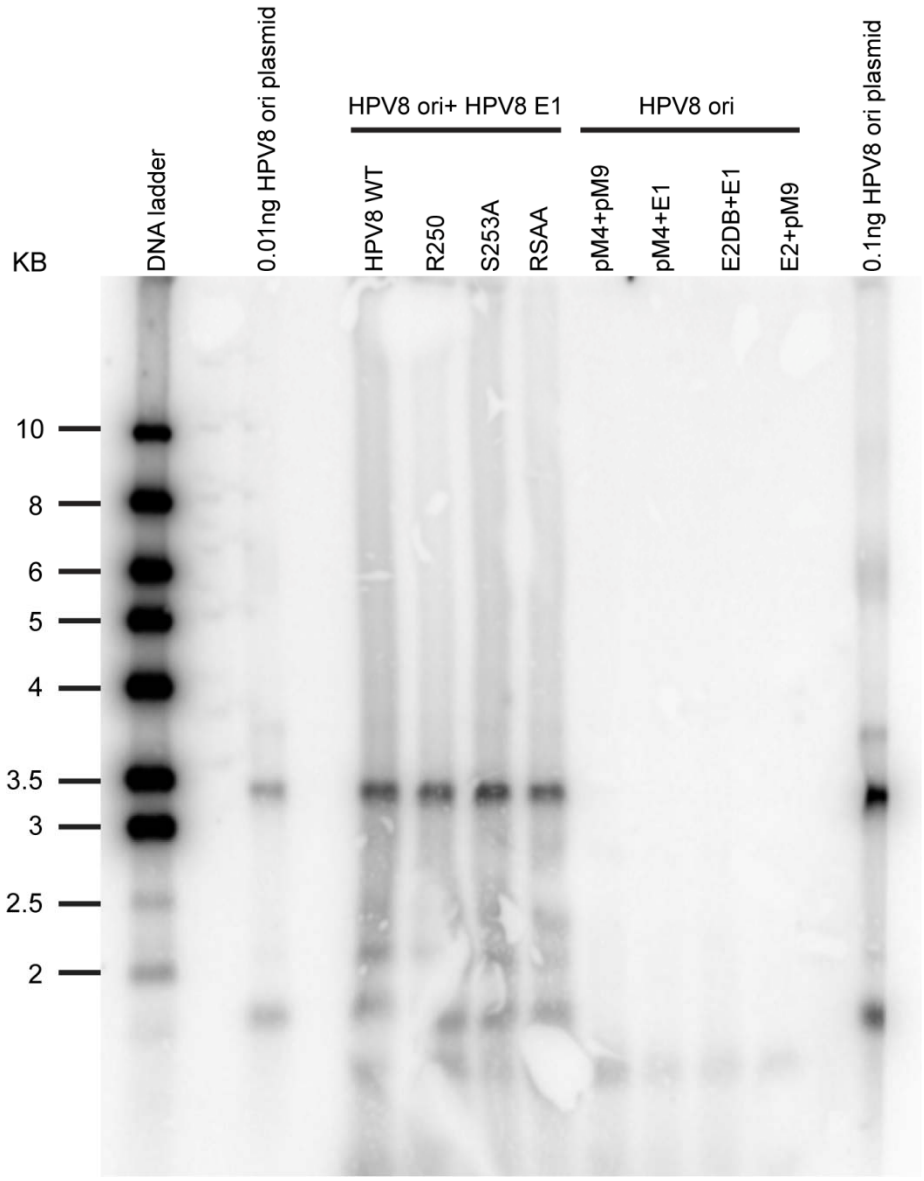

Figure 7.2
HPV8 ori plasmid can replicate in the presence of both R250A or S253A mutated E2 in U2OS cells. U2OS cells were co-transfected with HPV8 ori plasmid, wild-type HPV8 E1 and wild-type or mutated HPV8 E2 (R250A, S253A, RSAA, E2DB) expressing pMEP plasmids. RSAA is the HPV8 E2 protein carrying R250A and S253A mutations. Empty vectors pMEP4 (pM4) and pMEP9 (pM9) were used as negative controls. Low molecular weight DNA was digested with EcoRI and DpnI. Lanes labeled 0.01ng and 0.1ng marker represent the EcoRI linearized HPV8 ori plasmid. The DNA ladder used is the 1Kb ladder.

