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Abstract

Human papillomavirus are small DNA tumor viruses. The viral genome is a small circular double stranded DNA that replicates autonomously as an extrachromosomal plasmid. Occasionally infections by the high risk HPV viruses can lead to DNA integration and progression to cancer. How the HPV DNA is maintained and becomes established in the dividing host cells is not well understood. Additionally, as a double-stranded DNA virus it is likely that double-stranded DNA breaks in the viral genome, either from replication or random damage, will trigger a DNA repair response from the host cell. Such responses have been alternatively reported to be detrimental or helpful to viral DNA amplification for various DNA viruses. Interactions between HPV and the host cell's double-stranded DNA break repair machinery have not been examined. This dissertation investigates these two subjects.

The viral E2 protein is critical for viral DNA maintenance during cellular replication. The E2 proteins of HPV-11, 16 and -18 each localizes to the mitotic spindles. The first part of this research was to identify the specific regions in the carboxyl domain of HPV-11 E2 (E2C) necessary for this function. We found that a peptide of 23 residues in length which was highly conserved in HPV-11, HPV-16, 18,

and BPV-4 E2C. Mutations or deletions of this region rendered E2C unable to localize to the mitotic spindles. In BPV-1 E2C this region is highly divergent and we observed no localization of BPV-1 E2C to the spindles. We suggest this region is necessary and sufficient for the HPV-11 E2 protein's interactions with the spindle fibers.

The second subject of this research investigates interactions between viral replication and the cellular double-stranded DNA break repair complex, MRN. Other viruses have close interactions between components of the MRN complex and viral replication centers. We show that, during active viral replication, both Mre11 and Nbs1, two components of the MRN complex, relocalized to the replication centers. This relocalization is not necessary for efficient viral replication nor does it activate downstream mediators of DNA repair, most notably ATM. It is unclear as to what function Mre11 might play at the replication center.

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LIST OF ABBREVIATIONS

ATM	ataxia-telangiectasia-mutated
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bp	base pair
BPV	bovine papillomavirus
BS	binding site
C	carboxyl
CDK	cyclin-dependent kinase
CKII	casein kinase II
CRPV	cottontail rabbit papillomavirus
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DSB	double-stranded DNA break
E6AP	E6 associated protein
ERK	extracellular signal-regulated kinases
EV	epidermodysplasia verruciformis
G1	gap 1
G2	gap 2
GFP	green fluorescent protein

H	hinge
HAT	histone acetyltransferase
HDAC	histone deacetylase
HCMV	human cytomegalovirus
HLA-1	human leukocyte antigen-1
HPV	human papillomavirus
HR	high-risk
Hsp	heat shock protein
hTERT	human telomerase reverse transcriptase
IF	immunofluorescence
Kbp	kilo-base pair
LR	low-risk
MAPK	mitogen activated protein kinase
MAP	microtubule associated protein
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
MTOC	microtubule-organizing center
MRN	Mre11-Nbs1-Rad50
N	amino
ND10	nuclear domain 10
NES	nuclear export sequence
NLS	nuclear localization sequence

ORF	open reading frame
Ori	replication origin
PCNA	proliferating cell nuclear antigen
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PHK	primary human keratinocytes
PML	promyelocytic leukemia
PV	papillomavirus
RFP	red fluorescent protein
RNA	ribonucleic acid
RPA	replication protein A
RRP	recurrent respiratory papillomatosis
S	synthesis
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SV40	Simian virus 40
TFIID	transcription factor II D
URR	upstream regulatory region

Chapter 1

INTRODUCTION

The family of papillomaviruses is widespread, prevalent and can infect humans and a variety of vertebrate animals. The viruses are species specific, non-enveloped double-stranded DNA tumor viruses. The first papillomavirus isolated was from cotton tail rabbits (CRPV) (127). Since then, more than 200 papillomaviruses have been isolated and are grouped based on sequence homology. Over 100 types of human papillomaviruses have been isolated from patients (40). The phylogenetic relationship is reflected in their pathogenicity, their species specificity, and their tissue tropism for either cutaneous or mucosal squamous epithilium at different body sites (40).

Infections can cause epithelial hyperproliferation. The cutaneous HPV are mostly responsible for warts appearing on the skin. HPV-1 is the causative agent for plantar warts. HPV-2 and HPV-4 cause warts on the hands. Patients with certain immune deficiency and infected with HPV-5, -8 or closely related types can develop a rare disease called epidermodysplasia verruciformis (EV) (40). EV is characterized with numerous flat, reddish wart over the head, neck and trunk, which the patients are unable to resolve. Moreover, in sun exposed head and neck region, these lesions often progress to squamous cell carcinomas at an alarming frequency of 30% (82).

The mucosotropic HPVs infect epithelia of the anogenital and the aerodigestive tracks and can be sexually transmitted. These viruses are further classified into high-risk or low-risk groups based on their oncogenic potential. The high-risk groups, such as

HPV-16, HPV-18 and closely related types cause flat, potentially dysplastic lesions (condylomata plana) and have been consistently shown to induce cervical, penile, anal and head and neck cancers at a low frequency. Cervical cancer is the leading cause of cancer in women under the age of 35 (160). In contrast, HPV-11 and HPV-6 cause almost exclusively exophytic but benign condylomata acuminate of the anogenital tract and benign papillomas in the larynx (i.e. recurrent respiratory papillomatosis) (RRP) (45). Hyperproliferative epithelial growths on the larynx and along the upper respiratory tract can block airflow. Currently the most common treatment for RRP is surgical removal of the lesional tissue. However, surgical intervention results in rapid re-growth of infected cells comprising the wound margins, requiring further periodic surgical interventions (45).

Transmission of HPVs requires a cut or abrasion in the epithelium to allow the viral particles access to the basal cells. Viruses reproduce in the squamous epithelium and, as the cornified strata slough off from the outer layer of the warty lesions, progeny HPV particles are released (Figure 1). Infection is often asymptomatic or latent. Active infection usually regresses after 9-12 months and can be reactivated following wounding or during periods of immune depression (160).

Viral Genome Organization and Transcription

Papillomaviruses each contain a doubled-stranded circular DNA genome of approximately 8 Kbp in length (25). The viral genome codes for 8 open reading frames. The viral genomes are organized into a noncoding region or upstream regulatory region (URR), which contains viral promoters, enhancers, and the origin of replication, and two genetic blocks, the early (E) region and the late (L) region, each followed by an RNA

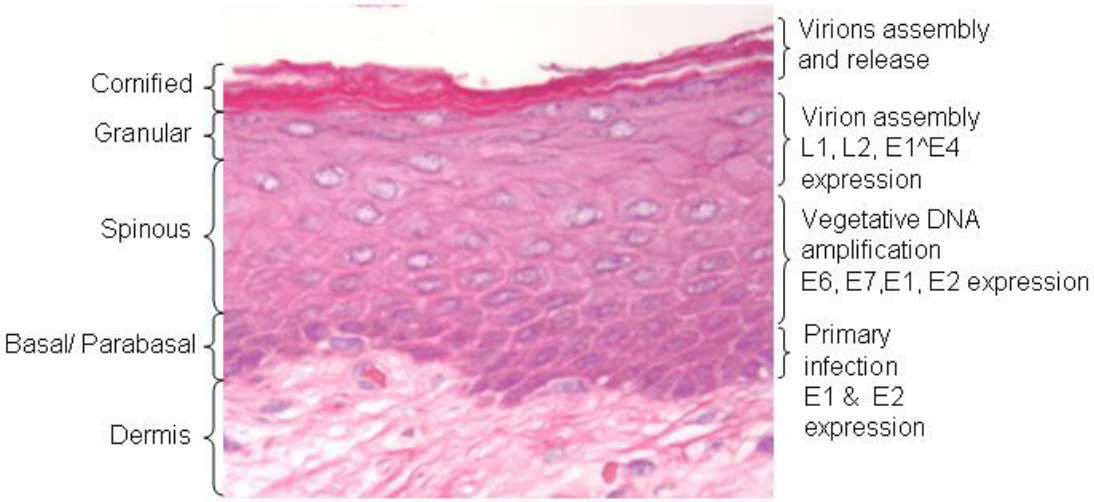
Cornified
Granular
Spinous
Basal/ Parabasal
Dermis
Virions assembly and release
Virion assembly L1, L2, E1^E4 expression
Vegetative DNA amplification E6, E7,E1, E2 expression
Primary infection E1 & E2 expression

Figure 1. Organization of the Epithelium and Corresponding Expression of Papillomaviral Genes. The epithelium is composed of multiple strata (listed on the left margin), basal and parabasal layers, multiple lower and upper spinous layers, a granular layer, and finally the superficial cornified layers. Only the basal and parabasal cells are capable of cycling, the cells of the other layers have exited the cell cycle. Papillomaviral gene expression and DNA replication are intimately linked to the different cellular layers (listed on the right margin). Initial infection occurs in the basal or parabasal layer, where E1 and E2 are transiently expressed to support brief viral replication and maintain low viral copy number. Upon progressive differentiation into the spinous layers, the differentiation- dependent URR promoter transcribes the early genes to promote re-entry of the differentiated keratinocytes into S-phase to enable vegetative viral DNA amplification. In the granular layer, the virus begins to express the late L1 and L2 proteins, respectively the major and minor capsid proteins, which self-assemble and then encapsidate the viral DNA during virion morphogenesis (Fig. 2) (24). Finally, as the cornified layers slough off the surface of the epithelium, the viral particles are released.

polyadenylation site. The E proteins (E1, E2, E4, E5, E6 and E7) are necessary to regulate

Transcription of the viral genome occurs from one strand. Messages are derived from several promoters and utilize one of two poly-adenylation sites in conjunction with multiple splice donor and acceptor site combinations (for a review, see Chow and Broker, 2007). Extensive investigations have been performed concerning regulation of the transcription of the E6 and E7 genes, as they encode two main viral oncoproteins. In all HPVs, a promoter immediately upstream of the E6 gene (the E6 or P1 promoter) is responsible for the transcription of E6 gene. In the LR HPVs, a P2 promoter embedded within the E6 open reading frame is responsible for the expression of E7 (25). In HR HPVs, the E7 gene is expressed from a polycistronic message derived from the E6 promoter (25). However, efficient production of the E7 protein is dependent on intragenic splicing within the E6 ORF. An unspliced RNA derived from a promoter in the E7 ORF encodes E1. In addition, an intragenic splice in this transcript results in the message for the E2 protein and an intergenic splice creates the mRNA for the E1^E4 fusion protein (119). All of these messages potentially encode the E5 protein(s) from an ORF located at the 3' end of the transcript. This promoter has low level activity in relatively undifferentiated keratinocytes and much higher activity following squamous differentiation (61, 119). This promoter is also responsible for abundant mRNAs encoding the late proteins when the transcripts are polyadenylated at the late polyA site. A twice-spliced late mRNA encodes E1^E4 and L1, whereas another mRNA encodes E1^E4 and L2 (25).

In addition to proteins evident from the E or L ORFs, some spliced mRNAs are deduced to encode truncated peptides or fusion peptides. Only a few of these proteins have been assessed for their functionality in reporter assays (to be described).

Upstream Regulatory Region (URR) or the Long Control Region (LCR)

The URR of approximately 700 bp in length contains no open reading frames and lies between the 3' end of the L1 gene and the 5' end of the E6 gene. The URR contains five major sequence elements: the late polyadenylation sites, three or four E2 protein binding sites, the E1 binding sites, one or more promoters, and regulatory elements that bind host transcription factors (23, 67, 77, 132, 134). The E6 promoter is relatively active in proliferating cells, which is thought to simulate the state of wound healing. Once the wound is healed and the stratifying epithelium returns to steady state growth and differentiation, the E6 and E1 promoters both revert to differentiation-dependency (23). Binding sites for many cellular transcriptional regulatory factors such as Sp1, Oct1, and AP1 are critical for high promoter activity, whereas chromatin remodeling by histone deacetylases is thought to down- regulate the viral promoter in the basal stratum (12, 50, 54, 110).

The viral E2 protein is a transcription factor and modulates viral promoter activity (23, 67). Specifically, the URR contains several E2 binding sites. Two of them are located in close proximity to the E6 promoter TATA box and an Sp1 binding motif at the downstream end of the URR. E2 bound to these E2BSs interferes with the binding of TATA binding protein and Sp1, leading to E6 promoter down-regulation (83). In addition to its role in viral transcription the E6 promoter proximal portion of the URR also contains the viral origin of DNA replication (ori). The origin of replication consists of

the E1 and E2 binding sites. For replication, at least three of the four E2 binding sites in the URRs of HPVs are essential. The arrangement and spacing of these binding sites are highly conserved among all papillomaviruses. The specific arrangement of E2 binding sites within the URR appears to be more important for viral replication than merely the number of sites (28, 134).

E2

The full-length E2 protein is a 42 kDa protein, which is involved in both replication and transcriptional regulation (21, 70, 77 Ustav, 1991 #1120, 111, 151). The full-length protein can be divided into 3 distinct domains, an N terminal transactivation domain, a flexible hinge region, and a C terminal DNA binding domain, both the N terminal and the C terminal domains are fairly well conserved in human papillomavirus. The N terminal domain is responsible for regulating E2's interactions with the E1 protein and the cellular transcription factor TF11D {Berg, 1997 #881, 152). In HPV-18 E2, the amino terminus can be ubiquinated, leading to proteasomal degradation (14). The hinge domain (H) is a flexible domain and variable in sequence among different E2 proteins. In HPV-11, this domain contains the main nuclear localization and matrix interaction sequence (158). This domain overlaps exactly with the E4 ORF in all PVs (29). In contrast, in bovine papillomavirus (BPV) E2, there are two known nuclear localization signals, with the first between amino acids 107 and 115 (KRCFKKGAR) in the N domain and the second between amino acids 339 and 352 (KCYRFRVKKNHRHR) in the C domain (128). The carboxyl terminal domain is the protein dimerization and DNA binding domain. All known E2 proteins bind to the palindromic sequence $ACCGN_4CGGT$, referred to as the E2 binding sites (E2BSs). In the mucosotropic HPVs,

there are 4 E2BS located within the URR, three of which flank a series of E1BS to constitute the origin of replication (ori) (26). In HPV-11, -18, -16, and BPV-4, the E2C region also associates with the mitotic spindle fibers, as demonstrated in this investigation (36, 144).

The primary role of E2 is to recruit the E1 protein and various host replication proteins to the ori. However, the E2 protein is no longer present in the active replication complex (21, 22, 85). HPVs and BPV-1 also encode several E2-related proteins that differ in their amino terminal sequences (see below). Since only the full length E2 can support replication, E2N is required to recruit E1 protein. Using chimeric BPV-1/HPV-11 E2 proteins, it was demonstrated that both the C-terminal and the N-terminal domains of E2 independently interact with the E1 protein (159).

Based upon indirect immunofluorescence visualization, full length HPV-11 E2 is localized to the nucleus in dot or speckle patterns in interphase cells. In some cells, the foci are few and large while, in other cells, the foci are small and numerous (136). In particular, E2 forms distinct nuclear foci that are associated with PML (promyelocytic leukemia oncogenic domains) or ND10 (Nuclear Domain 10) bodies (136). E2 colocalizes with E1 and host RPA and newly replicated ori-containing DNA, as revealed by BrdU (5'-bromo-deoxyuridine) incorporation and by in situ hybridization (136). The changing patterns of E2 foci might be associated with transcriptional or replication activities or be cell-cycle regulated. As the cell proceeds to mitosis, HPV-11 E2 relocalizes with the mitotic spindles and finally the midbody during telophase (36, 144).

E2-related proteins, including E2TR, E8-E2 (E2C) and E1M-E2C arise from alternative splicing and the use of alternative promoters (20). They differ in the amino

terminus but all contain the same DNA binding domain and bind to E2BSs (20, 119). Each is a transcription and replication repressor since each can compete with full-length E2 protein for binding to the E2BSs (131). BPV-1 E2TR uses an internal ATG in the E2 ORF and encodes a 249 amino acid peptide fused to a truncated version of E2N. E8^E2 results from a splice of 8 amino acids from a short E8 ORF which overlaps the E1 ORF, fused to the HC-domain of E2 (97, 114, 142). These repressors are analogous to those initially identified as E2C in HPV-11 and -6. The HPV-11 E2 protein also interacts with the cellular Brd4 protein to form a repression complex. It is thought that Brd4 binds to acetylated chromatin near the E6 promoter and helps to recruit E2 to its cognate binding sites (148-150) }. Once bound, the E2 protein prevents recruitment of TFIID and pol II to the promoter (149).

In addition to its role in viral replication and transcriptional regulation, E2 has recently been shown to be intricately involved in maintenance of the viral plasmid during cellular replication. Vegetative viral genome amplification occurs only in the spinous layers, where the cells are terminally differentiated and no longer cycling. However, initial and persistent infection occurs in the basal or parabasal layers of the epidermis. The cells of the basal layer periodically divide to replenish cells above that are lost to terminal differentiation, while those of the parabasal layer (transit amplifying cells) divide very often (~daily) to replenish the squamous epithelium. To maintain the viral genome in the cycling cells, papillomaviruses have evolved mechanisms that allow them to attach their genome to various host proteins during cell division.

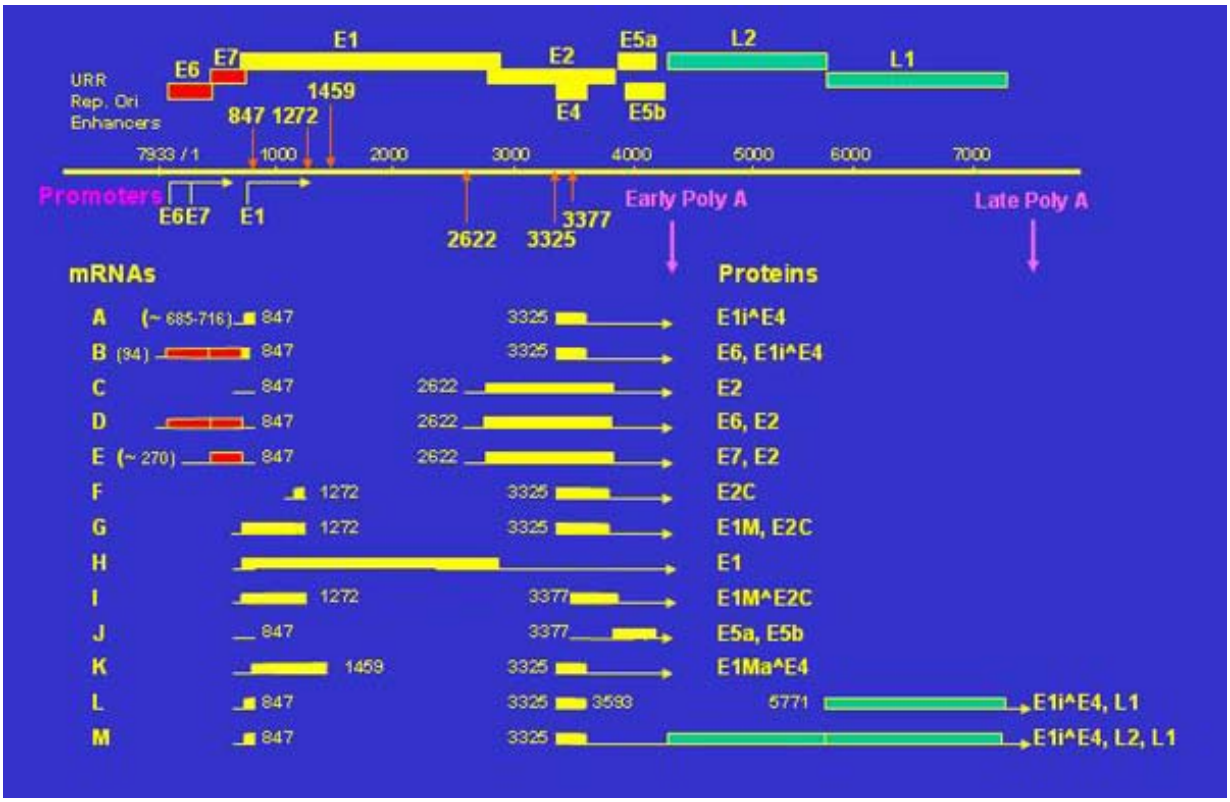
E1
E2
E5a
L2
L1
E6
E7
E4
E5b
URR
Rep. Ori
Enhancers
1459
847 1272
7933 / 1
1000
2000
3000
4000
5000
6000
7000
Promoters
E6E7
E1
2622
3325
3377
Early Poly A
Late Poly A
mRNAs
Proteins
A (~ 685-716) 847 3325 E1i^E4
B (94) 847 3325 E6, E1i^E4
C 847 2622 E2
D 847 2622 E6, E2
E (~ 270) 847 2622 E7, E2
F 1272 3325 E2C
G 1272 3325 E1M, E2C
H E1
I 1272 3377 E1M^E2C
J 847 3377 E5a, E5b
K 1459 3325 E1Ma^E4
L 847 3325 3593 5771 E1i^E4, L1
M 847 3325 E1i^E4, L2, L1

Figure 2. Human Papillomavirus Type-11 open reading frames, mRNA, proteins, and functions (Chow and Broker 1997). The circular genome of the 7933 bps is represented in a linear form. HPV RNA transcription is unidirectional. The noncoding region or URR contains the replication origin, the promoter, and the enhancer. All open reading frames are represented as colored boxes. The arrows indicate the direction of the transcript and the lines mark the 5' start points. Gaps in arrows signify spliced-out introns. The coding regions for each message are shown as open boxes over the mRNAs. The ^ symbol represents the fusion of different reading frames resulting from RNA splicing.

The mechanism of viral genome maintenance in dividing cells has been intensely studied for both human papillomaviruses and BPV-1. It is now clear that the different viruses utilize distinctly different strategies. Our studies of HPV-11 E2 demonstrate that the localization of the E2 protein during mitosis is dynamic (36). As mitosis begins, E2 localizes first to the centrosomes; during metaphase E2 then localizes with the mitotic spindle fibers. Finally, during telophase and cytokinesis the E2 protein relocalizes to the metaphase plate and the midbody (36). The region responsible for this was mapped to the C- terminal domain of HPV-11 E2; this region was found to be conserved in HPV-16, HPV-18 and BPV-4 (36). Localization studies of the E2 proteins of those papillomaviruses showed that they also localized to the mitotic spindles. Furthermore, in the presence of E2, an ori-containing tracking plasmid also localized to the mitotic spindles whereas, in the absence of E2 expression, the tracking plasmid had no discernable localization (36). Taken together the data indicate that for HPV-11 the E2 protein is necessary for viral DNA segregation.

In contrast to HPV-11 E2, the BPV-1 E2 localizes to the mitotic chromosomes (13, 153). It had been reported that the association of BPV-1 E2 with the mitotic chromatin was mediated by binding to the cellular protein Brd4 (1, 13, 154). Furthermore, inhibition of the association of BPV-1 E2 with Brd4 leads to viral genome loss in the tested cell lines (154). However, it was later reported that the association of the E2 protein with Brd4 was necessary for all papillomaviral E2 mediated transcriptional activation but not for viral genome maintenance (98, 125, 126, 150). Work by Parish et al. showed that, for BPV-1 and HPV-11 E2, the localization to the mitotic chromosomes is mediated by the cellular protein ChlR1. By mutational analysis, the investigators

showed that BPV-1 E2 W130R, an E2 protein that did not associate with ChlR1, abrogated the ability of the protain to localize with the mitotic chromosome; additionally this protein was deficient in genome maintenance when compared with wild-type E2 (107).

E1

The E1 protein is a multi-functional phosphoprotein, with a molecular weight of 68 kDa. Its activities include DNA helicase, ATPase, and ori binding (26). The E1 protein is composed of several functional domains: a localization regulatory region, a DNA binding domain, an oligomerization and helicase domain, and an E2 interacting domain (27 , 130). The E1 protein binds to the viral ori to establish the pre-initiation complex by recruiting DNA polymerase α/primase (4, 32, 94, 108). While E1 can bind the ori alone in vitro, the binding affinity is weak; in vivo, E2 is necessary to help recruit E1 to the ori. E1 protein also associates with topoisomerase 1 (31, 71), and replication protein A (RPA) (65, 89), both of which are required for E1 to unwind supercoiled circular DNA. E1 additionally interacts with WD 80 repeat protein, which is needed for proper maintenance of the viral genome in keratinocytes (33). E1 might further promote viral replication by interrupting the chromatin structure on the viral DNA (80).
X-ray crystallographic studies of BPV-1 and HPV-18 E1 proteins show that they initially assemble at the E1 binding site as a dimer (55, 86), which to serve as a nucleation point for further addition of E1 to form a trimer and then dihexamer. In contrast, electron microscopy reveals that the HPV-11 E1 protein binds to the ori primarily as a hexamer and in some cases as a dihexamer. The binding of E1 to the ori is enhanced by heat shock protein 70 (Hsp 70) (84, 86). It is thought that the presence of Hsp 70 may

facilitate E1 binding to the ori by dissociating E1 oligomers into monomers, which can then reassemble into a hexamer on the ori. The presence of Hsp40 then promotes efficient formation of a dihexamer on the ori (84, 86).

In BPV-1, nuclear import of the E1 protein is regulated by a bipartite nuclear localization sequence (NLS) within the amino terminal domain of the protein (16). In contrast, HPV-11 nuclear localization is dynamically regulated by bipartite NLS as well as a dominant nuclear export sequence (NES) (44). The localization of the HPV-11 E1 protein changes from the cytoplasm to the nucleus, depending on the phosphorylation state of the protein. The bipartite NLS is activated following phosphorylation of E1 at serines 89 and 93by catalyzed by various mitogen-activated protein kinases (MAPK). Once phosphorylated, the E1 protein translocates efficiently into the nucleus (44). However, to maintain its nuclear localization, the E1 NES must be inactivated by phosphorylation which is catalyzed by cyclin-dependent kinase 2 (CDK2) or cyclin-dependent kinase 1 (CDK1) on serine 107. The NES of HPV-11 E1 is a CRM-1 nuclear export sequence (44).

E4

The E4 open reading frame overlaps the hinge region of the E2 ORF. Roughly 90% of all viral transcripts are the spliced E1^E4 species, encoding the first four to six amino acids of E1 linked to the E4 ORF (24). In infected tissues, the cytoplasmic E4 protein exists as multiple species as a consequence of post-translational protein processing. In both patient-derived tissues and organotypic raft cultures, abundant E1^E4 is first detected in the mid and upper stratum of the epithelium in cells containing amplified viral DNA before expression of the capsid proteins (102). But the protein

persists into the cornified layers. Thus, colocalization of E4 and L1 in the upper spinous cells also observed. In fact, a bicistronic mRNA encodes both E1^E4 and L1 proteins (18, 30).

It is still unclear what role E1^E4 plays in the HPV reproductive cycle. E1^E4 is thought to facilitate release of newly formed viral particles by interacting with the cytoskeleton, thereby weakening the cornified cell envelope as well as inducing cell cycle arrest to facilitate viral replication (17). Cdk1/cyclin B is necessary for G2/M phase transition. In G2, cyclin B forms a complex with Cdk1, but is kept inactive via inhibitory phosphorylation, keeping it primarily in the cytoplasm. To initiate M phase the Cdk1/cyclin B complex is dephosphorylated and translocated into the nucleus. When over-expressed in cell lines from a strong surrogate promoter, E1^E4 of several HPV types can sequester cyclin B/cdk1 onto cytokeratin filaments, leading to G2/M arrest (37, 38, 101). Also in cell lines, HPV-1 E1^E4 interacts with the cellular protein Wee1, which phosphorylates Cdk1, to inhibit its activity (75).

The role which E1^E4 plays in viral genome amplification and induction of S-phase re-entry in infected cells has been controversial (49, 146, 147). In HPV-31, E1^E4 is necessary for efficient viral DNA amplification and was needed to induce S-phase reentry in suprabasal cells (113). Additionally, mutations that prevent the production of full length E1^E4 decreased the amount of late transcripts. Work with HPV-18 similarly showed that deletion of E1^E4 decreased the efficiency of viral DNA amplification and late gene expression. However, unlike HPV-31, deletion of E1^E4 did not inhibit S-phase re-entry of the suprabasal cells (41, 56). In contrast to both HPV-18 and HPV-31, work on HPV-11 showed the E1^E4 was not necessary for viral genome amplification,

and deletion of E1^E4 did not affect the levels of late gene production, indicating that the role of E1^E4 was after DNA amplification and coordinate with the expression of the late genes (49). There are several possible reasons for the different roles of E1^E4 in HPV-11 versus that of HPV-31 and HPV-18. These studies were conducted in immortalized cell lines, where wild type HPV DNA amplification and late gene expression are relatively limited, compromising the analyses of mutants.

E5

The E5 protein is a hydrophobic, trans-membrane protein found in the plasma membrane, Golgi, and endoplasmic reticulum. It associates with the growth factor receptors and causes MAP kinase (Erk1/2) activation that accelerates cell cycling (34, 74, 135). It has transforming activity in NIH 3T3 cells and can enhance the efficiency and immortalization functions of the HR HPV E6 and E7 proteins (93, 133, 138). When expressed in the basal cells of stratified squamous epithelia of mice, HPV-16 E5 induced epidermal hyperplasia and spontaneous skin tumors (96). Unlike HPVs for which the E6 and E7 are the main oncogenes, the BPV-1 E5 protein is the main oncogene (51, 60). BPV-1 E5 targets the platelet-derived growth factor (PDGF) pathway by binding to two PDGF β receptors (103). This complex then allows the receptors to autophosphorylate, thereby activating the PDGF signaling pathway (103).

In addition to its role in promoting cell proliferation, HPV-16 E5, as well as the E5 proteins of BPV-1, BPV-4, HPV-6 and HPV-2, also down-regulates cell surface expression of HLA-1 (5, 7, 9, 10, 19, 62, 92). HLA-1, also known as MHC-1, is a major cell surface molecule, presenting foreign antigens to the immune system to trigger immune response. The ability and mechanism of the E5 protein to down-regulate MHC-

1 expression varies among papillomavirus types. Each of the E5 proteins interferes with MHC-1 transport by retaining the MHC-1 molecule in the Golgi appartatus (7-10, 91). BPV-1 and BPV-4 down-regulate MHC-1 gene transcription and protein stability (7, 91). HPV-16 E5 is also thought to interact with calnexin (62), which is a chaperone that plays a major role in HLA-I maturation and surface transport. The interaction of HPV-16 E5 leads to an E5-calnexin-HLA-1 ternary complex that is unable to be further transported to the cell surface (62). The absence of peptide presentation by MHC I to cytotoxic T lymphocytes would allow the infected cells to evade the host cellular immune response and allow the lesions to persist or progress.

E6

E6 is one of the two major oncoproteins of HPVs. It is a 151 amino acid protein with two zinc finger domains. Alone, the E6 protein is unable to immortalize primary human foreskin keratinocytes; however, E6 is sufficient to immortalize human mammary epithelial cells (88). One of the major roles of E6 of the high-risk virus is the inactivation of p53 (121). p53 is a major tumor suppressor protein responsible for inhibiting the cell cycle phase transition following DNA damage (66). If the cell is unable to repair the DNA damage, then p53 induces caspase-mediated apoptosis (66). The HR HPV E6 prevents p53 activity in two ways. Schenffner et al. (121) demonstrated that E6 complexes with E6-AP act as the ubiquitin ligase for p53, promoting its degradation. E6 has prevents p53 from binding to its target sequences (64, 109, 157). In contrast, in vitro studies have shown that the LR HPV E6 does not have any effect on p53 degradation. Both HR and LR E6 can inactivate p53- targeted gene transcription by preventing p53 acetylation by p300, a histone acetlyase (140).

HR E6 protein also interacts with several other cellular proteins. The binding of HR E6 to its targets depends on two binding domains. To prevent apoptosis, HR E6 binds to the pro-apoptotic protein Bak and mediates its degradation via the proteasomal pathway (57). The association of E6 with Bak occurs in association with the E6-AP protein, much like E6 interactions with p53 (57). The E6-AP binding motif of the HR E6 is a seven-residue leucine-containing motif, LQELLGE; this consensus sequence has also been referred to as an LXXLL motif. Mutational analysis of this motif revealed the importance of the three highly conserved leucine residues, with substitutions of the leucines abolishing the ability of E6 to interact with E6-AP (76, 121).

The HR HPV E6 also plays a critical role in activating telomerase in infected cells. Upon E6 expression, E6/E6AP binds to and ubiquitinates NFX1-91 (59). NFX1-91 is a transcriptional repressor that is a key component in regulating the expression of the hTERT, the catalytic subunit of telomerase (59, 73). In normal cells NFX1-91 binds to the promoter of hTERT and recruits the deacetylase complex mSIN3A/HDAC to maintain the hTERT promoter in the repressed state (59). The absence of NFX-1 prevents the recruitment of the mSIN3A/HDAC to the hTERT promoter and allows for activation of the hTERT gene (59). Over-expression of hTERT allows the telomere ends to be maintained, thereby allowing the cells to avoid replicative senescence and become immortalized (73).

The carboxyl terminus of HR E6 associates with PDZ domains of many host proteins, including hScrib and hDlg (81, 99, 139). PDZ domains are approximately 90 amino acids in length and are found in a variety of signaling molecules (79). hScrib is associated with epithelial tight junctions and its loss is thought to prevent cell attachment

to the extracellular matrix. hDLG is responsible for maintaining cell-cell junctions and cellular polarity; its loss is thought to abolish cell polarity and inhibit cellular proliferation control (79).

In HR but not in LR HPVs, the majority of the transcripts spanning E6 have an intragenic or intergenic splice, abolishing the ability to encode the full-length E6 protein. In its place, truncated variants of E6, termed E6*I-E6*IV, are produced (46, 118). The significance of the E6* splices in the viral cycle is not clearly understood. Clearly, it reduces the amount of the E6 protein (63). The intragenic splice may facilitate translation of the downstream E7 and E1 proteins in the polycistronic mRNA protein (137). The E6* peptides may have functions. Unlike E6, which is steady during the cell cycle, expression levels of E6*I varies during the cell cycle. HPV-18 E6*I levels peak during G2/M (63). Over-expression of E6*I inhibits the ability of E6 to degrade p53, presumably by competitive interference with the interaction of E6 with E6AP (112).

E7

E7 is the other major oncogene expressed by HPVs. The HR HPV E7 protein binds to and destabilizes the major tumor suppressor protein, the retinoblastoma susceptibility protein (pRB). As with E6 interactions with p53, the affinity of the E7 protein of the low risk viruses is much lower than that of the high risk viruses and hence destabilizes pRB very inefficiently. This difference may be a reason why infections with the low risk viruses do not progress to high grade neoplasias and carcinoma, whereas infections with the high risk viruses can. Together the E6 and E7 proteins of the high risk viruses can immortalize primary human keratinocytes (PHKs) efficiently. Thus, it is necessary for the virus to tightly regulate the expression of its oncogenes.

It has long been thought that the primary method for inducing S-phase reentry was through the interactions of the viral E7 protein with the cellular pRB protein (24, 116). It had been unclear how the low risk viruses were able to induce S-phase re-entry in differentiated keratinocytes, since the E7 of low risk viruses are inefficient in degrading pRB (116).

Genovese et al. (2008) showed that, in normal epithelia, p130 is detected in the quiescent basal cells and in the differentiated suprabasal kerartinocytes, whereas the pRB is only detected in the proliferating parabasal cells (58). p130 is a member of the RB family and like pRb (p105) targets E2F to regulate the cell cycle, prevent S phase entry, and thereby block cellular proliferation. (106) In contrast, in HPV-infected benign lesions, p130 was also destabilized. Interestingly, in HPV-11 infected papillomatous tissues, pRB was elevated in the several layers of transit amplifying cells (11). Experimentally, both HR and LR HPV E7 destabilize p130 in post-mitotic, differentiated cells in organotypic raft cultures of primary human keratinocytes (PHKs); this ability is necessary for S phase reentry in these cells (58). The region of E7 that is responsible for p130 destabilization is localized to the pocket protein binding domain. The binding of p130 by E7 is greatly enhanced by casein kinase II (CKII) phosphorylation of serine residues in close proximity to the pocket protein binding motif (58). Based on this work, it is now thought that p130 is the key protein which prevents S-phase reentry in post-mitotic differentiated keratinocytes and that both the HR and LR HPVs induce S-phase re-entry by degrading p130 (58).

L1 and L2

L1 is the major capsid protein of papillomaviruses and its expression occurs only in the late stage of the viral life cycle. L2 is the minor capsid protein of HPV. HPV-33 L2 protein regulates and reorganises PML (53). The L1 protein can spontaneously self-assemble to form virus-like particles (VLP), which are very similar to the native virions (122). The formation of infectious viral particles requires both L1 and L2. L2 is not required for capsid formation; however, when co-expressed with L1 in the presence of a viral genome, L2 enhances the efficiency of viral DNA encapsidation (105). Aside from its role in mediating viral DNA packaging, the L2 protein is necessary for successful viral infection. It is thought that escape from the endosomal compartment by papillomavirions is mediated by cleavage of the L2 protein at its N-terminal end by furin, a cellular proprotein convertase (39).

One difficulty in generating effective HPV vaccines is that antibodies to antigenic linear L1 epitopes are non-neutralizing. Due to the ability of the L1 protein to self-assemble into VLPs (122), there have been intensive efforts to utilize them to produce prophylactic vaccines against HPV infections. Currently, Merck Vaccines is marketing a quadrivalent vaccine using VLPs of HPV-6, -11, -16, and -18, and GSK is marketing a bivalent vaccines incorporating HPV-16 and -18 VLPs. These vaccines have been highly effective at preventing the infections for 6 years and counting.

Productive HPV Infections and Oncogenesis

Epidemiological studies indicate that as much as 50-85% of the population harbors some type of mucosotropic HPV; however the vast majority of HPV infections are latent, showing few if any clinical symptoms (160). In infected tissues, viral DNA

normally replicates as extra-chromosomal nuclear plasmids. Papillomavirus initiates its reproductive program by infecting the basal level of the squamous epithelium after wounding. In the basal cells, the virus initiates a low level of transcription of early genes to establish infection, specifically expressing the E1 and E2 proteins that are required for genome amplification, and E6, E7 and E5 that promote short term expansion of infected cells (160). After wound healing is over, the basal cells likely return to quiescence and only E1 and E2 are expressed to maintain the viral genome copies when the basal cells periodically divide. The productive program takes place in a differentiation-dependent manner. Amplified viral DNA and abundant viral RNA are only detected in the mid and upper spinous cells, whereas the progeny are assembled only in the superficial cells just before they undergo programmed cell death. Virion release occurs as the stratum corneum sloughs off from the epithelium (160).

In high grade dysplasias and in carcinomas, the HR HPV DNA is often found integrated into the host chromosomes and progeny viruses are no longer produced. Integration followed by selection of surviving cells occur such that the E2 expression has been disrupted and E6 and E7 transcription is no longer regulated. Unregulated viral oncoprotein expression can then lead to progression to high grade dysplasia and carcinomas. All cervical carcinoma cell lines, such as CaSki, HeLa, and SiHa, continue to express abundant E6 and E7 proteins. Ectopic over-expression of E2 is able to induce senescence or apoptosis in these cells by repressing transcription of the E6/E7 message.

Viral DNA Replication

HPV DNA replication requires viral encoded E1 and E2 proteins, the origin sequences in a supercoiled template, reactivation of the synthesis of deoxyribonucleoside

triphosphate substrates, the host replication machinery, ATP, plus an active redox system (26) . Because of the highly conserved sequences and structures of papillomavirus E1 and E2 proteins and the origin of replication, matched pairs and certain mixed pairs of viral proteins can replicate all papillomavirus ori-containing plasmids, albeit with different efficiencies. Two basic assays have been developed in our laboratory as well as in the labs of other investigators to examine the mechanisms of HPV DNA replication. The first is a transient replication assay in transiently transfected cells. This assay entails transfecting the E1 and E2 expression plasmids plus an origin plasmid into the 293 cells. 48 hours post-transfection, low molecular weight DNA is harvested by alkaline lysis and the plasmids are linearized with a single-cut restriction enzyme. The restriction enzyme DpnI is used to fragment the input DNA in methylated sites in DNA that originated from *E. coli*. Plasmids newly replicated in eukaryotic cells are resistant to Dpn I digestion because they are not methylated at the Dpn I sites. The replicated DNA is then detected by Southern hybridization.

The second test system is a cell-free replication assay (21, 28). This is performed by using cellular extracts from human 293 cells, the HPV-11 ori plasmid, E2 and E1 proteins expressed in and purified from *E. coli* or from insect sf9c cells. The production of replication intermediates and full-length products is then determined upon autoradiography of ^{32}P-α-dXTP incorporated into products after separation by agarose gel electrophoresis (28, 77).

Using these methods, the mechanism of HPV replication has been partially elucidated. To initiate replication, the viral E2 protein binds to the viral ori at its cognate binding sites. Once bound to the ori, the E2 protein recruits the E1 helicase to its cognate

binding sites. In the absence of E2, there is little or no ori-dependent replication (21, 22, 85). Once bound to its binding site, E1 then recruits a number of cellular factors that are necessary for viral replication, including DNA polymerase (pol) α/primase, RPA and topoisomerase I. In addition, DNA polymerase δ and PCNA (the processivity factor or sliding clamp for DNA pol δ), RFC (the PCNA clamp loader), and topoisomerase II are also necessary (4, 32, 94, 108). The E2 protein is required only for the formation of the pre-intiation replication complex; once E1 has recruited its cellular partners, the E2 protein disassociates from the complex.

It has been well established, at the tissue level, that productive viral DNA replication occurs in differentiated squamous cells. However, it is not clear, at the cellular level, the precise location where viral DNA replication takes place. Work on both BPV-1 and HPV-11 E2 proteins indicates that E2 forms distinct nuclear foci that colocalize with the promyelocytic leukemia protein (PML), which is a major component of ND10 bodies (100, 136). In the case of BPV-1 E2, the localization to PML bodies is due to the presence of the minor capsid protein L2 (52, 100). Furthermore, work on HPV-1 indicates that the E4 protein, which was shown to interact with cellular keratin, induces relocalization of PML bodies to the periphery of nuclear E4 inclusions in productively infected primary keratinocytes (115). Other viruses, such as herpes simplex virus 1, human cytomegalovirus, and adenovirus, have also been shown to localize with PML bodies for efficient viral establishment and viral genome amplification (47, 48, 129). Taken together the data are suggestive that papillomavirus may also associate with the PML bodies for efficient viral genome amplification.