Additionally, these results also show that R250A and S253A mutations within the HPV8 E2 protein do not have any effects on the replication of the HPV8 ori plasmid in U2OS cells.

Discussion

In the studies presented in this chapter, we used a luciferase assay and a replication assay in U2OS cells to examine the effects of alanine substitutions of R250 and S253 on the HPV8 E2 functions of transcriptional activation and replication, respectively. Although, we did not observe significant differences in the functions of the wild-type and the E2 mutated proteins, it is premature to conclude that these mutations do not affect E2 functions other than chromosome binding. One of the best examples illustrating such a scenario was observed in the studies characterizing the phenotype of the BPV1 E2 mutation at phosphorylation residue, S301. Substitution of S301 with alanine within E2 in an expression plasmid did not affect any of the E2 functions. However, when the alanine substitutions were made in the E2 ORF in the context of the entire BPV1 genome, increased E2-specific transactivation and a high genome copy number phenotype were observed [166]. Similarly, in the case of the HPV8 E2 proteins, it is possible that the phenotypes observed with serine to alanine substitutions in the E2 gene expressed from an expression plasmid are different from those observed in the context of the entire HPV8 genome. We cannot rule out the possibility of E2 mutations alters its expression when expressed from the viral genome. As described previously, we have shown that both the R250A and S253A mutated E2 proteins are defective in phosphorylation and chromosome binding. According to our model explaining the

role of S253 phosphorylation in chromosome binding, we propose that as cells exit from mitosis during cell cycle, E2 proteins are dephosphorylated at S253 residue such that they are available to perform the transactivation function of the E2 protein. In such a scenario, it is possible that these mutations do not have an effect on the HPV8 E2 functions. However, further detailed studies in the background of the HPV8 genome are needed to conclusively demonstrate the effect of hinge mutations on the HPV8 E2 functions.

Chapter 8: Summary and general discussion

Summary of the findings described in the dissertation

A key challenge for all persistent viruses infecting replicating cells is to maintain their viral genomes in dividing cells. Several studies have shown that E2 proteins from different PVs bind mitotic chromosomes as a means to tether the viral genome; however, there are variations to this theme from one genus to another. Different E2 proteins show varied patterns of mitotic chromosome binding indicating that they associate with distinct chromosomal targets [191,205,262]. We assume that, analogous to other PVs, HPV8 E2-chromosomal association is crucial for the virus to tether its genome to host chromosomes during persistent infection.

To unravel the mechanisms of HPV8 E2 chromosomal interactions, we identified a 16 amino acid long region in the hinge that, when fused to CTD was crucial and sufficient for E2 chromosomal association. Further mutational analyses revealed that two specific residues in the mapped hinge region, R250 and S253, which lie within a common RXXS motif, are critical for the E2 mitotic chromosome binding. Notably, the identified RXXS motif, and residues R250 and S253, are completely conserved among the betapapillomaviruses that have been sequenced to date. Thus, the fact that this region is evolutionarily highly conserved in an otherwise divergent part of the beta E2 proteins, underscores its functional significance in the viral life cycle [235]. In addition to being essential for binding to host chromosomes,

it would be interesting to determine if this conserved hinge region plays additional roles in the biology of the beta PVs.

In comparison with other PVs, a unique feature of beta PV E2 proteins is the unusual composition and length of their hinge region. The hinge of beta PVs is 200 amino acids long and contains a large number of RG and RS dipeptide repeats. The RS dipeptide repeats of beta E2 proteins such as HPV5 and HPV8 are important for localization to nuclear speckles that are enriched in splicing factors and are adjacent to active transcription sites [141,235]. We observed that progressive removal of SR dipeptide motifs in the HPV8 E2 hinge results in loss of nuclear speckle localization. Additionally, for nuclear speckle localization the CTD of HPV8 E2 is essential.