MRN complex

The Mre11-Rad50-Nbs1 (MRN) complex plays a critical role in repair of double-stranded DNA breaks (DSBs). Several DNA viruses have evolved efficient mechanisms to inhibit and/or relocalize components of the MRN, presumably to promote efficient viral DNA replication. Adenovirus encodes three proteins that are able to interfere with the MRN complex. The E4orf3 re-localizes both Mre11 and Rad50 away from replicating viral genomes; however, Nbs1 was found to localize with the replicating centers. A complex of E4orf6–E1b55K targets the MRN complex for proteasome-mediated degradation. In the absence of MRN relocalization and degradation, the free ends of the linear viral genomes are concatomerized and are unable to be packaged (47, 87, 95, 123 , 124). In SV40, the T antigen interacts with ubiquitin ligase CUL7. This interaction is required for SV40-mediated cell transformation as well being necessary for degradation of the MRN complex. Whether CUL7 directly ubiquitinates MRN is still unknown (156). Our lab has recently found evidence that, in the presence of HPV-11, both Mre11 and Nbs1 relocalize to the replication center; the implications of this relocalization is currently being investigated.

Double-stranded breaks (DSBs) pose great risk to the genomic stability of cells. They can arise spontaneously during replication or be induced by a number of genotoxic agents. The detection of DSBs leads to arrest of cell cycle progression, either through G1/S phase arrest, S phase arrest, or G2/M phase arrest. To respond to and repair DSBs, all organisms have evolved mechanisms to recognize and repair the damage. Repair can be either through homologous recombination between sister chromatids or non-homologous end joining, where the broken ends are joined together. MRN is among the

earliest respondents to DSBs, and MRN mutations cause the human cancer predisposition diseases Nijmegen breakage syndrome and ataxia telangiectasia-like disorder (ATLD). The complex has four critical functions during the repair of double-stranded DNA breaks. First, the complex senses the presence of DSBs and is localized to the breaks to stabilize the broken ends. The complex signals to the cell that damage has occurred and prevents the cell from entering mitosis. Finally the complex serves as scaffolding to repair the broken DNA. To organize the MRN complex, the Mre11 exonuclease directly binds Nbs1, DNA, and Rad50. Once formed, the complex then initiates repair of the damaged DNA (6, 15, 35, 141, 143, 145).

Mre11 is a 708 amino acid protein with three major motifs, a phosphoesterase motif and two DNA binding motifs. It is responsible for binding to the broken DNA as well as forming a complex with Rad50 and Nbs1. The dimerization domain and the DNA binding domain have been mapped to the C-terminus of the protein, whereas the Rad50 interaction domain is located within the amino terminus of the protein. The multifunctional protein is a double-strand specific 3'-5' exonuclease and single-stranded DNA endonuclease, and it promotes DNA annealing and DNA unwinding. The exonuclease activity is Mn^{2+} dependent and the catalytically active site is located within the amino terminus. Point mutations of the Mn^{2+} binding region abolished the catalytic function of the protein but not the ability of the protein to bind to DNA, indicating that DNA binding and nuclease activity are two separate functions (6, 35).

The Rad50 protein is a 1,312 amino acid protein, composed of Walker A and Walker B motifs, a CXXC motif, and two coiled-coiled domains. It has ATP binding domains, which promote binding of Mre11 to the 3' overhangs of broken DNA. Current

models suggest that each Rad50 polypeptide bends upon itself, with the Walker A and Walker B motifs binding to DNA and Mre11, while the CXXC motif binds to another Rad50 protein. The association of the Rad50 polypeptides bridges the broken DNA ends and possibly sister chromatids for homologous recombination repair. Additionally, ATP binding and hydrolysis by Rad50 induces conformational changes in the MRN complex (143, 155).

The final component of the MRN complex is Nbs1, a 754 amino acid protein. As with Mre11 and Rad50, it is highly conserved in all eukaryotic cells. Also as most cell cycle check point proteins, it contains two domains that are associated with DNA damage response proteins: a fork-head associated (FHA) domain and a BRCA1 carboxyl-terminal (BRCT) domain, both at its amino terminus. At the carboxyl terminus, Nbs1 has an Mre11 binding motif as well as an ATM interaction domain (145). One consequence of MRN activity is the activation of ATM, which plays a central role in regulating cellular response to DNA damage (155). Upon detection and localization of MRN to the site of DNA damage, Nbs1 recruits ATM through the HEAT repeat domains of ATM. Upon recruitment to the DNA lesion, ATM becomes active through autophosphorylation.

Centrosomes and Mitotic Spindles

To establish viral persistence, the E2 various associates with mitotic chromosomes or various components of the mitotic apparatus, including the mitotic spindles as described previously (36, 144). During interphase, the microtubule fibers, composed of α and β tubulin heterodimers, serve as the cytoskeletal network of the cell. This network maintains the cell shape and serves as a highway for trafficking of cellular proteins. Two molecular motor proteins, kinesin and dynein, use the cytoskeletal

network for intracellular trafficking. Kinesin is an ATP-driven microtubule-based motor which is responsible the plus-end directed movement, from the center of the cell to the periphery. Dynein is also an ATP-driven microtubulue-based motor and is responsible for minus-end directed movement, from the periphery to the center of the cell (68). Dynein-mediated trafficking has been implicated in the activation of epidermal growth factor receptor signaling in *Drosophila* by interacting with the EGF receptor ligand, Spitz. Spitz ligand is produced as an inactive transmembrane precursor and is transported by dynein from the ER to the site of proteolytic cleavage in the Golgi or endosomal compartment for activation (117).

During mitosis, the mictrotubules reorganize into mitotic spindles. The role of the mitotic spindles is to capture the condensed chromosomes at the kinetochore. The spindles/microtubules are polar, with a plus end and a minus end. The minus end is anchored to the centrosome and the plus end projects outward. During both interphase and mitosis the microtubule ends are constantly in flux between active polymerization and depolymerization. During mitosis the plus end undergoes high rates of polymerization, which is regulated by several microtubule-associated proteins (MAPS), which help to control the dynamic instability of the microtubules (78, 90)}. Dynamic instability is thought to provide microtubules with the ability to search three-dimensional space more effectively than would be possible for a simple equilibrium polymer (69). Consistent with this idea is the observation that microtubule turnover increases dramatically during mitosis relative to interphase (120). This increase in microtubule turnover probably plays a crucial role in the assembly of the mitotic spindle (72).

The centrosome serves as the major microtubule organization center (MTOC); MTOCs serves as the site of nucleation for the microtubules and the mitotic spindles during interphase and mitosis, respectively. Microtubules and mitotic spindles are polymers of α and β tubulin dimers and grow or shrink in a GDP/GTP driven mechanism. During interphase all cells contains a single centrosome, consisting of two cylindrical centrioles, arranged perpendicularly to each other. The centrioles are surrounded by pericentriolar material, which contains many of the transient centrosomal interacting proteins. Studies of the centrioles show that they contain well over 100 polypeptides, including γ tubulin, centrin, cenexin, and tektin (42, 104).

Centrosomal replication is intimately linked to the cell cycle, with many of the same proteins necessary for cell cycle progression similarly necessary for centrosomal replication. The centrosome first begins to duplicate at the G1/S phase transition and is regulated by Cdk2/cyclin E for G1/S transition and by Cdk2/cyclin A for centrosomal replication. At the beginning of S-phase, each centriole duplicates itself resulting in two new centrosomes, each consisting of a mother and daughter centriole. During prophase the newly replicated centrosomes migrate to the opposite sides of the cells to function as poles for the mitotic spindles that pull mitotoic chromosomes apart. As mitosis progresses the centrosomes serves as the nucleation site for the formation of the mitotic spindle fibers (3, 104).

Questions Addressed in This Dissertation

This project addresses two main issues. The first deals with how papillomaviruses are able to stably maintain their genome in actively dividing basal cells of the epithelial in the absence of high level viral genome amplification. The second issue investigates the role of the DSB repair complex MRN in viral DNA replication.

Our lab had previously reported the localization of the E2 protein with the centrosomes and the mitotic spindles (142). We hypothesized that the association of the E2 protein with the spindles serves as the mechanism for maintenance of the viral genome. Using a tracking plasmid we were able to show that a plasmid containing the viral ori associated with the mitotic spindles in the presence of the E2 protein (142). In this dissertation, I report our efforts to further investigate this mechanism by identifying the domain on E2 that is responsible for its interaction with the mitotic spindles (36).

Several DNA viruses have been reported to interact with the DSB repair complex MRN. Adenovirus encodes two proteins; one which degrades Mre11 and another which induces relocalization of Nbs1 to the replication centers (47, 121, 122). It is thought that the degradation of Mre11 prevents concatermerization of the loose ends, though the role of Nbs1 relocalization is not well understood. SV40 induces relocalization of both Mre11 and Nbs1 to the replication centers as well as inducing degradation of both Mre11 and Nbs1 (154). It is thought that the relocalization is in response the appearance of DSBs in the viral DNA, during replication. In this dissertation I report our studies of the interactions of the MRN complex with the HPV-11 viral replication centers.

Dynamic Localization of the HPV-11 Origin Binding Protein E2 Through Mitosis While in Association with the Spindle Apparatus

by

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Chapter 2

Dynamic Localization of the HPV-11 Origin Binding Protein E2 Through Mitosis While in Association with the Spindle Apparatus

Abstract

Papillomaviral DNA replicates as extrachromosomal plasmids in squamous epithelium. Viral DNA must segregate equitably into daughter cells to persist in dividing basal/parabasal cells. We have previously reported that the viral origin binding protein E2 of human papillomavirus (HPV) type 11, 16, and 18 colocalized with the mitotic spindles. In this study, we show the localization of the HPV-11 E2 protein to be dynamic. It colocalized with the mitotic spindles during prophase and metaphase. At anaphase, it began to migrate to the central spindle microtubules where they remained through telophase and cytokinesis. It was additionally observed in the midbody at cytokinesis. A peptide spanning residues 285-308 in the carboxyl terminal domain HPV-11 E2 (E2C) is necessary and sufficient to confer localization to the mitotic spindles. This region is conserved in HPV-11, 16, 18 and BPV-4 E2 and is also required for the respective E2C to colocalize with the mitotic spindles. The E2 protein of the bovine papillomavirus type 1 is tethered to the mitotic chromosomes via the cellular protein Brd4. However, the HPV-11 E2 protein did not associate with Brd4 during mitosis. Lastly, a chimeric BPV-1 E2C containing the spindle-localization domain from HPV-11 E2C gained the ability to localize to the mitotic spindles, whereas the reciprocal chimera

lost the ability. We conclude that this region of HPV E2C is critical for localization with the mitotic apparatus, enabling the HPV DNA to establish persistence.

INTRODUCTION

Human papillomaviruses (HPV) cause epithelial proliferative diseases, such as cutaneous and anogenital warts and laryngeal papillomas. Infections by the high-risk genotypes, notably HPV-16 and HPV-18, can progress over time to cancers at a low frequency, whereas the low-risk types, such as HPV-11 and HPV-6 rarely cause cancer (60). The productive phase of the infection takes place only in suprabasal cells undergoing terminal squamous differentiation. But the initial viral infection is thought to occur in the basal or parabasal cells of the squamous epithelium, where the viral DNA is maintained as extrachromosomal nuclear plasmids in low copy numbers over long periods of time (13). To persist in the basal cells, HPV must ensure equitable segregation of its DNA into daughter cells during cell division. For HPV, the E2 protein is responsible for DNA segregation (48). The viral E2 protein of approximately 42 kDa is also critical for the assembly of the preinitiation complex on the viral origin of replication (ori) (11, 29, 47, 54). It is the origin recognition protein, it binds as dimers to multiple E2 binding sites in the ori, and recruits the viral E1 protein, which assembles into a dihexameric replicative DNA helicase (14, 19, 24, 31, 42, 43, 45, 46, 55). In turn, the E1 complex unwinds the ori and recruits the DNA polymerase α, the topoisomerase I, and the single-stranded DNA binding protein RPA to initiate replication (7, 15, 16, 33, 36, 39).

The E2 protein consists of three domains, an amino terminal *trans*-acting domain (abbreviated hereafter as the N domain), a flexible hinge (H) domain, and a carboxyl terminal (C) domain. Both the N domain of approximately 200 amino acids and the C domain of approximately 90 residues are relatively conserved in all papillomaviruses

while the H domain is variable in length and sequence. In HPV-11 E2, the H domain contains nuclear localization sequence and also associates with nuclear matrix (59). The E2N interacts with the helicase domain of the E1 protein; E2 protein lacking the N domain does not support HPV DNA replication (1, 2, 12, 32, 34). The E2C domain is responsible for dimerization and binding to the palindromic, consensus E2BS in the ori. The crystal structures of the dimeric C domains of several papillomaviruses have been elucidated (8, 20, 21). The dimer interphase contains an eight stranded β-barrel flanking two DNA binding α-helices, one from each monomer (See Fig. 2C).

Using fusion proteins to the enhanced green fluorescence protein (GFP), we have previously shown that the E2 proteins of HPV-11, -16 and -18 localize to the mitotic spindles (48). An epitope tagged, inducible HPV-11 E2 protein in a 293 cell line, 293 (11-E2), exhibits similar properties. Moreover, in these cells, a plasmid containing the HPV-11 ori is also localized to mitotic spindle fibers at metaphase, but only when the E2 protein is induced. Thus, this colocalization provides a mechanism for viral plasmid persistence in dividing basal and parabasal cells. Unlike HPV E2 proteins, the bovine papillomavirus type 1 (BPV-1) E2 protein associates with the mitotic chromosomes via its interactions with the cellular protein Brd4 to facilitate viral DNA segregation (25, 30, 44, 56). The region for this interaction has been localized to the N domain (4, 57). Association with the mitotic chromosomes as a mechanism for viral DNA segregation has previously been demonstrated for the Epstein-Barr virus, which uses the viral encoded nuclear antigen 1 protein, and the human herpesvirus 8, which uses the latency-associated nuclear antigen 1 protein (3, 28, 53)

For the HPV-11 E2 protein, both the C and N domains each independently associate with the mitotic spindles (48). In this work, we show that the localization of HPV-11 E2 is dynamic through different phases of mitosis and that it does not associate with Brd4. In addition, we delineate the segment in the HPV-11 E2C domain critical for mitotic spindle localization to a segment spanning residues 285-308. This spindle-localization motif is highly conserved in the C domain of the HPV-16, HPV-18, and BPV-4 E2 proteins. Deletion analyses demonstrated that this segment is also necessary for their spindle locazliation as well. Reciprocal chimeric E2C proteins of HPV-11 and BPV-1 supported this conclusion. We discuss the implication of these findings.

MATERIALS AND METHODS

Cell cultures. COS7 cells were maintained in DMEM supplemented with 10% FBS. Transfection was conducted by electroporation or lipofection (Invitrogen, Carlsbad, CA) as described (12). The 293(11-E2) tetracycline inducible cells were maintained in DMEM plus 10% FBS in the presence of 1 μg/ml tetracycline to repress E2 expression (48). Induction was achieved by culturing overnight in media containing 0.1 μg/ml tetracycline.

Plasmid construction and mutagenesis. pGFP-H11 E2C (spanning amino acid residues 285-367) was described previously (48). Plasmids expressing GFP fusions to E2C proteins of HPV-16 (pGFP-H16 E2C, residues 287-365), HPV-18 (pGFP-H18 E2C, residues 288-365), BPV-4 (pGFP-B4 E2C, residues 327-408), and BPV-1 (pGFP-B1 E2C, residues 327-410), as well as to deletion mutation of E2C proteins of these four viruses, pGFP-H11 E2C:285-308, pGFP-H11 E2C:285-318, pGFP-H11 E2C:285-328,

pGFP-H11 E2C:285-338, pGFP-H11 E2C:285-348, pGFP-H11 E2C:285-358, pGFP-H11 E2C:295-367, pGFP-H16 E2C:296-365, pGFP-H18 E2C:297-365, and pGFP-B4 E2C:338-408, were each constructed by PCR amplification of the appropriate E2 fragments and placed in frame into the *Bgl*II and *EcoR1* sites of the pEGFP-C1 plasmid (Clontech, Mountain View, CA). pGFP-H11 E2H+:202-295, which extends the HPV-11 E2H (residues 202-284) to include the first 11 residues of the C domain was similarly prepared. The plasmids expressing the GFP fusion of the point mutations in HPV-11 E2HC (residues 285-367), namely, pGFP-H11 E2HC:V287A, pGFP-H11 E2HC:Q288A, pGPF-H11 E2HC:L289A, pGFP-H11 E2HC:Q290A, and pGFP-H11 E2HC:S293A, were constructed using PCR mutagenesis. pGFP-H11 E2HC:A7 contained alanine residues at positions 287 through 293. All the above clones, except pGFP-11H+:202-295, HPV-11E2C:285-308, and HPV-11E2C:295-367, contained a STR tag at the carboxyl terminus as previously described (48). Expression vectors of chimeric E2C were generated by PCR mutagenesis. For GFP fusion to HPV-11/BPV-1 E2C (pGFP-H11/B1 E2C), the forward primer incorporated nucleotides encoding amino acid residues 285-295 of HPV-11 E2, replacing the corresponding sequence of BPV-1 E2C. The reciprocal chimera pGFP-B1/H11 E2C was constructed using a forward primer that contained nucleotides encoding amino acid residues 327-337 of BPV-1 E2 followed by nucleotides encoding HPV-11 E2C. GFP-Brd4 was generated by PCR. The Brd4 coding region was amplified using a forward primer GAATTCGTCTACCGAGAGCGGC, containing an EcoR1 restriction site, and a reverse primer GGATCCTCAAAAAAGATTTTCTTCAAATATTG, containing a BamH1 restriction

site. The PCR product was purified, digested with EcoR1 and BamH1 and then inserted in frame into the pEGFP-C1 vector (Clontech, Mountain View, CA) at the same sites.

Western blots. COS7 cells separately transfected with expression vectors of various pGFP-E2C fusion proteins were grown for 24 h and lysed with RIPA buffer (0.15 mM NaCl, 0.05 mM Tris-HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Sigma, St Louis, MO). Equal amounts of total proteins were separated on a 4–12% polyacrylamide/SDS gel (Fig. 2A) or on a 10% SDS/PAGE (Figs. 2B, 3C, 4C and 5B), and transferred to a nitrocellulose membrane for western blot. The nitrocellulose membranes were probed with a monoclonal antibody specific for GFP followed by an anti-mouse IgG antibody conjugated with peroxidase and developed with an enhanced chemiluminescence detection system (ECL; Amersham Pharmacia, Piscataway, NJ).

Fluorescence microscopy. For pre-extracted slides, the cells on multi-chamber slides (LabTek, Campbell, CA) were permeablized for 30s at room temperature with a microtubule stabilizing buffer, MTSB (BRB80, 80 mM PIPES pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) supplemented with 4 mM EGTA and 0.5% Triton X-100, to prevent degradation of the spindle fibers (51). The slides were then fixed in 4% paraformaldehyde for 10 minutes at room temperature. To stain for the mitotic spindles the slides were incubated with a Cy3-conjugted primary antibody to β-tubulin (Sigma, St Louis, MO) at 1:100 dilution for 1 h at 37°C. For immunofluoresence using the 293(11-E2) Tet-off cell line, the induced or uninduced cells were prepared as described above, following extraction and fixation, E2 was stained using a rabbit polyclonal anti-E2 antibody (1:100 dilution) (10) at 37°C for 1 hour. After incubation, slides were washed

and incubated with a 1:750 dilution of anti-rabbit Alexfluor 488 (Molecular probes, Portland, OR) and tubulin was stained as just described. Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI), and mounted in antifade (0.1 mM p-phenylenediamine in 100% glycerol, pH was adjusted to 8.0 with a carbonate buffer containing 4 mM Na_2CO_3 and 46 mM $NaHCO_3$). Alternatively, the FLAG-tagged E2 was detected with a monoclonal anti-FLAG M2 antibody (1:500) (Sigma, St Louis, MO) by incubation at 37°C for 1 hour. The slides were then washed, incubated with a 1:750 dilution of anti-mouse Alexfluor 488 (Molecular probes, Portland, OR) at at 37°C for 1 hour, and then washed 3x with PBS + 0.1% Tween-20 for 5 minutes each. Mitotic spindles were stained with a Cy3-conjugted primary antibody to β-tubulin(1:10) (Sigma) for 10 minutes. Nuclei were stained with DAPI, and mounted in antifade. For colocalization studies with GFP-Brd4, GFP-Brd4 was transfected into the 293(11-E2) cells. Expression of the E2 protein was induced by culturing the cells in tetracycline-free media overnight. The cells were permeabilized and fixed in 4% paraformaldehyde with 0.5% Triton X-100. Mitotic spindles were stained with Cy3-conjugted primary antibody to β-tubulin as just described. Alternatively, the slides were incubated as described with the E2 primary antibody followed by a 1:1000 dilution of TRITC-conjugated anti-rabbit antibody (Sigma, St Louis, MO) for 1 h at 37°C. The slides were washed and mounted in Antifade after staining the nuclei with DAPI. All images were acquired with a 100x objective lens with an Olympus AX70 fluorescence microscope with Speicher filters (Chroma, Rockingham, VT) and a Zeiss Axiocam digital camera (Thornwood, NY). Processing and assembly were accomplished with PHOTOSHOP (Adobe Systems, Mountain View, CA).