Regulation of the HPV8 E2 chromosome binding function

In our attempt to understand the regulation of the HPV8 E2 chromosome binding function, we investigated the role of S253 phosphorylation. We demonstrated that S253 is most likely phosphorylated by PKA. Further S253 phosphorylation specifically increased during the S-phase of the cell cycle and remained high through mitosis, suggesting that phosphorylated E2 bound mitotic chromosomes. Our finding that PKA phosphorylates S253 in S-phase is in accordance with studies that have shown that PKA is active in the S-phase during which it is required for chromosomal DNA replication [53]. Notably, various PV proteins appear to utilize the cellular PKA pathway to influence the process of malignant progression. For example, PKA is involved in HPV16 E7 mediated transformation by promoting the process of cellular alkalinization [42]. Likewise, HPV16 E5 utilizes the PKA pathway to stimulate

degradation of the Bax protein and thereby, inhibits apoptosis of transformed epithelial cells [189]. Additionally, the PDZ binding motif of high-risk HPV E6 proteins is phosphorylated by PKA [138]. The expression of the E6 and E7 proteins is regulated by the E2 protein, with increased E2 expression resulting in repression of E6 and E7 [70]. Moreover, E2 protein has been shown to bind to both E6 and E7. Binding of E2 to E7 increases the stability of E7 but reduces its transforming potential [87]. Similarly, binding of E2 to E6 results in inhibition of E6 mediated degradation of PDZ proteins [100]. Since, E2 potentially interacts with E6 and E7, it is intriguing to think how the interaction of E2, E6 and E7 proteins will be affected by PKA during the course of viral infection when the levels and activities of each of these proteins changes. Likewise, it is essential to examine whether there is a differential regulation of PKA activity as cells transition from basal layer to differentiated layers. Analyzing the effect of PKA activity on E2 proteins in the presence of other PV proteins will ultimately help to determine how all these findings fit in the context of viral life cycle. Our findings clearly add another dimension to the role of PKA in the HPV lifecycle.

14-3-3 proteins are members of a highly conserved family of small acidic proteins [3]. These specifically bind to phosphorylated proteins and alter the sub-cellular localization, protein-protein interactions and enzymatic properties of the target proteins [3]. These proteins are involved in regulating a myriad of cellular processes including cell cycle regulation, apoptosis, cell proliferation, chromatin structure, metabolism, nucleolar function, among others [276]. A proteomics study conducted to identify interacting partners of 14-3-3 proteins during interphase and

mitosis identified a number of novel protein partners [172]. Among the proteins identified, one protein of interest was a protein kinase A anchoring protein (AKAP) [172]. AKAP plays an important role in regulating PKA activities in cells by controlling their spatial distribution. AKAP95 is responsible for recruitment of PKA to mitotic chromosomes and is important for chromatin condensation at mitosis [50,142]. Moreover, the consensus binding site of 14-3-3 proteins, RSXpSXP, somewhat resembles the R-X-X-S motif observed in the HPV8 E2 chromosome binding region (reviewed in [112]). To determine whether 14-3-3 proteins interact with the phosphorylated E2 protein we can perform protein immunoprecipitation assays and probe for the different isoforms of 14-3-3 proteins. Thus, examining whether 14-3-3 proteins are involved in regulating the HPV8 E2 chromosome binding function will provide further insights into the regulation of chromosome binding function. Additionally, recent studies have identified 14-3-3 sigma (also called stratifin) as the isoform that is expressed in keratinocytes (reviewed in [112]). It is believed to be a tumor suppressive gene and is silenced in many carcinomas (reviewed in [112]). Co-culturing of keratinocytes with fibroblasts results in secretion of statifin in differentiated keratinocytes that in turn results in increased expression of matrix metalloproteinase 1 (MMP1) (reviewed in [112]). Importantly, HPV8 E2 protein was recently shown to upregulate expression of another metalloproteinase, MMP9 in keratinocytes by binding to the E2 binding sites within the MMP9 promoter [5]. It would therefore be interesting to determine if there is any crosstalk between E2, 14-3-3 sigma and MMP proteins in the HPV8 lifecycle.