RESULTS

The localization of HPV-11 E2 is dynamic during mitosis and is independent of Brd4. To determine the localization through mitosis of the epitope-tagged, tet-regulatable HPV-11 E2 protein in the 293(11-E2) cell line, we extracted the uninduced or induced cells under conditions which stabilized the mitotic spindles. This procedure removed E2 protein which was not attached to any stable subcellular structures. The remaining E2 was clearly and easily visualized by staining with a polyclonal antibody (green fluor). Mitotic apparatus was visualized using antibody to β-tubulin (red fluor) and host chromosomes were stained with DAPI (blue). Uninduced cells yielded little or no E2 signal at any phase of the cell cycle (Fig. 1Aa and data not shown). Upon induction, the localization of E2 was dynamic through the different phases of mitosis but it remained colocalized with the mitotic spindles fibers. During prophase, E2 was localized to the aster fibers that extended from the centrosomes (Fig. 1Ab). At metaphase, the E2 protein was seen to colocalize with mitotic spindles as described previously (Fig. 1Ac). During anaphase, the E2 protein remained localized to the mitotic spindle fibers, which have started to pull the chromosomes towards the spindle poles. However we also began to see an increased concentration of the E2 protein in the mid plane of the cell where the central spindle microtubules assembled during progression to telophase (Fig. 1Ad). At late anaphase, telophase and cytokinesis, the E2 protein was primarily localized to the central spindle microtubules (Fig. 1Ae, f). At cytokinesis, the E2 protein was additionally observed in the midbody in some of the cells (Fig. 1Af). The E2 protein expressed in the 293 (11-E2) cell lines was tagged with the FLAG epitope at its amino terminus. To verify the observations made with the polyclonal E2 antibody, we repeated the

experiment by using a monoclonal FLAG M2 antibody. The dynamic distribution patterns observed were essentially identical to those seen with the polyclonal E2 antibody (compare Fig. 1B to 1A). In the uninduced cells, no FLAG antibody reactivity was detected (Fig. 1Ba).

Brd4 is a cellular protein with bromodomains. It binds to acetylated histones and coats the chromosomes in a diffused manner throughout the cell cycle (17). In the presence of the BPV-1 E2 protein, Brd4 colocalizes with E2 and forms distinct foci on the mitotic chromosomes (4, 56). To examine the localization of the HPV-11 E2 protein relative to Brd4 through mitosis, we generated a GFP fusion construct of Brd4 and analyzed its localization pattern in transiently transfected human 293(11-E2) cells. The cells were then probed either with the HPV-11 E2 antibody or with the β-tubulin antibody. With or without prior induction the HPV-11 E2 protein expression, GFP-Brd4 diffusely coated the mitotic chromosomes as described previously (35), while the mitotic spindles formed normally. We observed no Brd4 foci formation on the chromosomes during prophase, metaphase, anaphase, telophase, and cytokinesis (Fig. 1Ca, and data not shown). Fig. 1Ca is a set of images of an induced anaphase cell where the central spindle microtubules had clearly formed. As just described, the distribution of HPV-11 E2 protein was dynamic and remained localized to the spindle fibers (Fig. 1Cb, and data not shown). In this anaphase cell, the E2 protein was observed to concentrate at the central spindle microtubules. No colocalization of E2 and Brd4 was observed. Thus, we conclude that, during mitosis, HPV-11 E2 and Brd4 do not associate and do not influence each other's localization.

Residues 285-308 of HPV-11 E2 are necessary for localization to the mitotic spindles. To localize the region of HPV-11 E2C (residues 285-348) responsible for its colocalization with the mitotic spindles, we generated a series of carboxyl terminal truncations of GFP-H11E2C, yielding the following clones: GFP-H11 E2C:285-338, 285-328, 285-318, and 285-308. Each expressed the fusion protein of expected length in Western blots (Fig. 2A). The expression clones were each transfected into COS-7 cells. The cells were extracted with microtubule stabilizing buffer and stained for mitotic spindles using an anti-β-tubulin antibody as described above. Each of the truncated fusion proteins was seen on the mitotic apparatus, suggesting that the spindle-localization domain was within residues 285-308 (Fig. 2Ca and data not shown). As inferred from the crystal structure of the HPV-16 (Fig. 2D), residues 285-295 of HPV-11 E2 are expected to form a β-sheet with residues 296-308 constituting the DNA binding helix. We reasoned that the DNA binding helix is not likely to be involved in spindle localization as well. Thus, the peptide spanning residues 285-295 is a logical candidate. To test this hypothesis, we generated two additional fusion proteins. GFP-H11 E2C:295-367 deleted the first 11 residues of E2C. It no longer colocalized with the spindles fibers (Fig. 2Cb). HPV-11 E2H (spanning residues 201-284) does not colocalize with mitotic spindles (48). However, the extended hinge region (GFP-H11 E2H+:201-295) gained the ability to localize to the mitotic spindles (Fig. 2Cc). The expression of both fusion proteins was confirmed by Western blot analysis (Fig. 2B). Collectively, these results demonstrate that, for HPV-11 E2C, residues 285-308, possibly as short as residues 285-295, are necessary and sufficient for localization to the mitotic spindles.

Mutational analysis of the HPV-11 E2C spindle localization domain. We generated a number of single point mutations in HPV-11 E2HC (Q288A, L289A, Q290A, D292A, S293A, and N294A) as well as a clone containing 7 alanines (HPV-11 E2HC:A7) in the spindle localization motif delineated above, each as a GFP fusion protein (Fig 3A). We observed that none of the individual point mutations abrogated the ability of the fusion protein to colocalize with the mitotic spindles (Fig 3Ba,b and data not shown). In contrast, HPV-11 E2HC:A7 no longer colocalized with the mitotic spindles, confirming the importance of the region (Fig 3Bc). Western blot analysis confirmed that the mutated proteins were synthesized in transfected cells (Fig. 2C).

HPV-16 E2C, HPV-18 E2C and BPV-4 E2C colocalize with the mitotic spindles. The E2 proteins of HPV-16 and the HPV-18 colocalize with the mitotic spindles (48). The spindle localization domain of HPV-11 E2C is highly conserved among these three viruses (Fig. 4A). To test if the conserved region in HPV-16 E2C and HPV-18 E2C also mediates this localization, we generated GFP fusion constructs of the E2C domains of these HPVs as well as truncations that removed the segment spanning the putative mitotic spindle localization domain. Only the fusions with the full-length E2C proteins, H16 E2C:287-365 and H18 E2C:288-365, colocalized with the mitotic spindles (Fig. 4B, rows a, c), whereas the truncated fusion proteins H16 E2C:296-365 and H18 E2C 297-365 did not (Fig. 4B, rows b, d). Western blot showed that all 4 GFP-E2C fusion proteins were synthesized in the transfected cells (Fig. 4C).

Interestingly, the E2C of the bovine papillomavirus type 4 (BPV-4) also contains a sequence very similar to the spindle-localization domain of HPV E2C proteins (Fig. 4A). We were curious as to whether BPV-4 E2C also colocalizes with the mitotic apparatus.

To test this possibility, we generated GFP fusion constructs of BPV-4 E2C (pGFP-B4 E2C, residues 327-408) as well as a truncation BPV-4 E2C:336-408. The full-length E2C protein was able to colocalize with the mitotic spindles (Fig. 4B, row e), whereas the truncated form did not (row f). Western blot showed that both proteins were expressed (Fig. 4C). These observations were corroborated by using an epitope-tagged BPV-4 E2C protein (data not shown). Collectively, these results support the notion that BPV-4 E2C colocalizes with the mitotic spindles and that GFP had no role in this localization.

A chimeric HPV-11 and BPV-1 E2C colocalizes with the mitotic spindles. The N domain of BPV-1 E2 mediates the association with the mitotic chromosomes via the cellular protein Brd4, whereas the C domain is not involved (57). We constructed a GFP fusion to the E2C domain of BPV-1 (GFP-B1 E2C). Indeed, in the absence of extraction, it did not associate with either the mitotic spindles or the mitotic chromosomes but was diffusely distributed throughout the cell with a reduced intensity in areas occupied by the chromosomes due to an excluded volume effect (data no shown). Upon extraction, little GFP-B1 E2C remained in the cell (Fig. 5Ca). Sequence alignment indicates divergence in the HPV-11 E2C spindle-localization domain compared to the corresponding region in BPV-1 E2C (Fig. 4A). To examine if this difference contributed to the distinct localization patterns of the HPV-11 and BPV-1 E2C proteins, we generated two chimeric E2C proteins. In the GFP-H11/B1 E2C chimera, the first eleven amino acids from BPV-1 E2C were replaced with the spindle-localization domain of HPV-11 E2C. In the reciprocal chimera, GFP-B1/H11 E2C, the spindle- localization domain of HPV-11 E2C was replaced with the corresponding region from the BPV-1 E2C. Western blot confirmed the synthesis of GFP-BPV-1 E2C, GFP-H11/B1 E2C and GFP-B1/H11 E2C

(Fig. 5B). The chimeric proteins were analyzed for their ability to colocalize with the mitotic spindles in transfected cells after extraction to remove any unattached GFP-E2C protein. Chimera GFP-H11/B1 E2C was observed to colocalize with the mitotic spindle fibers (Fig. 5Cb), whereas the reciprocal chimera GFP-B1/H11 E2C (Fig. 5Cc) exhibited no discernable patterns of localization, similar to GFP-BPV-1 E2C (Fig. 5Ca). These experiments verify our conclusion that residues 285-295 of HPV-11 E2C are critical for mitotic spindle localization.

DISCUSSION

Throughout mitosis the mitotic spindles constantly dissociate and reform as the mitotic chromosomes are pulled/pushed towards the two poles and the cell divides into two at the mid plane. We have demonstrated that the distribution of the HPV ori binding protein E2, as revealed by either an antibody to E2 or an antibody to the FLAG epitope tag, is also dynamic and its localization pattern changes as the cell progresses through mitosis, but always seen in association with the spindle fibers. During prophase, metaphase, and early anaphase, E2 colocalizes with mitotic spindles in close proximity to the mitotic chromosomes. But strikingly, E2 then relocalizes to the central spindle microtubules during late anaphase, telophase and cytokinesis. It was additionally colocalized with the midbody during cytokinesis in some cells. Mass spectrometry of E2-containing complexes pulled down from a cell line harboring a previously described inducible, tagged HPV-11 E2 protein (48) detected the presence of β-tubulin as well as β-actin, a component of the midbody (our unpublished results). In addition, this dynamic E2 localization is reflected by the distribution of a self-tracking plasmid containing the

HPV-11 origin in the presence but not in the absence of the E2 protein (R. Carter, T.R. Broker, and L.T. Chow, unpublished results).

It is intriguing that a very similar and dynamic localization during mitosis has been reported for human Orc6, one of the subunits of the cellular origin recognition complex, the counterpart of the HPV E2 protein. Chromatin-bound Orc complexes serve as sites for the assembly of the pre-replication complexes (5). During early metaphase, the Orc6 protein localizes to the kinetochores but at anaphase and telophase as the chromosomes segregate, Orc6 relocalizes to the central spindle microtubules. Finally during cytokinesis the Orc6 protein can be seen in the midbody (41). The dynamic E2 localization during mitosis suggests that E2's apparent association with the spindle fibers might be mediated by one or more passenger proteins. Indeed, in vitro translated E2 protein did not bind in vitro translated β-tubulin (our unpublished observations). Passenger proteins are involved in coordinating events during mitosis and cytokinesis, regulating the movements of both the chromosomes and the mitotic apparatus. Several passenger proteins, including Aurora B (6) and Polo-like kinase (50), exhibit dynamic localization with some similarity to that observed for the E2 protein. Notably, Aurora B, which forms a complex with INCENP during mitosis, localizes to the kinetochores during prometaphase and then relocalizes to the central spindle fibers during anaphase and telophase (6). Interestingly, Aurora B plays a crucial role in regulating plasmid segregation for the Epstein-Barr virus (EBV) genome. The EBV nuclear antigen 1 (EBNA-1) associates with the mitotic chromosomes, in a process mediated by the human EBNA-1 binding protein 2 (hEBP2) (53). Silencing Aurora B caused hEPB2 to

disassociate from the metaphase chromosomes, implicating this kinase in the segregation of EBV DNA along with hEBP2.

By deletion and point mutations, the mitotic spindle-localization domain of HPV-11 E2C has been delineated to residues 285-308, possible as short as residues 285-295. The region as a whole is involved in mitotic spindle association because individual point mutations had no effect on this property. Furthermore this domain is conserved, both in sequence and function in the E2C peptides of HPV-16, HPV-18, and BPV-4, and is necessary to confer the ability to localize to the spindle apparatus. We propose that as HPV-11, HPV-16, HPV-18, and BPV-4 may also use the ability of the respective E2 protein to associate with mitotic spindles as a means to maintaining their extra-chromosomal plasmids during cell division. Upon completion of mitosis, the viral plasmid DNA would presumably travel along the microtubules back into the nuclei of the daughter cells.

These patterns of localization of HPV E2 are different from the BPV-1 E2 which is tethered to the mitotic chromosomes via an interaction with Brd4 (4, 56). Through chimeric proteins, we showed that this spindle-localization domain of HPV-11 E2C is sufficient to confer spindle localization to BPV-1 E2C, which by itself did not do so. In agreement, we have investigated the localization patterns of HPV-11 E2 through different phases during mitosis relative to GFP-Brd4. No colocalization of the FLAG-tagged HPV-11 E2 and GFP-Brd4 was observed throughout mitosis and Brd4 remained associated exclusively with the chromosomes.

Intriguingly, HPV-16 and HPV-18 can induce carcinomas. We have previously suggested that, in addition to the oncogenic properties of the HPV E6 and E7 proteins,

the association of the E2 protein with the mitotic spindles might play an important role in the carcinogenic conversion of the infected tissues (48). Oncogenic transformation by the high-risk HPV types results when the viral oncoproteins E6 and E7 is over-expressed in the dividing basal cells. High levels of E7 lead to extended periods of cellular proliferation by inactivating the pRB family of proteins, whereas over-expression of E6, which degrades the p53 tumor suppressor, leads to the survival of cells harboring chromosomal damages by abrogating various check points and apoptosis (60). Moreover, both E6 and E7 can independently induce chromosome instability (18). Intriguingly, BPV-4 can also induced carcinomas, but by a hit-and-run mechanism (9)

How might the E2 protein contribute to carcinogenesis? It is our thought that, initially, both E1 and E2 proteins might be expressed from intact copies of integrated HPV genomes. The repeated reinitiation of replication from the viral ori could generate fragile sites susceptible to breakage. More importantly, the viral origin in integrated viral genomes may act as a viro-centromere (48). During mitosis, the E2 protein would be able to bind to the viral origin as well as the mitotic apparatus. If the viro-centromere and the chromosomal centromere, on which the kinetochore assembles, are pulled to opposite poles during chromosome segregation, a break in the DNA might occur. Broken DNA ends are prime targets for recombination by non-homologous end joining, thereby generating di-centric chromosomes and initiating cycles of breakage-union-bridge (37). Breakage could also occur when the virocentromere-bound E2 relocalizes to the central spindle microtubules at telophase and cytokinesis away from the rest of the chromosomes. Chromosome fragments could be lost when they fail to be included in the newly reformed nucleus of the daughter cells. Any of these events would result in

genome instability and accelerate viral oncogenesis. Our hypothesis explains why, in patient lesions and in a cell line, HPV DNA integration is invariably and quickly accompanied by host chromosome instability (23, 38, 40).

Our hypothesis would also account for additional attributes consistently observed in HPV-induced cancers, cancer cell lines, and keratinocytes immortalized in vitro by HPVs. For instance, singly or tandemly integrated viral genomes are always disrupted in the E1 or the E2 gene, disrupting the expression of both genes (52). A predominant viral oncogene transcript, which contains downstream host sequence and polyadenlyation site, was invariably detected in these cells (58). Such chimeric RNAs are thought to confer higher mRNA stability than the normal viral mRNA, leading to elevated levels of viral oncoprotein expression and a growth advantage to the cell (26, 27). Indeed, in situ hybridization studies demonstrated that, in HPV immortalized cells, cancers, and cervical cancer cell lines, there is usually one transcription center regardless of the copy number or number of integration sites. Moreover, in tandemly integrated viral genomes, the active copy was always located at the downstream integration site which could not express an intact E2 protein. All the upstream copies, each containing an intact E2 gene, were silenced by DNA methylation (49). We suggest that E2 expression from integrated viral DNA has to cease in the cancer or immortalized cell which eventually emerges to avoid mitotic catastrophe induced by the association of E2 with the mitotic apparatus.

However it must also be noted that HPV-11 does not induce carcinomas in infected patients. This difference can be readily explained by the fact that the HPV-11 E6 and E7 proteins do not inactivate p53 and pRB as efficiently as those of the high-risk viruses and in particular, the E6 does not cause the degradation of p53. Thus, the low risk HPV

oncoproteins would not be able to overcome the various mitotic checkpoints or prevent apoptosis. The hypothesis that E2 contributed to the initial stage of HPV oncogenesis remains to be tested.

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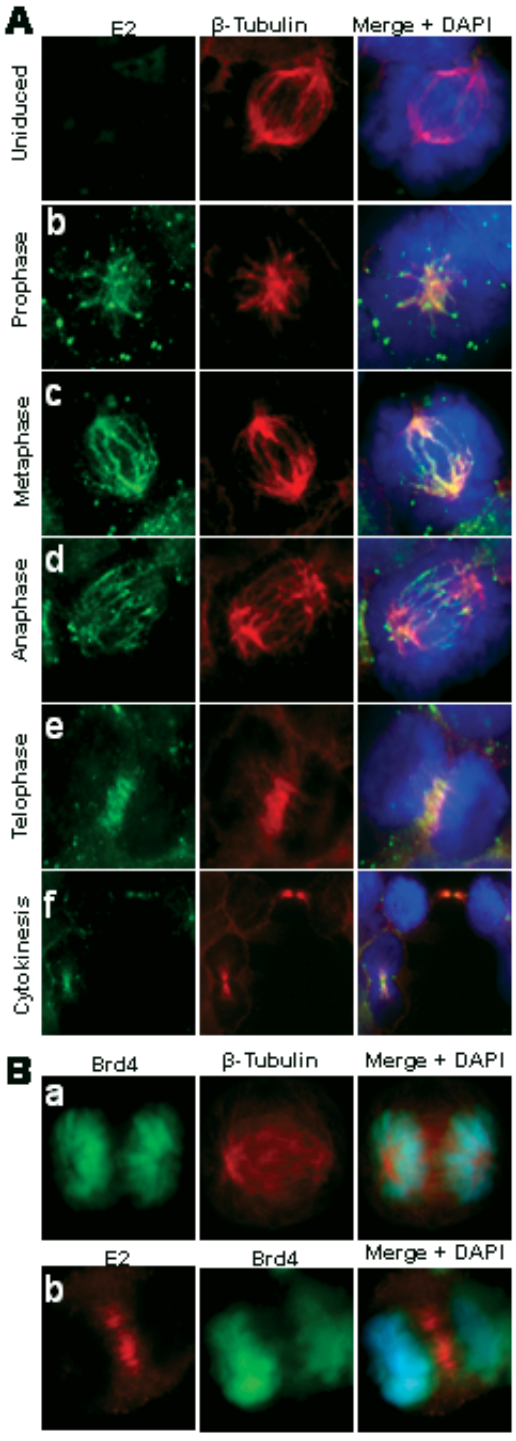
Fig. 1


A
E2
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Cytokinesis
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Brd4
β-Tubulin
Merge + DAPI
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E2
Brd4
Merge + DAPI
b

Fig. 1. HPV-11 E2 localization is dynamic during mitosis and does not colocalize with Brd4. (A) through **(C)** Three images are presented for each cell and each cell is denoted by a lower case letter. (**A)** & (**B)** HPV-11 E2 localization is dynamic throughout the different phases of mitosis. Human 293(11-E2) cells that harbor FLAG-tagged HPV-11 E2 under the control of a tetracycline regulated promoter were probed (**A**) with antibodies to HPV-11 E2 and β-tubulin. Left column, E2 (Alexa488); middle column, β-tubulin (Cy3) and DAPI-stained chromosomes (blue); right column, merged images. (**B)** with antibodies to the FLAG epitope and β-tubulin. Left column, FLAG (Alexa488); middle column, β-tubulin (Cy3) and DAPI-stained chromosomes (blue); right column, merged images. (**C)** E2 does not associate with GFP-Brd4. 293 (11-E2) cells were transfected with pEGFP-Brd4 and induced for HPV-11 E2 expression. The cells were stained with an antibody to β-tubulin (a) or an antibody to E2 (b). Both cells were in the late anaphase. Row a: left panel, GFP-Brd4; middle panel, β-tubulin (Cy3) with DAPI-stained chromosomal DNA (blue); right panel, merged images. Row b: left panel, E2 (Cy3); middle panel, GFP-Brd4; right panel, merged images with DAPI-stained chromosomal DNA (blue).