HPV 8 E2 phosphorylation and E2 stability

One of the observed effects of PKA phosphorylation of HPV8 E2 is that it enhanced both chromatin binding and protein half-life. However, it is unclear whether E2 phosphorylation directly regulates protein half-life or whether phosphorylation regulates chromatin binding and chromatin bound E2 has a much longer half-life than unbound protein. Nonetheless, our findings are supported by a recent report that states that HPV16 E2 has both increased phosphorylation and extended half-life in S-phase [129]. Another recent study showed that the EBNA1 chromosome binding GR repeat regions inhibit its proteasomal degradation [52]. These findings are in fact in accordance with the hypothesis that phosphorylation of S253 regulates chromatin binding and chromatin bound E2 has a much longer half-life than unbound protein as was observed in the pulse-chase experiments in chapter 4. It is important to keep in mind that our studies were conducted in the background of the truncated HPV8 E2 protein that consists of the 16 residues long chromosome binding hinge region fused to the CTD. In preliminary studies conducted in our laboratory, we observed that the full length HPV8 E2 protein appears to have a number of elements distributed within the hinge region that are responsible for regulating E2 stability (Alison McBride, personal communication). It is unlikely that the 16 amino acid long hinge region alone is responsible for regulating E2 stability. We believe that the enhanced phosphorylation of S253 residue within the chromosome binding region of HPV8 E2 results in increased chromatin binding and thus, longer half-life of chromosome bound E2 protein.

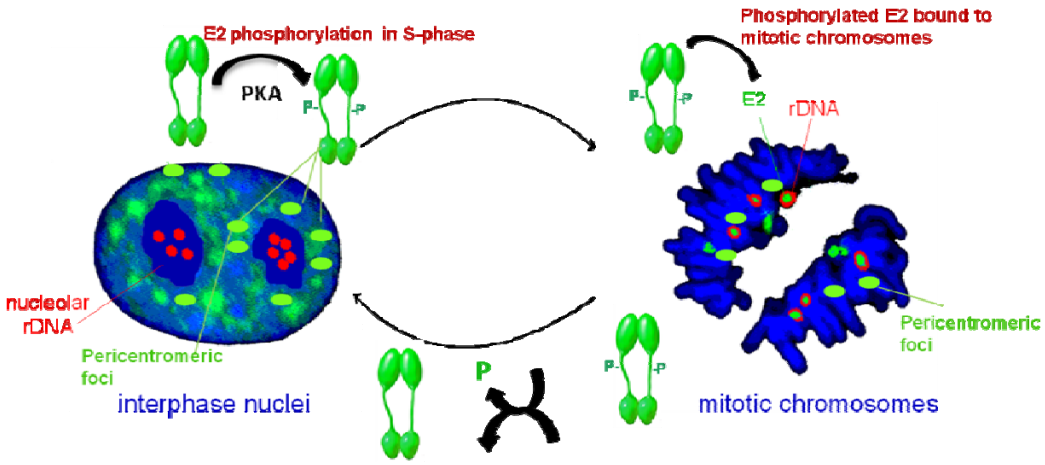

Figure 8.1
Model to explain the regulation of the HPV8 E2 chromosome binding by PKA phosphorylation. PKA phosphorylates the E2 protein at S253 residue during S-phase, resulting in a stabilized population of E2 that binds to chromatin strongly and can be observed as E2 associated pericentromeric foci in interphase. In mitosis, phosphorylated E2 remains bound as pericentromeric foci and unphosphorylated E2 is degraded. Adapted from [235].

Based on our findings, a simplified model explaining the role of PKA in regulating the chromosome binding function of the HPV8 E2 protein is shown in figure 8.1. During S phase of the cell cycle, PKA phosphorylates E2 protein at S253 residue, resulting in a stabilized population of E2 that binds to chromatin strongly and can be observed as E2 associated pericentromeric foci in interphase. As cells transition to mitosis, the phosphorylated E2 population remains bound to chromosomes whereas the unphosphorylated and chromatin unbound population is labile and degraded. When cells exit from mitosis, the phosphorylated E2 proteins undergo dephosphorylation and are available to perform the transcription function of E2 during interphase.

Similarities between the tethering proteins E2, EBNA1 and LANA

Similar to PVs, the gamma herpesviruses also cause long term infection of their hosts and maintain their genomes as extrachromosomal elements in infected cells. Comparison of the chromosome binding regions of the gammaherpesvirus tethering proteins, LANA and EBNA1 with the HPV8 E2 protein reveals that there are sequence similarities within these regions in all three proteins as shown in figure 3.9 in Chapter 3 [235]. Interestingly, the identified RXXS kinase motif is also present in the chromosome binding regions of the LANA and EBNA1 tethering proteins. The two chromosome binding domains of EBNA1 have been mapped to the N-terminal domain and are called domain A (residues 33-89) and domain B (residues 328-378) [160]. These regions have been shown to carry A-T hook motifs that are required for chromosome binding and partitioning [232]. Notably, several of the A-T hook motifs overlap with the RXXS motifs. Furthermore, the serine residues within these RXXS

motifs are phosphorylated and substitution of these serines disrupts the EBNA partitioning function [240]. In the case of KSHV LANA, the chromosome binding motifs have been mapped to the N-terminus (residues 5-22) [13]. Additionally the LANA CTD can associate with mitotic chromosomes at pericentromeric and telomeric regions [133]. In LANA, substitution of residues overlapping the RXXS motif also disrupts episomal persistence, chromosome binding and interaction with histones H2A and H2B [13]. Thus, from these observations we can propose that although the three tethering proteins may have different chromosomal targets, a common mechanism regulates their chromosome binding functions.

Importantly, similarities among the three tethering proteins do not end with similarities in their chromosome binding regions. Despite no strong sequence similarity between the proteins, E2, EBNA1 and LANA CTDs form similar structures. The EBNA1 protein has been shown to form a similar dimeric beta-barrel structure to that of the E2 CTD [29]. The LANA DBD has also been predicted to fold into a similar structure as the EBNA1 CTD [101,105]. Thus, it is tempting to speculate that the similarity in CTD structures of the three proteins further supports the hypothesis of common mechanism of regulation of chromosome binding. Our mapping studies demonstrate that the HPV8 E2 CTD is also essential for the chromosomal association function of the HPV8 E2 protein [235]. However, the contribution of the CTD in the E2 chromosomal association function is not completely clear. The CTD could be involved in mediating E2-chromosome association either through direct DNA binding or protein-protein interactions. From the RNAse treatment experiments, it appears that E2 chromosome binding is not

mediated through RNA interactions. Due to the similarity between the CTD structures of EBNA1 and E2 and the predicted structure of LANA, using homology between EBNA1 and E2, a computer model of the LANA DNA binding domain can be developed. Based on a study by Kelley-Clark *et. al* wherein they identified LANA CTD residues important for chromosome association, the homology model would serve as a powerful tool to identify residues in analogous position in the HPV8 E2 CTD and determine whether they are required for HPV8 E2 chromosomal interaction.

General Discussion

The members of the betapapillomavirus genus share a number of unique characteristics that distinguish them from other PVs. Beginning with their genomic organization to composition and features of the viral proteins they express, beta PVs are different. The fact that the genome size of beta PVs is smaller (7654 bp) and the LCR is shorter (350-500 bp) compared to other genera of PVs underscores their uniqueness (reviewed in [144]). The LCR has unique transcriptional regulatory elements, which indicates a different regulatory mechanism of the early promoter region of beta PVs (reviewed in [144]). Likewise the genome of these viruses does not encode the E5 gene. The E5 ORF encodes a small hydrophobic protein in cutaneous and genital alpha PVs that is believed to contribute to hyperproliferation of cells together with E6 and E7 [65]. E5 protein is primarily localized to the endoplasmic reticulum and is believed to play a role in transit of signaling factors [146,210] thereby augmenting the oncogenic activities of E6 and E7. High expression of HPV16 E5 in transgenic mice resulted in tumor formation in the skin [164]. Studies

have shown that E5 proteins play an important role in many PV pathogenesis [186]. An interesting question to ponder over is how the lack of E5 might affect the beta PV biology. Loss of E5 gene function in the HPV31 genome was reported to result in inactivation of late viral functions in differentiating cells [79]. A provocative speculation could be that the beta E2 proteins because of their unique characteristics can compensate partly for the loss of the E5 ORF in the beta PV life cycle. The fact that beta E2 proteins seem to possess oncogenic potential underscores this possibility. One means to check for the ability of E2 to compensate for lack of E5 would be to compare the cellular targets of E5 with beta E2s and examine if they share any protein interactions.

Several studies on beta E2s are throwing light on their unique properties. The E2 proteins of PVs are not thought to be oncogenic. In fact, they are usually pro-apoptotic as seen in case of many high-risk E2s such as HPV18 and HPV 16 [63]. However, HPV8 E2 proteins appear to possess oncogenic potential. Recent studies expressing either the HPV8 early gene regions or HPV8 E2 protein under the kertain-14 promoter in transgenic mice resulted in formation of skin tumors in mice [203,221]. Additionally, UV treatment of transgenic mice results in rapid onset of tumor development [203]. These studies suggested that they are two possible explanations for the enhanced E2 induced tumor development. One possibility is that increased oncogenesis is due to increased E2 expression as a consequence of upregulation the keratin-14 promoter. The E2 protein is a transactivator and it is possible that it could upregulate expression of cellular genes. For example, HPV8 E2 can enhance the transcriptional activity of C/EBPβ and modulate keratinocyte