Fig. 2

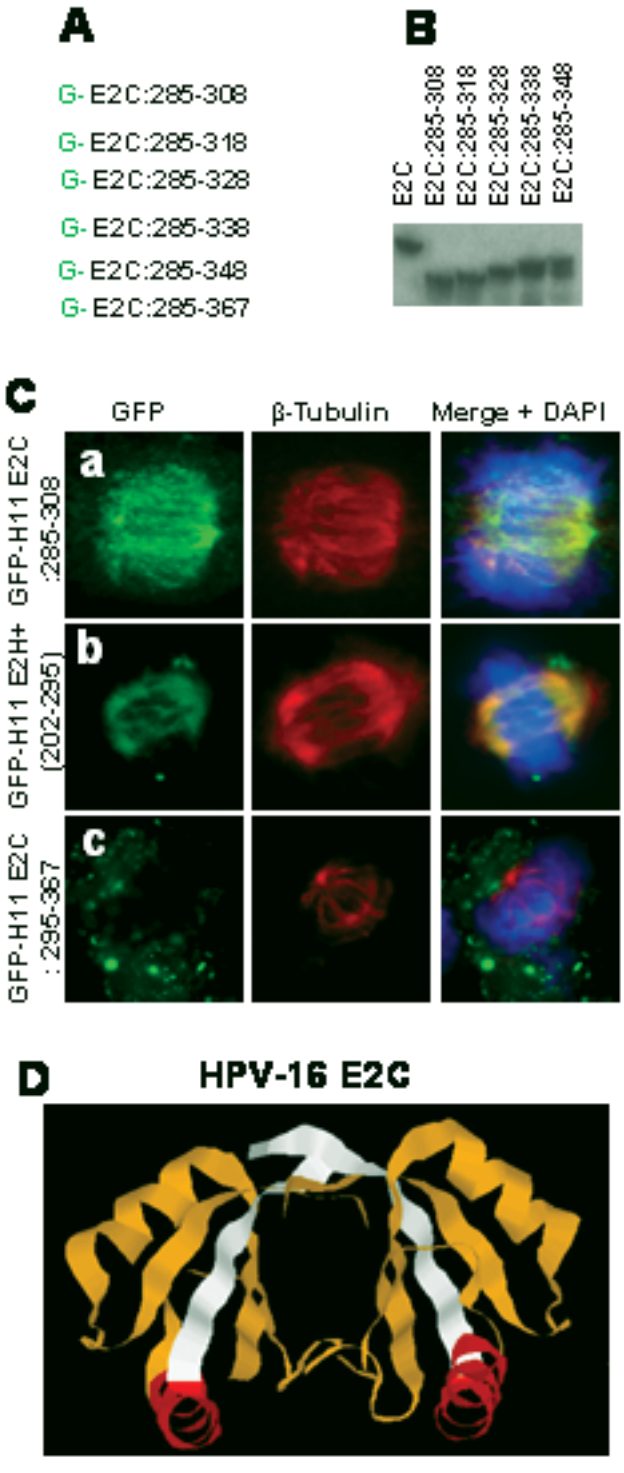
A
G-E2C:285-308
G-E2C:285-318
G-E2C:285-328
G-E2C:285-338
G-E2C:285-348
G-E2C:285-367
B
E2C
E2C:285-308
E2C:285-318
E2C:285-328
E2C:285-338
E2C:285-348
C
GFP
β-Tubulin
Merge + DAPI
GFP-H11 E2C :285-308
GFP-H11 E2H+ (202-295)
GFP-H11 E2C : 295-367
a
b
c
D
HPV-16 E2C

Fig. 2. Delineation of the mitotic spindle localization domain in HPV-11 E2C. (A) & (**B)** Western blots of SDS-PAGE of transfected COS-7 cell lysates by using an antibody to GFP. (A**)** an SDS-4%-10% polyacrylamide gradient gel. **(B)** an SDS-10% polyacrylamide gel. (**C)** Localization of selected GFP fusion peptides. Left column, GFP-E2C; middle column, β-tubulin (Cy3) with DAPI-stained chromosomal DNA (blue); right column, merged images. The cells were in prometaphase (a) or metaphase (b, c). (D**)** Crystal structure of the HPV-16 E2C dimer. The DNA binding domain is colored red and spindle-association domain white (adapted from ref. 22).

Fig. 3

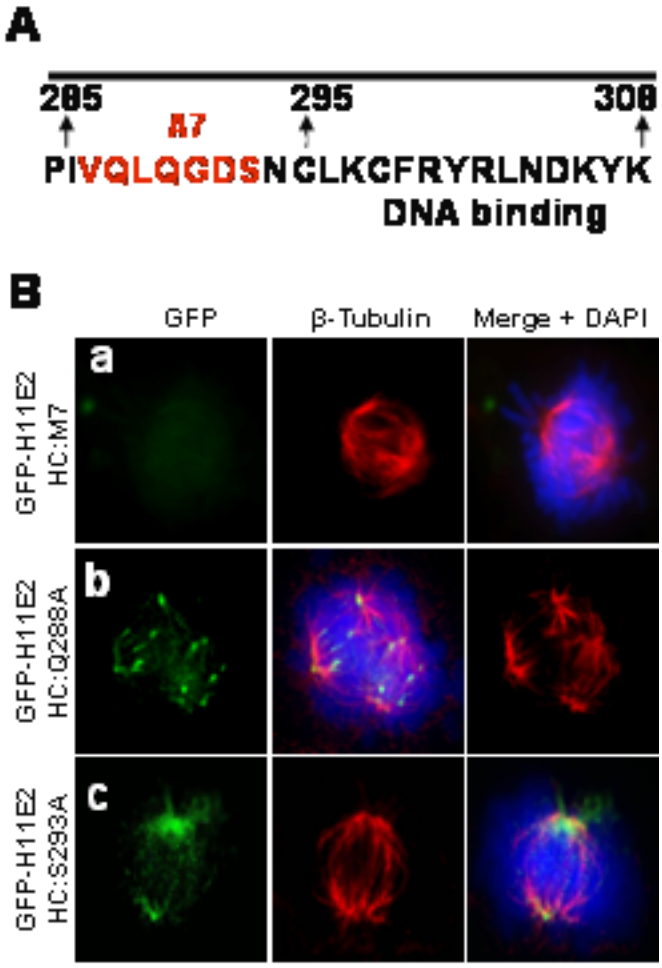
A
285
295
308
M7
PIVQLQGDSNCLKCFRYRLNDKYK
DNA binding
B
GFP
β-Tubulin
Merge + DAPI
a
b
c
GFP-H11E2 HC:M7
GFP-H11E2 HC:Q288A
GFP-H11E2 HC:S293A

Fig 3. Site-directed mutagenic analysis of the spindle localization domain in HPV-11 E2C. (A) Sequence of and mutations at the amino terminal region of E2C. In GFP-H11E2-HC:A7, residues 287-293 were replaced with 7 alanine residues. In others, individual residues were separately mutated as described in the **RESULTS**. (**B)** Localization of representative mutated fusion proteins. Left column, GFP-E2C; middle column, β-tubulin (Cy3) with DAPI-stained chromosomal DNA (blue); right column, merged images. The cells were approaching metaphase. (**C)** A western blot of transfected COS-7 cell lysates with an antibody to GFP.

Fig. 4

A

```
HPV-18:  P I I H L K G D R N S L K C L R
HPV-16:  P I V H L K G D A N T L K C L R
HPV-11:  P I V Q L Q G D S N C L K C F R
 BPV-4:  P V L L L Q G A A N T L K C F R
 BPV-1:  C F A L I S G T A N Q V K C Y R
```

B

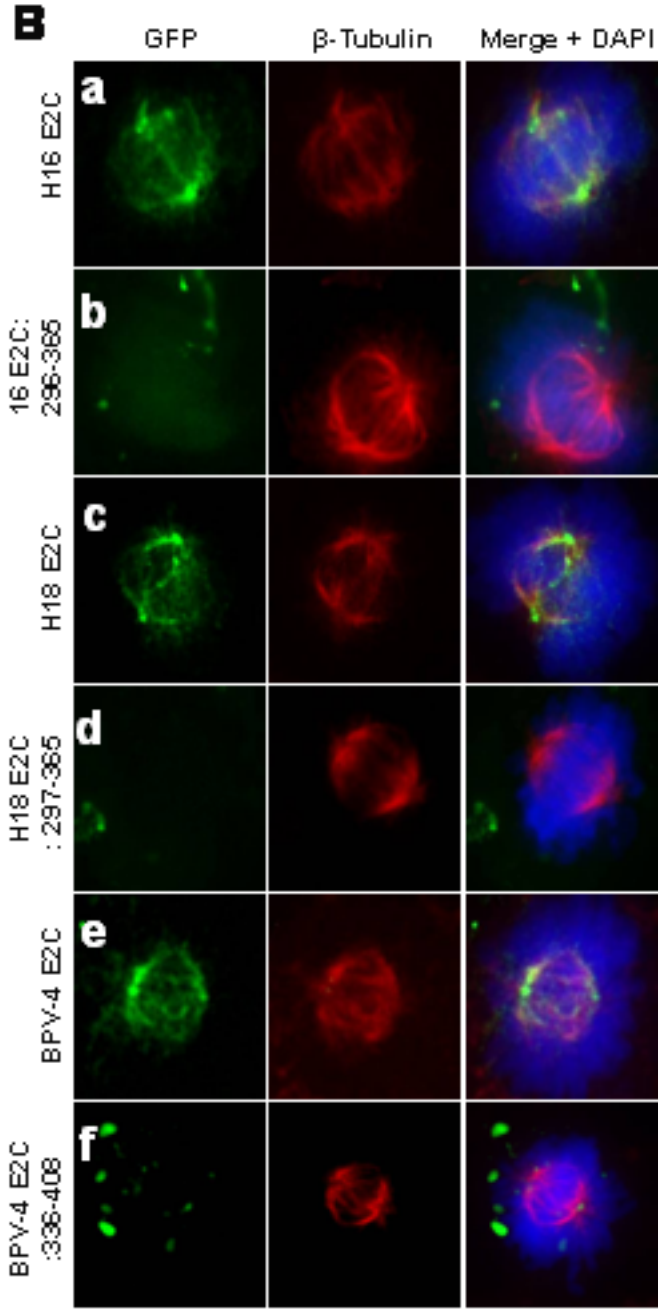

Fig 4. Conservation of the spindle-localization domain in E2C proteins of HPV-11, HPV-18, HPV-16, and BPV-4, but not in that of BPV-1. (A) A sequence comparison of the amino terminal portion of E2C implicated in colocalization with the mitotic spindles from papillomaviruses examined in this report. (**B)** Localization of GFP fused to E2C domain of HPV-16, HPV-18, or BPV-4 (a, c, e) and mutations deleted of the putative spindle localization domain (b, d, f). Left column, GFP-E2C; Middle column, β-tubulin (Cy3) with DAPI-stained chromosomal DNA (blue); and right column, merged images. The cells were in prometaphase (b, c, e) or approaching or in metaphase (a, d, f). (**C)** A western blot of transfected COS-7 cell lysates with an antibody to GFP.

Fig. 5

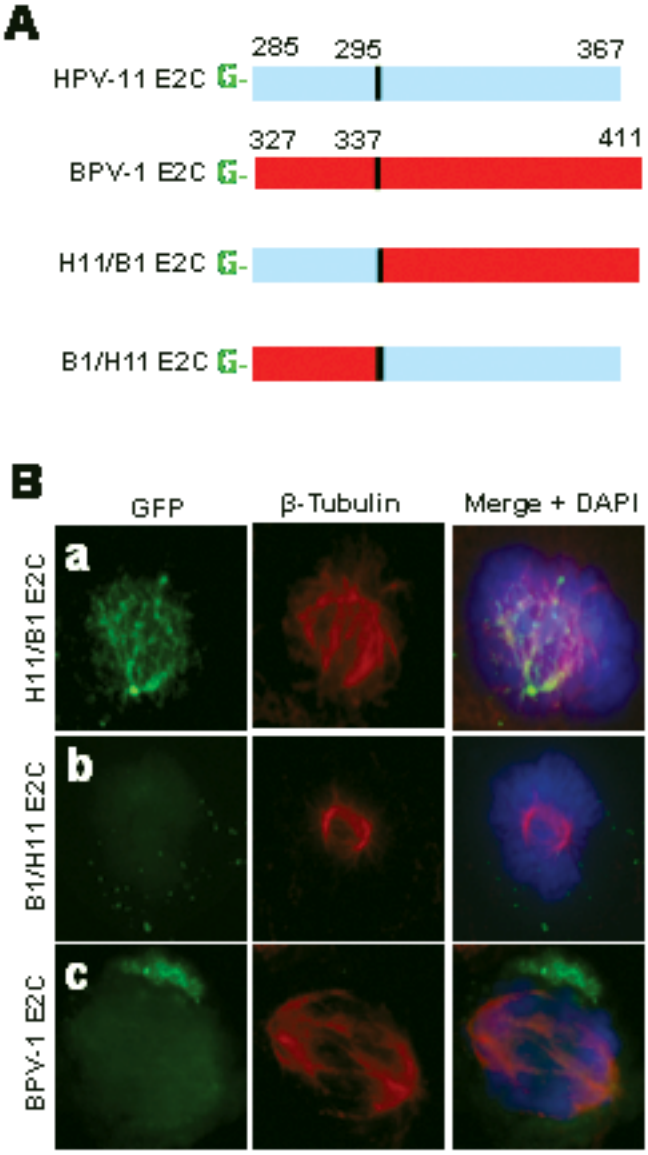
A
285
295
367
HPV-11 E2C
G-
327
337
411
BPV-1 E2C
G-
H11/B1 E2C
G-
B1/H11 E2C
G-
B
GFP
β-Tubulin
Merge + DAPI
a
H11/B1 E2C
b
B1/H11 E2C
c
BPV-1 E2C

Fig 5. Structure and properties of reciprocal chimeric HPV-11 and BPV-1 E2C fused to GFP. (A) A schematic diagram of GFP fusions of HPV-11 E2C, BPV-1 E2C, and the reciprocal chimeric H11/B1 E2C and B1/H11 E2C. (**B)** A western blot of transfected COS7 cell lysates using an antibody to GFP. (**C)** Localization of the fusion proteins in transfected cells. Left column, GFP-E2C; middle column, β-tubulin (Cy3) with DAPI-stained chromosomal DNA (blue); right column, merged images. The cells were in prometaphase (b), metaphase (c), or anaphase (a).

Relocalization of the MRN Complex by HPV-11 E1 Protein Is Not Essential for Viral Ori-specific Replication

by

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Chapter 3

Relocalization of the MRN Complex by HPV-11 E1 Protein Is Not Essential for Viral Ori-specific Replication

Abstract

A number of DNA viruses encode proteins that cause the degradation and relocalization of the MRN complex to the replication centers. These interactions are regarded as crucial to viral DNA amplification. The MRN complex composed of Mre11, Rad50, and Nbs1 proteins is the main repair complex for double-stranded DNA breaks. In this study, we examined the interaction between human papillomavirus type 11 (HPV-11) origin (ori)-specific replication and the MRN complex in transiently transfected PHKs (primary human keratinocytes). HPV replication requires virus-encoded E1 and E2 proteins and the viral ori. All other replication proteins are supplied by the host. Using a replication-competent GFP-E1 fusion protein, we showed that Mre11 and Nbs1 are both recruited to the HPV replication centers. Recruitment requires active ori replication. However, we detected no degradation of either host protein. Moreover, we identified a motif in E1 which interacts with Mre11 protein. An E1 protein mutated in this binding motif abolished association with Mre11 and the recruitment of Mre11 and Nbs1 to the replication centers. Nonetheless, this mutant form of E1 supported ori

replication as efficiently as the wild type protein. Furthermore, the relocalization of Mre11 and Nbs1 did not lead to activation of ATM at the replication centers, as measured by γH2AX distribution. Thus, we conclude that HPV replication does not depend on the MRN complex, unlike the other DNA tumor viruses. Moreover, as with other DNA viruses, some of the replication centers were juxtaposed to a subset of PML bodies. This distribution was independent of Mre11 or Nbs1.

INTRODUCTION

Each member of the large family of papillomaviruses has a circular double-stranded DNA genome of about 8 kbp in length. These ubiquitous viruses infect cutaneous and mucosal epithelia, generally causing hyperproliferation. Over 120 genotypes of human papillomaviruses (HPVs) have been cloned from patient specimens (9). Infection occurs through skin wounding, which provides access of the viral particles to the cycling basal and parabasal (transit-amplifying) keratinocytes of the epithelia. A low copy number of the viral genome is established in the nuclei of dividing cells during healing of the wound. However, vegetative viral amplification, viral assembly, and release of virions only occur during the stratification and differentiation of the squamous epithelia. Because of this absolute dependence on squamous differentiation, mechanistic studies of the viral DNA replication have been significantly limited but have mainly been conducted in transiently transfected cells or in cell-free systems (5). Only two viral proteins are required to support replication of viral origin (ori)-based plasmids: the E1 protein and the E2 protein. All other replication proteins and substrates are supplied by the host cells (5)

The ori sequences are conserved among papillomaviruses and comprise a series of E1 protein binding sites (BSs) flanked by E2BS. The E1 and E2 proteins are also largely homologous both in sequence and in function among papillomaviruses. The E2 protein is 42-kDa and has multiple functions. It is the origin binding protein and is necessary for initiation of viral DNA replication (3, 8, 19) and is responsible for viral plasmid partitioning and maintenance in dividing cells {McPhillips, 2005 #1158, 28, 30, 38, 41, 45-47). It also regulates viral gene transcription, both positively and negatively (11, 16,

27, 35). To initiate replication, the E2 protein binds as a dimer to multiple copies of the E2BS, which consists of the palindromic sequence $ACCGN_4CGGT$ (15). The three E2 dimers bound to the three E2BS in the HPV-11 ori establish a hexameric disc (32). The bound E2 then recruits the 68 kDa replicative helicase E1 protein to the E1BS via an interaction between the amino terminus of E2 and the carboxyl terminus of E1 (2, 54). Thereupon, the E1 protein assembles into a dihexameric bidirectional helicase on the ori (24). For HPV-11 E1, dihexamer formation is facilitated by the presence of heat shock proteins Hsp70 and Hsp40 (24). Additionally, E1 recruits the DNA polymerase α/primase, and topoisomerase I, and RPA, enabling the initiation of DNA replication (6, 14, 25). In vitro, the E1 dihexamer functions as an efficient helicase on supercoiled plasmids in the presence of topoisomerase I, replication protein A, and ATP (22). E1 is required throughout initiation and elongation of viral ori-specific replication, whereas E2 is only required for initiation, at the stage of the assembly of the preinitiation complex (23).

During DNA replication, the viral DNA would suffer a double-stranded DNA break (DSB) if one of the template strands has a single-stranded nick. The presence of DSBs is one of the most serious problems a eukaryotic cell could encounter. As such, all eukaryotic organisms have evolved a complex mechanism to detect, respond to, and repair the DNA lesions. The main DSBs repair complex is the MRN complex which is composed of three proteins: Mre11, Rad50, and Nbs1 (7, 40). This complex is conserved from yeast to mammalian cells. It is one of the earliest detected protein complexes at the site of DNA damage. The MRN complex has four main roles: detecting the damage, localizing to the site of damage, inducing cell cycle checkpoints, and

repairing the DSBs. Mre11 is responsible for mediating the actual repair of DSBs. Mre11 has 3'-5' nuclease activity and, in conjunction with Rad 50, it tethers the broken ends and holds them in proximity. Nbs1 is responsible for the recruitment of ATM, a cell cycle checkpoint protein (1, 39, 48). The activation of ATM further recruits additional MRN complexes to the site of damage. The local accumulation of MRN complexes forms large foci observable by indirect immunofluorescent detection with antibodies and serves as a validated` marker for activation of the repair pathway (7, 40, 42, 50, 52).

Several DNA viruses encode proteins that interact with the MRN complex. Adenovirus E4orf3 protein mediates relocalization of the MRN complex, with Mre11 forming nuclear 'tracks' and Nbs1 relocalized to the viral replication centers (12). With adenoviruses mutated in E4orf3, Mre11 is bound to the viral genome in an Nbs1 dependent manner (26). Degradation of Mre11, following relocalization, is mediated by the E4orf6–E1B 55-kDa protein complex (31). As adenovirus has a double-stranded linear genome, it is thought that the relocalization and degradation of Mre11 would prevent concatamerization of linear viral genomes. Similarly, the SV40 large T-antigen (Tag) promotes a decrease in both Mre11 and Nbs1. The remaining Mre11 and Nbs1 are relocalized to the SV40 replication centers. This recruitment is thought to be important for SV40 replication as the associated ATM activity is necessary to support SV40 ori replication (51). Studies of Herpes simplex type-1 yielded very different results for the role of Mre11 in viral replication, which was greatly decreased in cells containing mutations of Mre11 when compared with the same cells complemented with an Mre11 expression plasmid (20). Yet there are conflicting reports on whether late stage viral

replication results in degradation of Mre11 and also on the identity of viral proteins responsible for interaction with Mre11 (13, 20).

In this present study, we examined the potential interactions between HPV-11 replication complexes and the MRN repair complex. Our results show that Mre11 and Nbs1, but not Rad50, are recruited to HPV-11 replication centers that are positive for the HPV-11 E1 protein, cyclin A, and RPA. This relocalization of Mre11 and Nbs1 is dependent on active ori replication, for a replication-incompetent mutant form of the E1 protein did not recruit any of the host proteins. However, unlike adenovirus or SV40, we also demonstrate that the interaction between E1 and Mre/Nbs appears not essential for ori replication in the transiently transfected cells. We have identified a putative Mre11 interaction motif in the C-terminus of E1. Wild type E1, but not an E1 mutated in this motif, coimmunoprecipiated with Mre11. This mutated E1 supported transient HPV ori-dependent replication as well as did the wild type E1, but it did not induce relocalization of Mre11 or Nbs1 to the viral replication centers. Furthermore, the recruitment of Mre11 and Nbs1 was not accompanied by an activation of ATM, as we observed no elevated concentrations of γH2AX, a target of ATM activity, at the replication center.

MATERIALS AND MATHODS

Plasmids pUC7730-99, an HPV-11 *ori* containing plasmid, pMT2-11E2, and pEGFP-11E1dm wild type have been described (Chiang et al., 1992; Kuo et al., 1994; Deng et al., 2003; Yu et al., 2007). The mutational inactivation of the E1 RNA splice donor site in E1dm greatly increases the production of the E1 protein. p11URR-E1dmL465PH466R was generated by swapping the E1 region of pEGFP-E1dmL465PH466R with the wild-

type E1dm region of p11RR-EGFP-11E1dm (Deng et al., 2003). pEGFP-HA was made by inserting the HA tag sequence (ATG TAC CCA TAC GAT GTT CCG GAT TAC GCT) into the pEGFP-C1 vector (Clontech, Mountain View, CA) between BglII and EcoRI restriction sites. The GFP-HA tagged 11E1 constructs were cloned by swapping the wild type or mutation 11E1dm ORF into pEGFP-HA between Bam HI and Kpn I cleavage sites. pmCherry-11E2 was made by swapping EGFP in pEGFP-11E2 (Zou et al., 2000) with the PCR product of the mCherry ORF (Shaner et al., 2004) between BglII and Eco47III sites. All constructs were verified by DNA sequencing.

Western Blots HEK293 cells were cultured in DMEM plus 10% FBS and 5% CO_2 at 37°C. The cells were transfected by electroporation of 5 μg of expression plasmid for pEGFP-HA-11E1dm or pEGFP-HA-11E1dmW440A alone or together with 5 μg of pMT2-11E2 and 1 μg of pUC7730-99. Possessing the ori sequence in the URR, p11URR-E1dm or p11URR-E1dmL465PH466R was cotransfected with pMT2-11E2 alone. As controls, we also performed transfection with pEGFP-HA vector or used untransfected 293 cells. The transfected cells were then transferred to 10 cm plates and grown for 48 hours. The cells were harvested with 200 μl SDS lysis buffer (60 mM Tris-C1 pH 6.8 and 1% SDS) and sonicated. Protein concentrations were determined and 100 μg of total protein of each sample were separated by 10% SDS-PAGE followed by western blot. Mre11 and Nbs1 were detected using specific polyclonal rabbit antibodies (Novus Biologicals, Littleton, CO). Protein loading was verified by HRP-conjugated goat anti-actin antibody (Santa Cruz Biotech, Santa Cruz, CA).

Co-immunoprecipitation Two μg of GFP-HA vector or 5 μg of each of the GFP-HA tagged 11E1dm constructs were transfected into HEK293 cells, respectively. 48 hours

post-transfection, cells were harvested by TGH buffer supplement with proteinase and phosphatase inhibitors, as described previously (49). The protein concentrations of lysates were determined by bicinchoninic acid reagent (Pierce, Rockford, IL). The same amount of total protein from each cell lysate was mixed with mouse monoclonal anti-HA affinity matrix (Covance, Philadelphia, PA) for co-immunoprecipitation. The precipitates were washed by TGH buffer and separated by 10% SDS-PAGE followed by western blot. Mre11 and Nbs1 were detected using polyclonal rabbit anti-Mre11 and anti-Nbs1 antibody, respectively (Novus Biologicals). GFP-HA-11E1 were detected by monoclonal mouse anti-HA antibody (12CA5) (a gift from Dr. Hengbin Wang, UAB). The protein loading was verified by HRP-conjugated goat anti-actin antibody (Santa Cruz Biotech, Santa Cruz, CA).).

Transient Replication Assay This assay was performed as described previously (4, 10)). Briefly, 1 μg of GFP-HA-11E1dm expression plasmid, 5 μg of pMT2-11E2, and 0.5 μg of pUC7730-99 were co-transfected into HEK293 cells. 48 hours post-transfection, cells were harvested, and the low molecular weight DNA was isolated as described previously (4) and subjected to BglII or BglII + DpnI digestion. Southern blot analysis was performed to identify the newly synthesized DNA (49).

Fluorescence microscopy Early passage primary human keratinocytes (PHKs) were plated on multi-chamber slides (LabTek, Campbell, CA) 24 hours pre-transfection in keratinocyte serum- free medium (Invitrogen, Carlsbad, CA). PHKs were then transfected with the appropriate plasmids using Fugene 6 (Roche Applied Science, Indianapolis IN). The slides were subsequently fixed in 4% paraformaldehyde for 10 minutes at room temperature and permeabilized with 1X PBS-0.5% Triton-X treatment

for 10 minutes at room temperature. After blocking with 5% goat serum for 30 minutes at 37°C, the cells were probed with rabbit polyclonal antibodies specific for Mre11 and Nbs1 or mouse monoclonal antibodies specific for RPA, cyclin A, PML, or yH2AX (all primary antibodies used at 1:100 dilution) at 37°C for 1 hour. After incubation, slides were washed and incubated with a 1:250 dilution of anti-rabbit Alexa Fluor 647 (Molecular Probes, Eugene, OR) and 1:750 dilution of anti-mouse Alexa Fluor 555 (Molecular Probes, Eugene, OR). Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI) and mounted in *SlowFade* Gold antifade (Invitrogen, Carlsbad, CA). To correlate with western blots conducted with 293 cell lysates, selected key experiments to detect replication foci were also repeated in 293 cells. All images were acquired with a 60x or 100x objective lens on an Olympus AX70 fluorescence microscope equipped with Speicher filters (Chroma, Rockingham, VT) and a Zeiss Axiocam digital camera (Thornwood, NY). Processing and assembly were accomplished with PHOTOSHOP (Adobe Systems, Mountain View, CA).

RESULTS

Visualization of HPV-11 replication foci via eGFP-E1 dm foci

Previously we demonstrated that HPV-11 E2 protein recruits HPV-11 E1 protein into small nuclear foci and that large foci formed only in the presence of an HPV-11 ori-containing plasmid (37, 49, 53). We identified these large foci to be DNA replication centers by virtue of the colocalization of E1, E2, RPA, BrdU and ori DNA in various combinations using indirect double immunofluorescence detection and in situ hybridization (37). In this new investigation, we examined replication foci using a

replication-competent GFP-tagged HPV-11 E1dm fusion protein (10). HPV-11 GFP-E1dm encodes eGFP fused to the wild type E1 protein. The dominant splice donor site in the E1 mRNA has been mutated (dm) to abolish the predominating intragenic splicing such that the eGFP signal is virtually synonymous with eGFP-E1 protein rather than a truncated variant of eGFP protein. This approach facilitates the detection of replication foci. Indeed, E1dm alone forms numerous tiny punctate dots throughout the nucleus but was relocalized with mRFP-11E2 into small foci in cotransfected COS7 cells (49). This pattern of E1 localization held true in transfected primary human keratinocytes (PHKs) using GFP-11E1dm and mCherry-11E2 (Fig. 1A).

To verify that the distribution of GFP-E1dm can be used to reveal HPV ori replication foci, we transfected PHKs with pGFP-11 E1dm, an HPV-11 ori plasmid, along with pMT2-11E2, an HPV-11 E2 expression vector. 48 hr post-transfection, we observed large GFP-positive foci that colocalized with RPA (Fig. 1B, bottom row), in agreement with our previous report. The large GFP-E2dm/RPA foci were also positive for the S phase cyclin A (Fig. 1C, bottom row). Previous work in our lab identified the need for cyclin dependent kinases (cdks) to phosphorylate E1 on S107, inactivating the dominant NES in order to maintain E1 in the nucleus (10). Inhibition of cdk activities by the inhibitor p21cip1 or roscovitine results in cytoplasmic E1 (10). In contrast, in controls without HPV ori replication, RPA or cyclin A, when detected, formed numerous small foci in the nucleus, consistent with cellular DNA replication (Fig. 1B and C, top rows). The formation of large foci positive for GFP-11E1/RPA/cyclin suggests that host DNA has already replicated and cells are transiting toward late S/G2 phases, as we previously suggested (37). Interestingly, very little to no cyclin E was present at the

replication foci (data not shown). Cyclin E is a late G1 cyclin and it decreases sharply upon progression into S phase. However, the cyclin E/cdk2 complex is required for initiation of replication in the cell-free system (21). It is possible that cyclin E/cdk2 initially promotes E1 nuclear retention. However, upon initiation of replication, cyclin E is quickly degraded and cyclin A becomes the predominant cyclin responsible for phosphorylating E1 on S107 to maintain it in the nucleus. In the absence of the E2 protein, GFP-E1dm did not form large foci or colocalize with RPA or cyclin A (Fig. 1B and C, middle row). Transfection with an eGFP-expression vector alone did not result in large RPA or cyclin A foci. These results support our interpretation that the large GFP-E1dm foci represent HPV ori replication complexes.

Components of the MRN complex relocalize with the viral replication centers

To determine whether HPV-11 ori replication induces the relocalization of components of MRN to the HPV-11 viral replication centers, we transfected PHKs with pGFP-E1dm, pMT2-11E2, and an ori plasmid. Cells were then stained with antibodies against Mre11, RPA, and Nbs1 or Rad50. Both Mre11 and Nbs1, but not Rad50, were relocalized from a diffuse pattern as seen in the peGFP-C1 transfected control cells (Fig. 2 A and B, top rows and data not shown), to discrete foci that colocalized GFP-E1dm and RPA (Fig 2 A and B, bottom rows). Without the ori plasmid, pEGFP-11E1dm did not form large replication foci in the presence or in the absence of pMT2-11E2, as described previously (Fig. 2A and B, middle rows). In the absence of viral replication, E1dm, Mre11 and Nbs1 each formed numerous punctate dots within the nuclei (Fig. 2A and B, middle rows), too numerous to ascertain colocalization status accurately. It is only in the presence of viral replication when foci were formed that one can clearly see a

relocalization of Mre11 and Nbs1 to the viral replication centers. These foci were observed in cells analyzed at 24 hours and 48 hours post-transfection. However, at 24 hours post-transfection, there were fewer and smaller HPV-11 replication foci (data not shown). These results were not unexpected because, in transient HPV replication assays, robust HPV-ori plasmid replication as revealed by Southern blot hybridization was detected at 48, but not at 24 hr, post-transfection (3). Collectively, these results show that Mre11 and Nbs1 were recruited to the HPV-11 replication complexes.

Relocalization of MRN Complex is Dependent on Viral Replication

To validate our interpretation that Mre11 and Nbs1 were both relocalized to the replication complex in large nuclear foci, we generated a p11URR-E1dm construct p11URR-GFP-E1dmL465PH466R which also contains the ori sequence. This mutant form of E1 was replication incompetent in transient assays (Fig. 3A). When transfected into PHKs, GFP-E1dm L465PH466R, in the presence of pMT2-11E2, (referred as 11URR-E1dmPR-Rep in Fig. 4) was found to be diffuse throughout the nucleus. Notably, it did not form small or large nuclear foci as did the wild type GFP-E1dm protein, indicative of a defect in its interaction with the E2 protein and in ori replication (Fig. 3B, bottom row), in agreement with transient replication assays (Fig. 3A). There was no relocalization of Mre11 or Nbs1 (Fig. 3B, bottom row, middle and right columns). To confirm that the incorporation of the viral ori into the GFP plasmid would still allow for replication, we used p11URR-GFP-E1dm, which encodes wild-type E1dm, as a positive control. This construct was able to form large foci as seen before, and these foci colocalized with both Mre11 and Nbs1 (Fig 3A middle row, middle and right column). However, because much more GFP-E1dm protein was expressed from the replicating

plasmid, there was diffuse GFP signal beyond the foci than when the GFP-E1dm expression plasmids did not contain the ori. Taken together, these observations demonstrate that the relocalization of the both Mre11 and Nbs1 requires active viral ori replication and that E2 is not responsible for recruiting Mre11 or Nbs1.

E1 helicase recruits Mre11 to the replication foci

Since SV40 large T-antigen (T-ag) interacts with Nbs1 (44), we were curious to determine whether the functional homolog in HPV, E1 DNA helicase, also plays a role in recruiting the MRN components. We found no conserved motif in HPV-11 E1 similar to the MRE11-binding motif present in Nbs1 (NFKXF) (48). However, a p53 mutation, R248W, which is commonly found in cancer, has been shown to gain the ability to interact with Mre11 (33). In so doing, the mutant form of p53 protein abrogates ATM activation by preventing the MRN complexes from binding to the dsDNA broken ends (33). The Mre11 interacting motif in this mutated p53 (GGMNW^{248}RPIL) turns out to be very homologous a sequence we identified as conserved among several HPV E1 proteins, but not present in E2 proteins (Fig 4A).

To test the functionality of the putative Mre11 binding motif in E1, the codon for tryptophan 440 was mutated to encode alanine. Both wild type E1dm or E1dm W440A were then tagged with GFP-HA and tested for their abilities to bind to Mre11 in transfected HEK 293 cells. HEK 293 cells were used in these assays because of their high transfection efficiency. Furthermore, we confirmed that Mre11 and Nbs1 proteins also colocalized with the GFP-E1dm replication foci, as observed in PHKs (data not shown). GFP-HA was used as a control. As demonstrated in Fig. 4B, wild type E1 binds to Mre11, whereas neither the W440A mutation nor GFP-HA was able to bind to Mre11.

Therefore, E1 appears to recruit Mre11 to the replication foci through this apparent Mre11 binding motif. In the same experiments, we did not detect Nbs1 binding to GFP-HA-11E1dm. Thus, the recruitment of Nbs1 to the replication foci is mediated by an interaction with Mre11.

HPV-11 replication induced no reduction in Mre11 or Nbs1 protein levels

In SV40, the relocalization of Mre11 and Nbs1 to the viral replication center is accompanied by a marked decrease in the cellular concentration of both proteins. The interaction of the SV40 T-antigen with Mre11/Nbs1 and the E3 ubiquitin ligase Cul7 is thought to mediate the degradation of both Mre11 and Nbs1. To examine whether the levels of Mre11 and Nbs1 were similarly decreased during HPV-11 replication, we transfected into 293 cells expression vectors of GFP-HA-11E1dm alone, GFP-HA vector alone, or three plasmids: the ori plasmid, pMT2-11E2 and the pGFP-E1dm or E1dmW440A. Alternatively, cells were cotransfected with pMT2-11E2 and p11URR-E1dm or p11URR-E1dmL465PH466R. 48 hours post-transfection, the cells were harvested and the levels of Mre11 or Nbs1 were analyzed by western blotting. We observed no reduction of either host protein relative to levels detected in non-transfected cells or cells transfected with GFP-HA only (Fig. 5). We thus conclude that the HPV replication does not lead to the degradation of Mre11 and Nbs1. However, we cannot rule out the possibility that a small reduction in cells in which ori plasmid replicated was masked by the relative high levels of these host proteins in the majority of untransfected cells or in cells not cotransfected with all three plasmids.

HPV-11 replication centers do not colocalize with γH2AX

In response to MRN activation during double-stranded DNA damage response, histone H2AX becomes phosphorylated (γH2AX) by ATM. The phosphorylation of H2AX leads to a feedback loop which further enhances MRN recruitment to and activation at the site of damage. In turn, more γH2AX accumulates. However, we were not able to detect any ATM signals by indirect immunofluoresence (IF). Thus, we used γH2AX as a surrogate marker to investigate whether the presence of Mre11 and Nbs1 at the HPV ori replication center represents an active MRN complex.

We first examined the level of γH2AX by western blotting. pGFP-HA or pGFP-HA-E1dm was transfected into COS7 cells or 293(11-E2), a 293 cell line in which the expression of HPV-11 E2 is negatively regulated by doxycyclin (43). We observed little or no γH2AX upon induction of E2 subsequent to removing doxycyline (Fig. 6A). However, there was a small increase in γH2AX in response to the transfection of pGFP-HA. In cells transfected with pGFP-HA-E1dm, the level of γH2AX was dramatically increased over that of the control (Fig 6A). In agreement, by fluorescence microscopy in PHKs, γH2AX was elevated and diffuse throughout the nucleus in the presence E1dm alone relative to cells expressing GFP (Fig. 6B, first and second rows). It is probable that the degradation of the bulk of the transfected DNA may have triggered the MRN and ATM activation. However, due to the small size of the plasmid DNA, no obvious foci were discernable. Interestingly, when HPV ori replication took place, some strongly positive γH2AX foci were observed. However they did not colocalize with any of the replication centers (Fig. 6B, third and fourth rows). Thus, there was no elevated ATM

activity at the replication centers as a consequence of the recruitment of Mre11 and Nbs1, as might have been expected from the presence of active MRN complexes.

Mre11 is not required for HPV viral DNA replication in transient replication

Since the above observation suggests that the recruitment of Mre11 and Nbs1 to the replication centers appears not to represent an activation of the MRN complex, we then asked whether the recruitment of the MRN component is important for viral ori replication, as shown for other DNA viruses. We conducted transient replication assay in 293 cells with the GFP-HA-E1dmW440A mutant form of E1; it supported the replication of the HPV ori plasmid as efficiently as the wild type GFP-E1dm (Fig. 4C). Moreover, in transfected PHKs, neither Mre11 nor Nbs1 was recruited to HPV ori replication centers formed in the presence of GFP-HA-E1dmW440A (Fig. 4D, middle and bottom panels). Therefore, it appears that the recruitment of Mre11 and Nbs1 to the viral replication foci in HPV is not essential for transient replication of the HPV ori plasmid.

HPV-11 replication centers do not exclusively colocalize with PML nuclear domains

Many DNA viruses have been reported to establish replication centers at the periphery of promyelocytic leukemia protein-containing nuclear bodies (PML bodies, or ND10, for nuclear domain 10). Early after infection by adenovirus, SV40, HSV-1, and cytomegalovirus, the viral genomes are found adjacent to PML bodies. Indeed, many of these viruses encode proteins that directly interact with the PML (12 , 17, 18, 34). The adenovirus E4orf3 protein interacts with and disrupts the localization PML from discreet nuclear foci to track-like structures, which colocalize with the viral proteins E1B-55 kD, E4orf3, and components of the MRN complex (12, 17). SV40 replication centers colocalize with PML bodies (18, 51), but SV40 DNA replication occurred equally well in

cells transfected with siRNA against PML as in mock-transfected cells (18). Similarly, in HCMV, the absence of PML bodies did not affect viral replication (34).

HPV-11 E2 protein can be found colocalized with PML in punctate dots (37). To determine whether transient HPV-11 replication induces alterations in PML bodies, PHKs were transfected with various plasmids. In cells negative for GFP, PML bodies were small and their number varied. Expression of GFP or GFP-E1dm alone did not induce any changes in the number or sizes of PML bodies and they did not colocalize with Mre11 (Fig. 8, top two rows, shown in low magnification to visualize more cells). When HPV ori replication took place, we did not observe a consistent change in the size or number of PML bodies. Interestingly, some of the PML bodies were found in juxtaposition to the replication foci positive for GFP-E1dm, Mre11 or Nbs1. The reverse was also true: only some of the replication foci were located close to PML bodies (Fig. 7, bottom two rows). When the E1dmW440A was analyzed, we did not see colocalization of the replication centers with either Mre11 or Nbs1 as expected, whereas the juxtaposition of the replication foci and PML remained unchanged (data not shown). Thus, there appears to be no connection between the MRN complex and PML bodies. Our observations suggest that HPV ori replication does not always occur at or near the PML bodies. This conclusion agrees with studies of BPV-1 and HPV-16. Transient replication assays of BPV-1 in cells without PML revealed little difference in replication levels to that seen when PML was ectopically expressed from a cDNA (29). Furthermore, in organotypic cultures of NIKS cells, PML bodies were lost in the differentiated strata where viral DNA amplifies (29). Similarly, during early time points, HCMV viral replication centers precisely coincide with PML bodies. However at

late time points, some viral replication centers were found to be adjacent to the PML bodies whereas others were not localized to PML, and vice versa (34).

Discussion

Double-stranded DNA breaks represent a major obstacle for cell survival. Accordingly, most organisms have evolved intricate strategies to detect and repair the damage. In eukaryotic cells, the major repair mechanism for DSBs is the MRN complex. The Mre11 protein is responsible for carrying out the actual repair of the broken DNA, recognizing structures such as double-stranded DNA ends, single-stranded DNA, and hairpin structures (7). Recent studies have demonstrated that certain viral proteins encoded by adenoviruses and SV40 interact with the MRN complex, causing their relocalization and degradation. These interactions are critical for their genome amplification (26, 7, 36). In this paper we provided evidence for a different relationship between MRN and HPV-11 ori-specific replication .

Using a replication-competent GFP-E1-dm protein in a transient replication assay, we show that, in the presence of HPV-11 ori and E2 protein, the GFP-E1, the Mre11 and Nbs1 proteins all undergo nuclear redistribution, each transitioning from diffusely punctate nuclear dots to very discreet larger foci. We believe that these foci are replication centers, for they colocalize with RPA and do not form when a replication-incompetent mutant form of GFP-E1 is present (Fig. 2 A, B). This result was similar to that seen with both SV40 and adenovirus. However, unlike these other small DNA tumor viruses, we show that these interactions do not lead to a reduction of Mre11 or Nbs1 protein levels (Fig. 5) Furthermore, this recruitment of Mre11 or Nbs1 to the replication centers is not necessary for transient amplification of an HPV-11 ori plasmid

(Fig. 4C). We additionally demonstrate that HPV-11 E1 protein can be co-immunoprecipitated with Mre11 via a conserved motif we identified in the helicase domain (Fig. 4B). However a mutant form of GFP-E1dm, GFP-E1dmW440A, which is unable to associate with Mre11 by co-IP (Fig. 4B) nor able to recruit Mre11 or Nbs1 to the replication foci (Fig. 4D), is capable of supporting transient replication as well as does the wild type GFP-E1 (Fig. 4C).

An additional observation with HPV-11 ori replication supports this difference from SV40 with regard to the role of MRN. SV40 viral replication requires ATM activity and, as a result, the replication centers colocalize with phosphorylated H2AX (51). Western blots and indirect immunofluorescence assays show that γH2AX is significantly up-regulated upon expression of the GFP-E1dm alone or following cotransfection of all three plasmids, when compared to pGFP-C1 transfection (Fig. 6A B). However, γH2AX was not elevated in replication foci positive for E1dm, Mre11, or Nbs1 (Fig. 6B). These results suggest that the Mre11 and Nbs1 recruitment might not represent an activation of the MRN complex. This conclusion is further supported by the lack of Rad50 in the replication foci (data not shown). A correlate is that there were no significant numbers of double-stranded breaks in the replication foci.