differentiation [103]. The second possibility is that E2 interferes with the UV induced damage response pathway and thus, induces cell proliferation by inhibiting apoptosis. This is supported by reports that have shown that HPV8 E2 interacts with a number of UV DNA damage response factors such as TopBP1, p53 and Poly (ADP-ribose) polymerase (PARP) [169]. Hence, these studies clearly indicate that the HPV8 E2 protein is different from the alpha E2s. It is not clear as to what interactions of the HPV8 E2 protein or which domains of the E2 protein are responsible for the observed oncogenic potential. It would not be out of context to speculate that the unique composition of the HPV8 E2 hinge plays a role in the oncogenic potential of the virus. It would be critical to determine whether other beta HPV E2 proteins such as HPV5 E2 also show similar carcinogenic capabilities.

Like other PVs, betapapillomaviruses cause persistent infection of the host, the clinical manifestations of which are especially seen in immunocompromised individuals. For instance, EV patients that show a high susceptibility to infection with cutaneous HPVs like HPV5 and HPV8 specifically but not to other genera of PVs such as alpha PVs [192]. There is wide speculation about the contribution of EVER genes in exerting intrinsic immunity against beta PV infections. Moreover, it has been suggested that lack of E5 gene could be compensated for by EVER mutations during induction of EV lesions [186]. Another aspect linked to the phenomenon of host restriction of beta PVs is their inability to replicate in normal human keratinocytes. Both EVER genes are transcribed in normal human skin [208]. It would be worthwhile therefore, to determine whether down regulation of EVER genes allows for successful replication of beta PV genomes in keratinocytes. Notably, UV is an

important co-factor for a number of pathological effects of beta PVs. Treating keratinocytes with UV before transfecting the beta PV genomes would be an interesting approach to test for successful replication.

Use of PV vectors in therapy

The recent advent of the two prophylactic HPV vaccines is believed to significantly reduce the burden of HPV associated infections. Nevertheless, due to the fact that these vaccines are not effective against pre-existing infections and infections with viral types other than the ones included in the vaccines, there is still a need for specific antiviral therapy. The multifunctional E2 proteins of papillomaviruses are indispensable to the viral life cycle and hence, it makes them an attractive target for antiviral therapy. One line of therapy that could potentially interfere with the viral life cycle is to use compounds that would inhibit the binding of E2 to the E2 binding sites. However, there are important considerations to keep in mind while employing such a strategy. Many times during viral integration of high risk HPV genomes, the E2 ORF is lost during the process and hence, such an approach would be beneficial only prior to integration. Moreover, interfering with E2 binding can also upregulate E6 and E7 expression, which would lead to tumor progression. However, this approach might be beneficial against low risk E2s. A class of polyamides was specifically found to interfere with the binding of HPV18 E2 to its E2BS in vitro [220]. But one of the major limitations in using these compounds has been their limited bioavailability in cells. Recently an HIV DNA vaccine has been developed based on the nuclear retention and binding capacity of BPV1 E2 protein [161].

Our studies aimed to understand the molecular interactions of E2 proteins with host chromosomes and have added to our knowledge of how E2 proteins can act as a molecular bridge to bind chromosomes. However, it is important to determine if the chromosome binding region of the HPV8 E2 protein can contribute to plasmid maintenance by efficiently tethering and maintaining a reporter plasmid. Developing a papillomavirus partitioning assay has been challenging because of the role that the E2 protein plays in transactivation and replication initiation. In different approaches that have been tried in the laboratory, the reporter readout for plasmid maintenance has been problematic since we are unable to determine if the reporter readout observed is due to plasmid maintenance or due to E2-mediated transactivation. However, the regions required for chromosomal association of HPV8 are different from that of other E2s and do not include the transactivation domain. Thus, the read out of the partitioning assay will likely be much clearer with the truncated HPV8 E2 proteins in the background of 240-255 amino acids of the hinge fused to the CTD. If we can successfully test the role of the chromosome binding region in the tethering and partitioning assay, it would prove to be a useful tool in the development of vectors for gene therapy.

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