Collectively, the above results show that E1 binds to Mre11 which in turn recruits Nbs1, but not Rad50, to the replication foci. These interactions, however, did not lead to local activation of ATM and enrichment of γH2AX. In addition, this interaction was not essential for HPV ori-dependent DNA replication in this transient replication. However, at present we cannot rule out that, in the context of genomic viral DNA and in the setting

of differentiated keratinocytes, the activation of ATM could play an important role for efficient viral DNA amplification. This possibility remains to be tested.

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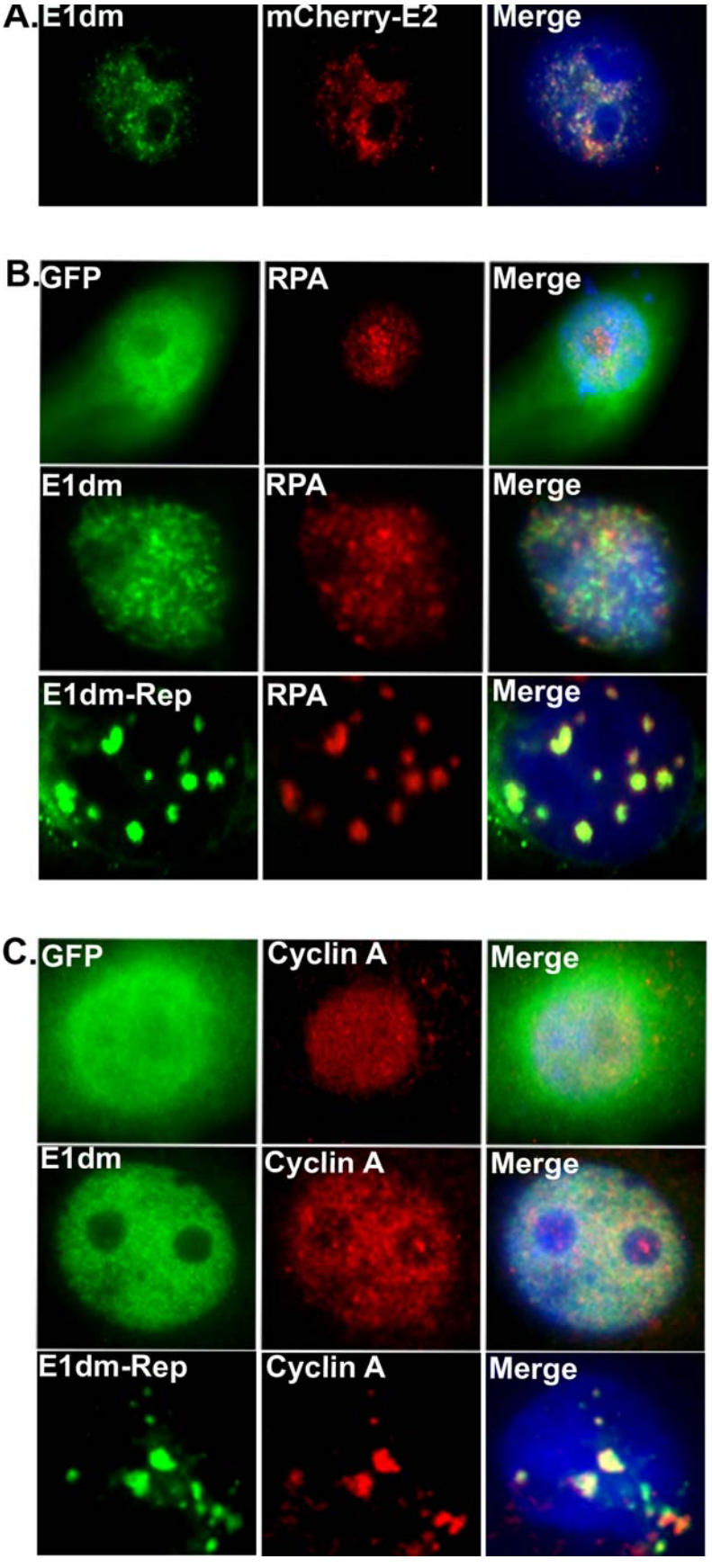
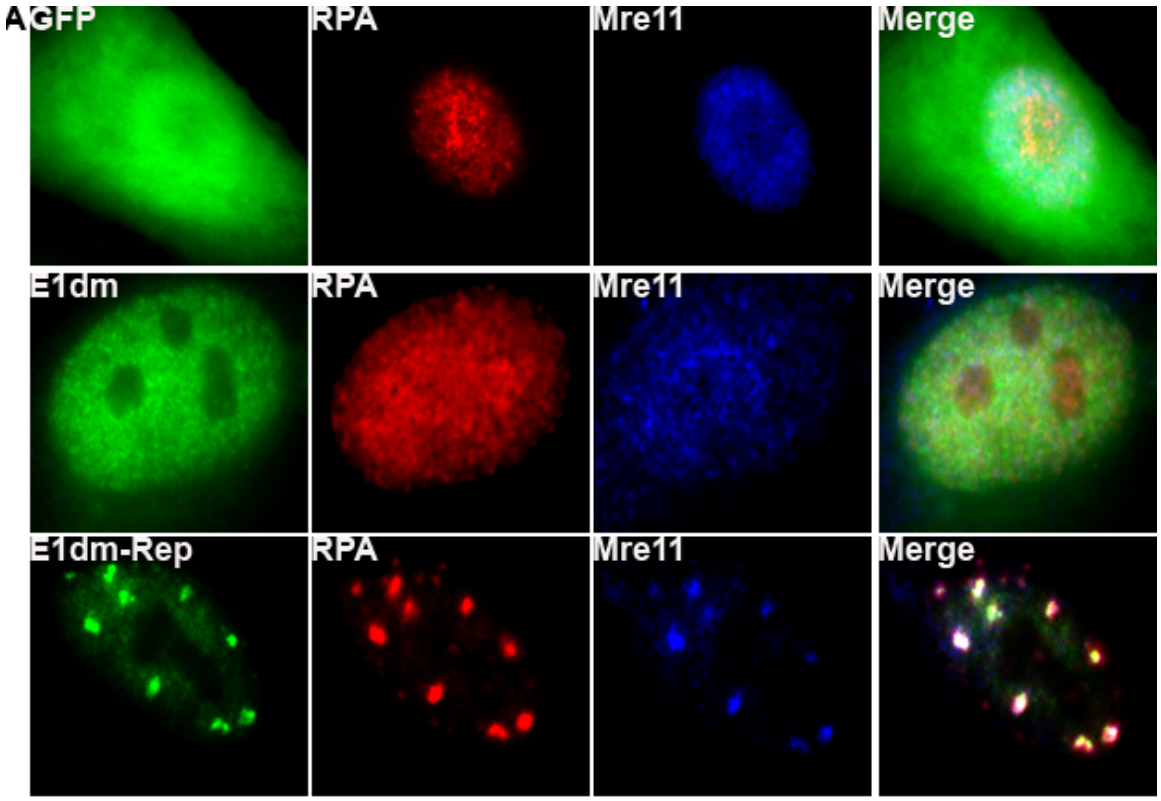

A.
E1dm
mCherry-E2
Merge
B.
GFP
RPA
Merge
E1dm
RPA
Merge
E1dm-Rep
RPA
Merge
C.
GFP
Cyclin A
Merge
E1dm
Cyclin A
Merge
E1dm-Rep
Cyclin A
Merge

Fig. 1**. pGFP-E1dm forms large replication foci in the presence of E2 and an ori plasmid in transiently transfected PHKs at 48 hr post-transfection** (for A, B, and C). **A,** pGFP-E1dm (green, left panel) and pmCherry-11E2 (red, right panel) colocalize in small nuclear foci as shown in the merged images (right panel). Cells were transfected with pGFP-C1 (top row), pGFP-11E1dm (middle row), or pGFP-11E1dm, pMT2-11E2 and an ori plasmid (bottom row, labeled -Rep in this and the subsequent color images); specimens were probed with antibodies to RPA or cyclin A. Left column, GFP; middle column, RPA (**B**) or cyclin A (**C**) **(**red, Alexa Fluor 555); right column, merged image with DAPI stained nuclei. Large GFP-11E1dm foci positive for RPA or cyclin A were observed only under ori replication conditions.


A
GFP
RPA
Mre11
Merge
E1dm
RPA
Mre11
Merge
E1dm-Rep
RPA
Mre11
Merge

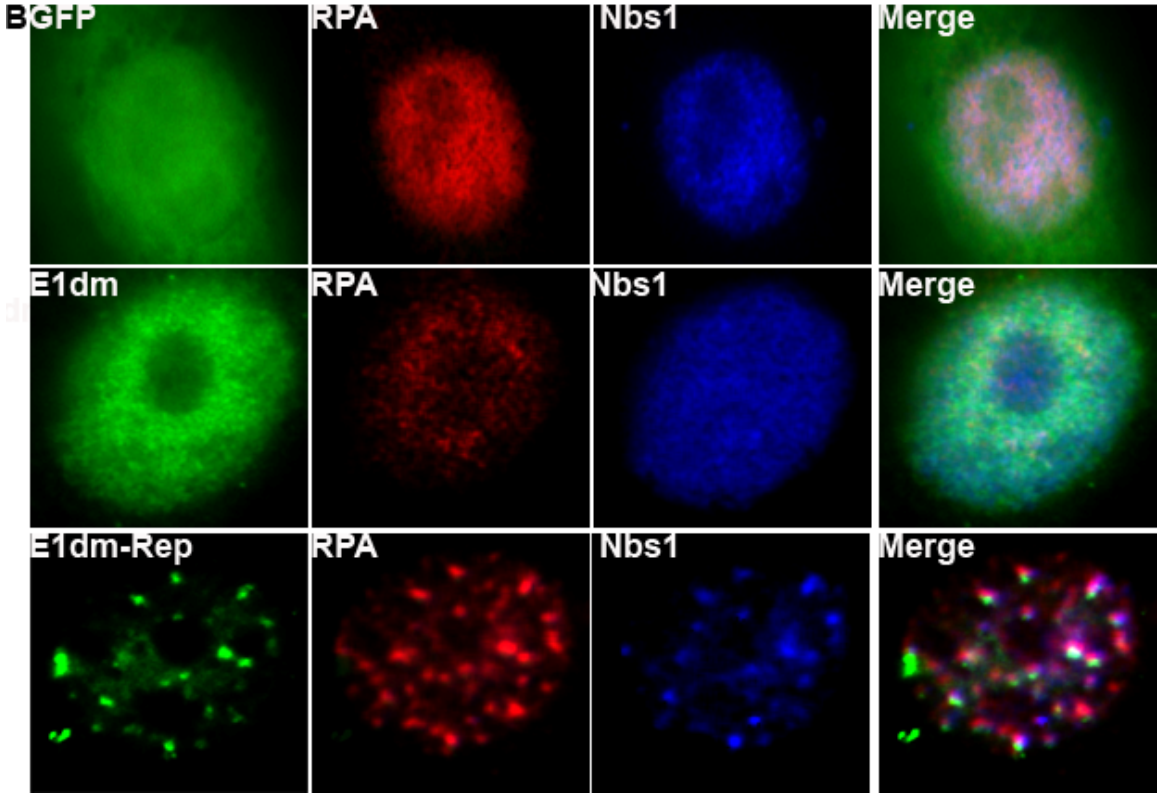
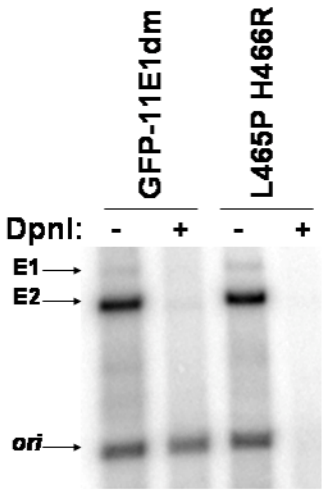

B
GFP
RPA
Nbs1
Merge
E1dm
RPA
Nbs1
Merge
E1dm-Rep
RPA
Nbs1
Merge

Fig. 2. **HPV-11 DNA replication induces relocalization of Mre11 and Nbs1 to the replication foci**. PHKs were transfected with pGFP-C1 (top panels), pGFP-11E1dm (middle panels), or three plasmids for ori replication (bottom panels), as indicated. At 48 hr post- transfection, cells were probed with a mouse antibody against RPA (**A** and **B,** second column from left) and rabbit antibodies against Mre11 (**A**, third column from left) or Nbs1 (**B**, third column from left). Secondary antibodies were α-mouse (red, Alexa Fluor 555) and α-rabbit (Alexa Fluor 647, pseudo-colored blue). Four images are presented for each cell, with GFP or GFP-11E1dm fusion protein in green, RPA in red, and Mre11 and Nbs1 pseudo-colored in blue. Merged images are shown in the right columns.

A


GFP-11E1dm
L465P H466R
DpnI: - + - +
E1
E2
ori

B

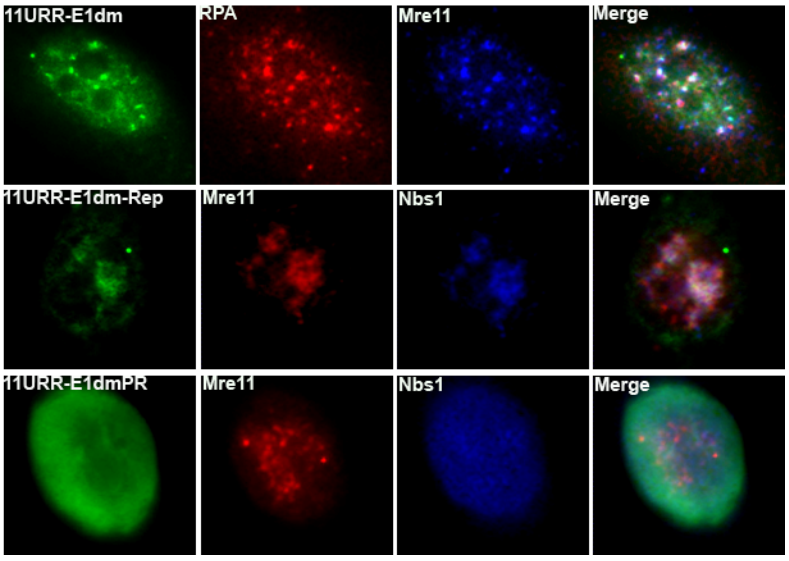

11URR-E1dm
RPA
Mre11
Merge
11URR-E1dm-Rep
Mre11
Nbs1
Merge
11URR-E1dmPR
Mre11
Nbs1
Merge

Fig. 3. **Replication-deficient HPV-11E1dm does not colocalize with MRN complexes**. (**A**) HEK293 cells were transfected with pMT2-11E2 and an ori plasmid along with pEGFP-11E1dm wild type or the GFP-11E1dmL465PH466Rmutation. 48 hours post-transfection, low molecular weight DNA was isolated and subjected to *HindIII* restriction digestion, first and third lanes, or *Hind III* and *Dpn1* digestions, second and fourth lanes. The DNA was then subjected to gel electrophoresis, and *DpnI*-resistant ori DNA was detected by Southern blot hybridization. The E1L465PH366 mutation did not support transient replication. (B) PHKs were cotransfected with pMT2-11E2 and pURR-11E1dm wild type or pURR-11E1dmL465PH466R that also harbors the HPV-11 ori located in the upstream regulatory region (URR). Bottom row: there were no replication foci and no Mre11 and Nbs1 relocalization when GFP-E1dmL465PH466R was present. When wild type E1dm was present, there were replication foci in which GFP-E1dm wild type, Mre11 and RPA (top row) or Nbs1 (middle row) were colocalized. Probes: GPF-E1dm in green, RPA or Mre11 in red (second column from left), Mre or Nbs1 pseudo-colored in blue (third column from left), and merged images (right column).

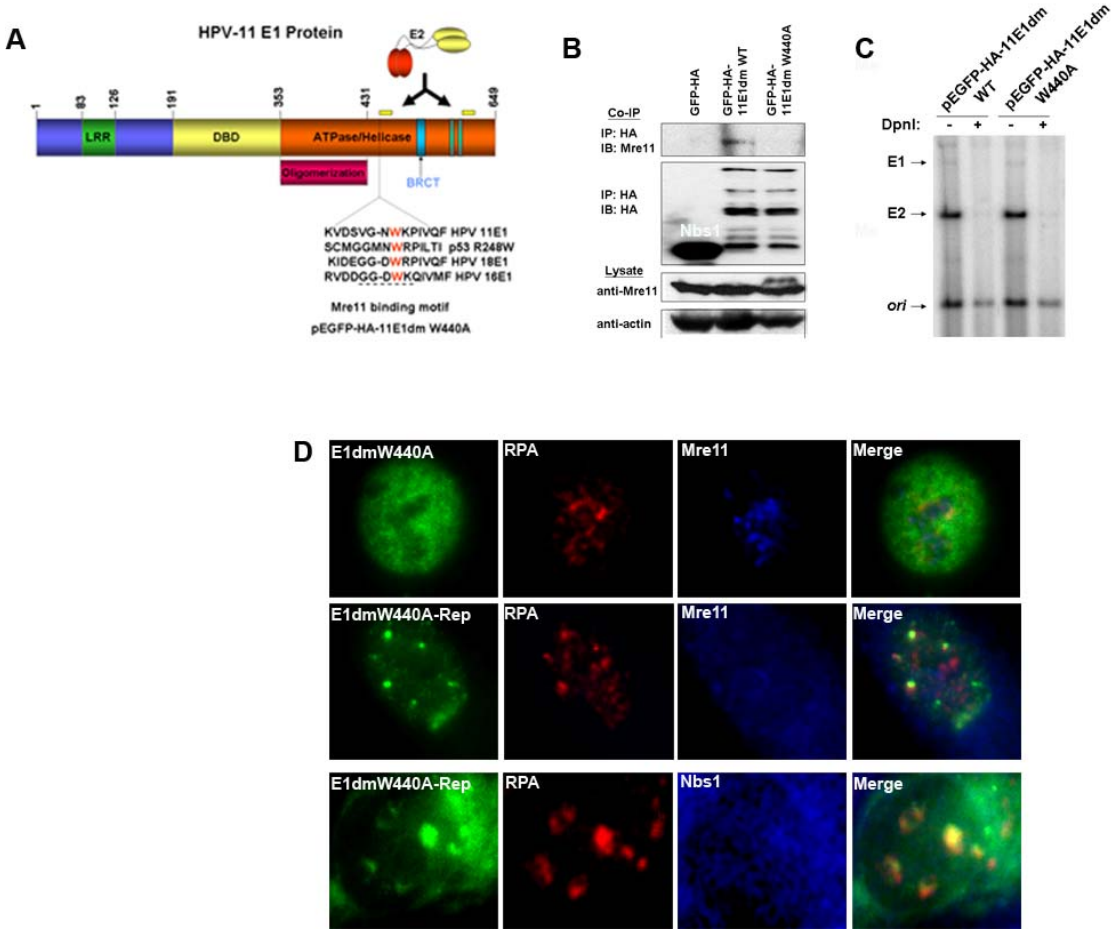

A
HPV-11 E1 Protein
E2
1
83
126
191
353
431
649
LRR
DBD
ATPase/Helicase
Oligomerization
BRCT
KVDSVG-NWKPIVQF HPV 11E1
SCMGGMNWRPILTI p53 R248W
KIDEGG-DWRPIVQF HPV 18E1
RVDDGG-DWKQIVMF HPV 16E1
Mre11 binding motif
pEGFP-HA-11E1dm W440A
B
GFP-HA
GFP-HA-11E1dm WT
GFP-HA-11E1dm W440A
Co-IP
IP: HA
IB: Mre11
IP: HA
IB: HA
Nbs1
Lysate
anti-Mre11
anti-actin
C
pEGFP-HA-11E1dm WT
pEGFP-HA-11E1dm W440A
DpnI:
- + - +
E1
E2
ori
D
E1dmW440A
RPA
Mre11
Merge
E1dmW440A-Rep
RPA
Mre11
Merge
E1dmW440A-Rep
RPA
Nbs1
Merge

Fig. 4. **GFP-E1dmW440A is replication competent but does not interact with Mre11**. (**A)**. Schematic diagram of HPV-11 E1 protein features, with the Mre11 binding site underlined and the location of W440A in red; this critical tryptophan residue is highly conserved in many HPV types. (**B**) HEK293 cells were transfected with pEGFP-HA, pEGFP-HA11E1dm wild type or the GFP-HA11E1W440A mutation. 48 hours post-transfection, the cells were harvested and equal amounts of protein from each sample were used to perform co-IPs using α-HA antibodies. The samples were then separated by SDS-PAGE and analyzed by Western blot. Mre11 was revealed using an α-Mre11 antibody. **(C)** HEK293 cells were transfected with pMT2-11E2 and an ori plasmid along with pEGFP-11E1dm wild type or the GFP-11E1W440A mutation. 48 hours post-transfection, low molecular weight DNA was isolated and subjected to *HindIII* digestion (first and third lanes), or *Hind III* and *Dpn1* digestion (second and fourth lanes). The DNA was then separated by gel electrophoresis and *DpnI*-resistant ori DNA was detected by Southern blot hybridization. (**D)** PHKs were cotransfected with pGFP-E1dmW440A alone (top row) or along with pMT2-11E2 and an ori plasmid (middle and bottom rows). Only under replication conditions (-Rep) did GFP-E1dmW440A form replication foci positive for RPA (compare middle to top rows) but the foci were not positive for Nbs1 or Mre11 (middle and bottom rows). Probes: GFP-E1 fusion protein in green, RPA in red, and Mre11 and Nbs1 pseudo-colored blue, as labeled. Merged images are shown in the right columns

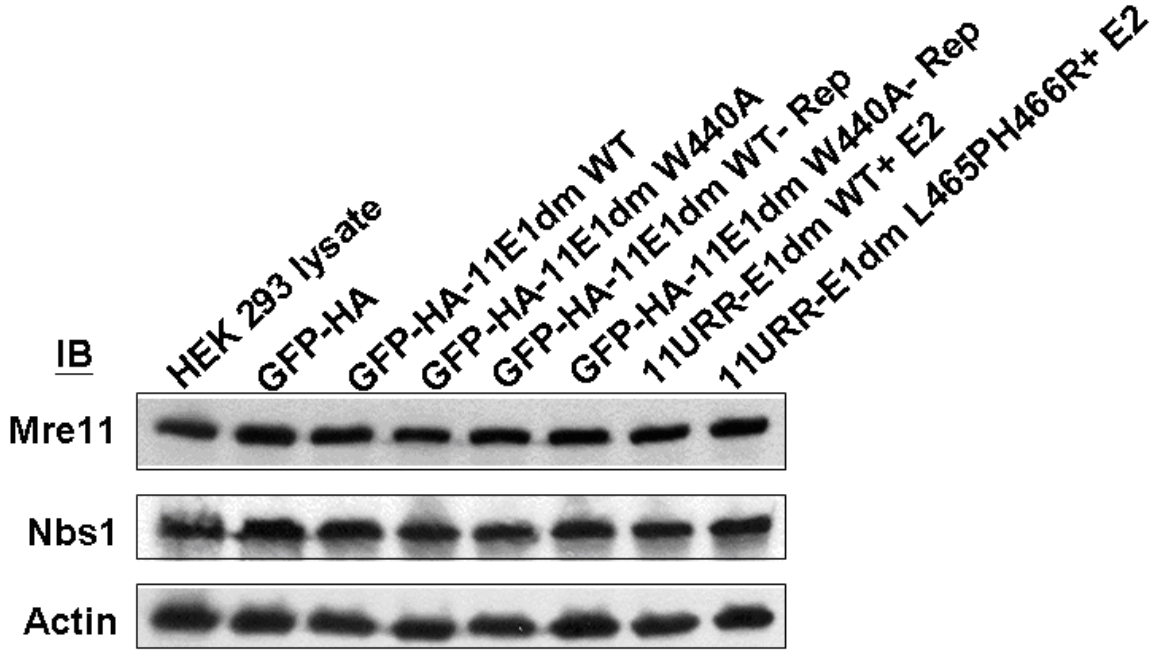
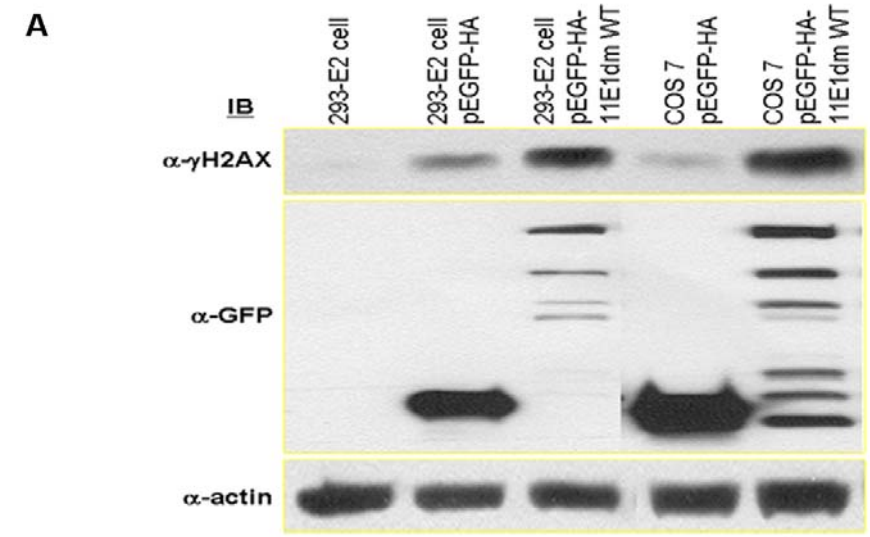

IB
HEK 293 lysate
GFP-HA
GFP-HA-11E1dm WT
GFP-HA-11E1dm W440A
GFP-HA-11E1dm WT- Rep
GFP-HA-11E1dm W440A- Rep
11URR-E1dm WT+ E2
11URR-E1dm L465PH466R+ E2
Mre11
Nbs1
Actin

Fig. 5. **HPV-11 replication does not induce degradation of Mre11 or Nbs1.** 293 cells were transfected with the indicated plasmid or plasmids. 48 hours post-transfection, the cells were harvested and lysed. Equal amounts of lysates were loaded and separated by SDS-PAGE. Mre11, Nbs1 and actin were detected using rabbit polyclonal antibodies.


A
IB
293-E2 cell
293-E2 cell pEGFP-HA
293-E2 cell pEGFP-HA-11E1dm WT
COS 7 pEGFP-HA
COS 7 pEGFP-HA-11E1dm WT
α-γH2AX
α-GFP
α-actin

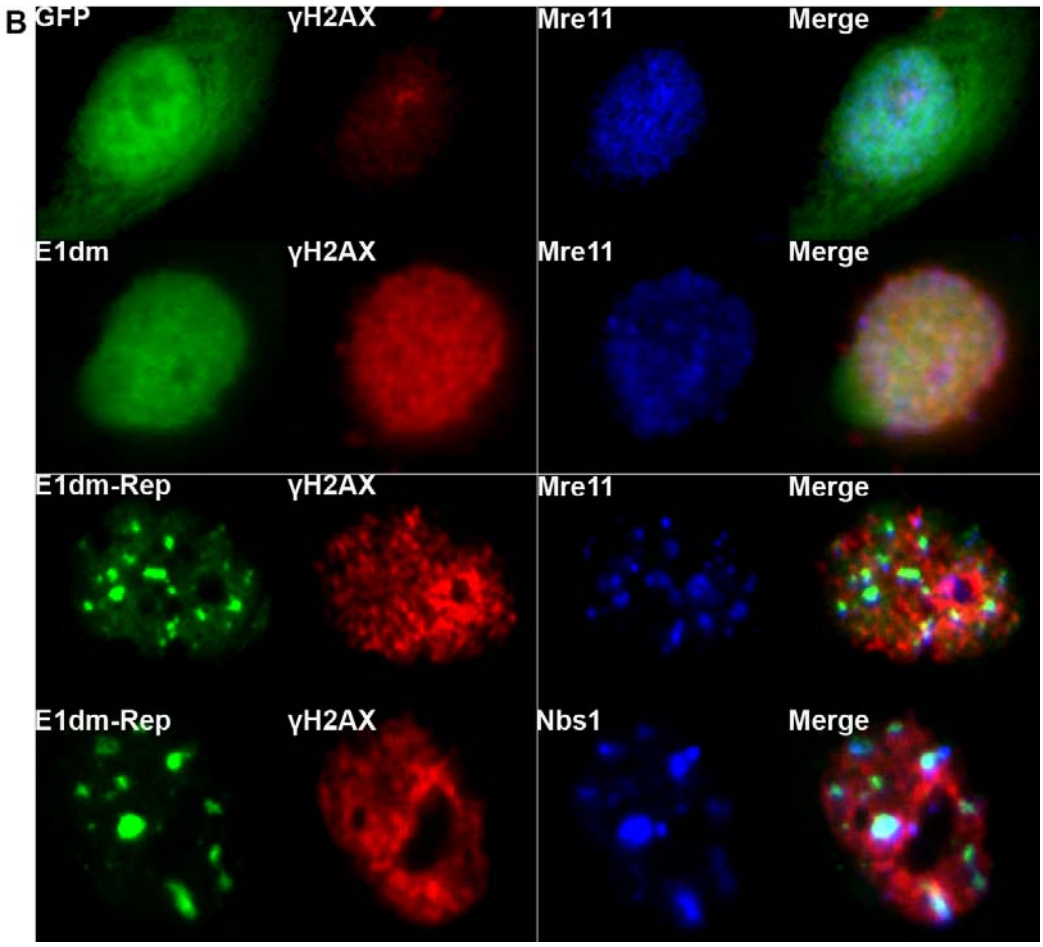

B
GFP
γH2AX
Mre11
Merge
E1dm
γH2AX
Mre11
Merge
E1dm-Rep
γH2AX
Mre11
Merge
E1dm-Rep
γH2AX
Nbs1
Merge

Fig. 6**. HPV-11 E1dm induced elevated γH2AX in a diffuse manner**. **A**, western blots of lysates of 293-E2 cells or COS7 cells 24 hr after transfection with pEGFP-HA or pEGFP-HA11E1dm. γH2AX, GFP, and actin were detected using specific antibodies. **B**, Immunofluorescence detection of γH2AX (red) relative to GPF-E1dm in PHKs 48 hr post- transfection. Relative to cells transfected with pGFP-C1 (top row), the signals of γH2AX (red) were increased in the presence of GFP-E1dm alone (second row from the top) or under replication conditions (bottom 2 rows, labeled –Rep). The replication foci, as revealed by colocalization of GFP-E1d (green) and Mre11 or Nbs1 (pseudocolored blue), did not have enhanced γH2AX signals.

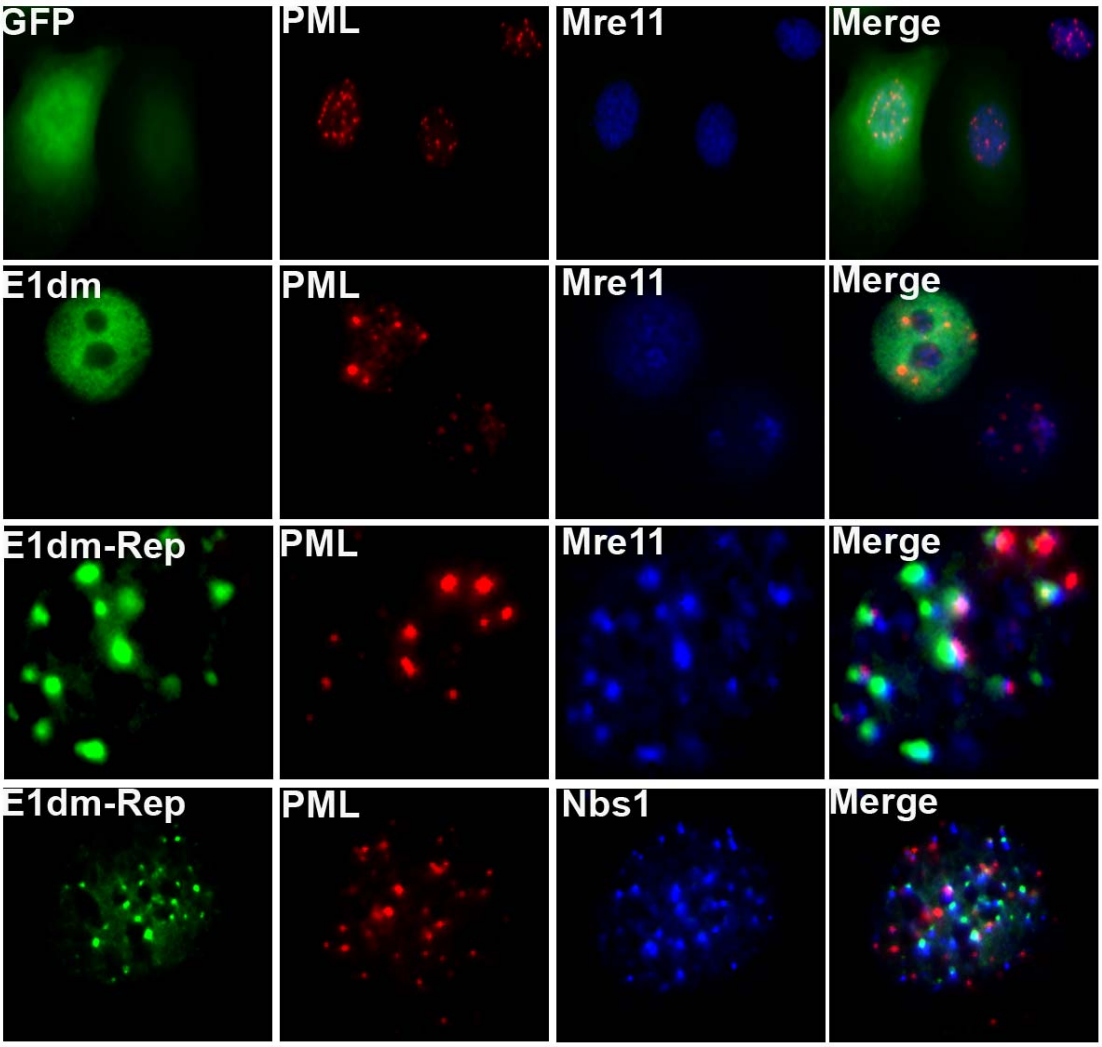

GFP
PML
Mre11
Merge
E1dm
PML
Mre11
Merge
E1dm-Rep
PML
Mre11
Merge
E1dm-Rep
PML
Nbs1
Merge

Fig. 7 **HPV-11 E1dm does not colocalize exclusively with the PML nuclear bodies**. Immunofluorescence detection of PML localization (red) relative to GPF-E1dm replication foci in PHKs 48 hr post-transfection. PML bodies (red) formed punctate dots found throughout the nuclei in PHKs transfected with GFP (top row), GFP-11E1dm (green) alone (second row), or under replication conditions (bottom 2 rows, labeled – Rep). Under replication conditions, PML nuclear bodies (red) were found to colocalize with only some of the replication centers (bottom 2 rows), whereas Mre11 and Nbs1 (pseudocolored blue) were always localized to the viral replication centers (third and fourth rows, respectively).

Chapter 4

SUMMARY

Human papillomaviruses are prevalent and widespread pathogens which cause benign proliferation of infected epithelia. Infrequently, infections with the HR HPV genotypes can progress from a state of benign proliferation to cellular immortalization, transformation and carcinogenesis (160). The papillomaviral infection cycle is closely tied to the differentiation stages of the keratinocytes forming squamous epithelia, with vegetative amplification of the viral genome occurring only in well-differentiated cells. In the basal layer of the epithelia, where initial PV infection occurs, the keratinocytes are generally quiescent until division is needed to replenish senescing cells comprising the transit amplifying layer, or following wounding to promote healing.

Viral replication requires the virus encoded E1 replicative helicase and E2 origin binding protein, as well as the sequences comprising the viral origin of replication. In the basal cells, the viral DNA is maintained at low copy numbers, approximately 10-50 copies per cell. To persist, the virus must replicate its genome and have a mechanism to ensure that the genomes are equitably distributed to each daughter cell during cell division. The mechanisms by which various papillomaviruses maintain their genomes in the dividing basal cells have been investigated by several labs in the last few years. All point to a role of the viral E2 protein

During mitosis the BPV-1 E2 protein associates with the mitotic chromosomes, mediated by its interaction with the cellular bromodomain protein Brd4 through the E2N

domain (1, 2, 13, 153). During mitosis, BPV-1 E2 forms foci on the mitotic chromosomes that colocalize with Brd4. In contrast, previous work in our lab demonstrated that the E2 proteins of HPV-11, -16, and -18 colocalize with the mitotic spindle fibers during mitosis. This observation was further confirmed with biochemical data showing HPV-11 E2 was able to co-immunoprecipitate β-tubulin, which is a component of the spindle fibers. Furthermore, in the presence of the HPV-11 E2 protein, an ori-containing plasmid localizes to the mitotic spindles (144).

Studies of a number of DNA viruses show that viral DNA replication is intimately linked to the MRN complex, the cellular machinery which repairs double-stranded breaks. One of the major specific aims of my dissertation research was to provide an understanding of mechanisms that regulate HPV-11 viral DNA replication and viral plasmid partitioning.

In the first part of my dissertation, HPV-11 DNA partitioning during mitosis was investigated. We first examined the distribution of a FLAG-HA-tagged E2 protein by indirect immunofluoresence detection with α-FLAG or α-E2 antibodies using a cell line in which the transcription of HPV-11 E2 is regulated by doxycyclin. Antibody to β-tubulin, a component of the mitotic spindles, was used to mark the difference stages of mitosis. Our results showed that, during prometaphase and metaphase, the E2 protein was localized exclusively to the mitotic spindles, in agreement with observations made with GFP-E2 in transfected COS7 Cells (144). As the cells progressed into anaphase, the E2 protein was still localized to the mitotic spindles, but we also observed increased concentration of the E2 protein in the middle of the cell where the metaphase plate and midbody would eventually form. During the final phases of mitosis, telophase and cytokinesis, the majority of the E2 protein was redistributed from the spindles to the

metaphase plate and the midbody. At no time did we observe an association between the E2 protein and GFP-Brd4 which was ectopically expressed in these cells (see discussion below).

BPV-1 E2 protein is localized to the mitotic chromosomes. To understand the basis for this significant difference, we next localized the spindle association domain of HPV-11 E2. Using C-terminal deletions, we delineated the spindle localization motif within the E2C domain of HPV-11. This domain, spanning residues 285-308, is highly homologous to comparable regions of HPV-16 and 18 E2 proteins that also localize to the mitotic spindles (144). To identify the specific residues in this region responsible for spindle localization, we examined the protein crystal structure of the HPV-16 E2C and found a promising candidate. Based on this structure, site-directed mutagenesis in a domain immediate upstream of the DNA binding motif, which spans residues 296-308, show that multiple residues are involved in spindle association whereas individual point mutations have no effect. Additional studies of E2 protein of HPV-16, HPV-18 and BPV-4 revealed that each E2C also associated with the mitotic spindles, but only when the corresponding and homologous spindle association domain was present.

In contrast, the BPV-1 E2 protein interacts with the mitotic chromosomes and the amino terminal domain of BPV-1 E2 mediates this association (13). The amino acids comprising the spindle association domain of HPV-11 E2 are not conserved in BPV-1 E2. Indeed, GFP-BPV-1 E2C alone exhibited no discernable localization pattern and was diffuse throughout the cell. We then generated two chimeric E2C fusion proteins, where we swapped the first 10 amino acids of the HPV-11 E2C domain for the corresponding BPV-1 E2C region, and vice versa to form pGFP-H11/B1E2C and pGFP-B1/H11E2C.

When these proteins were analyzed for spindle localization, we observed that pGFP-H11/B1E2C, which has the HPV-11 E2 spindle localization domain fused to BPV-1 E2C, was able to localize to the spindles, whereas pGFP-B1/H11E2C, which has the first 10 amino acids of BPV-1 E2C, was not able to associate with the mitotic spindles.

These studies clearly demonstrated that two different mechanisms are used by E2 proteins of different papillomaviruses to promote equitable viral plasmid partitioning during mitosis. However, it is still unclear whether this is the sole mechanism for viral DNA segregation. It had been reported that for BPV-1 and HPV-16 the E2 protein interacted with the cellular protein Brd4 during mitosis as a mechanism for viral genome maintenance. However, recent work on E2 proteins of BPV-1, HPV-16 and HPV-11 discounts this hypothesis. Using yeast-two hybrid approach, Parish et al. identified ChlR1, an ATP-dependent DNA helicase which associates with cohesin during mitosis, as an E2 binding partner during mitosis (107). These authors have demonstrated that the interaction of BPV-1 E2 with ChlR1 is necessary for maintenance of the viral genome, whereas a mutant BPV-1 E2 protein, W130R which no longer binds to ChlR1, does not confer viral DNA persistence. It could be that for HPV-11 E2, which has a dynamic localization pattern during mitosis, an association with both the spindles and ChlR1 is necessary for proper viral plasmid segregation (107). Nevertheless, we did not detect any HPV-11 E2 on the mitotic chromatin itself. One possible technical reason for this could be that the GFP-E2 signal was too intense and prevented us from discerning discrete chromosome-associated E2.

Previous work in our lab has shown that plasmids containing Gal-4 binding sites and the viral ori associated with the mitotic spindles during mitosis in the presence of the

E2 protein, when visualized with a GFP-Gal4 fusion protein. We have not, however, tested whether mutations of the spindle association domain in the context of the full E2 protein are able to abrogate the ability of an ori plasmid to associate with the spindle. This is because the amino terminal domain can independently associate with the mitotic spindles, but for this we have not been able to delineate the responsible residues (unpublished results) (144). In fact, the deletion mutation suggests that large regions of E2N contribute to spindle association. Since the amino terminus is necessary for HPV ori replication, it would be extremely difficult to construct an E2 mutation completely devoid of spindle association without affecting its ability to support ori replication.

The second part of this study deals with the interactions between the HPV replication complexes and the DSB repair complex MRN. Investigations of SV40 and adenovirus showed that proteins of both viruses interacted with components of the MRN complex, leading to relocalization of Mre11 and Nbs1 to the viral replication centers and the degradation of residual MRN proteins. Recruitment of the MRN complex to the replication centers also resulted in downstream recruitment and activation of ATM, leading to phosphorylation of H2AX which also colocalizes with the viral replication centers. It is thought that T-ag-bound SV40 replicating minichromosomes are recognized as damaged chromosomes that have to be repaired. In adenovirus, the relocalization and degradation of Mre11 and Nbs1, appears to be necessary to prevent the concatomerization of the linear genome which cannot be packaged into viral capsids. We investigated whether the MRN complex also interacts with HPV replication centers, and our findings are distinct from those from studies of SV40 and adenovirus. In our studies, we took advantage of a replication-competent GFP-HPV-11 E1 protein which

harbors a splice donor mutation in the mRNA, GFP-11E1dm, described by a previous graduate student in the lab (43). This mutation did not affect protein coding but allows for high levels of GFP-E1 protein to be produced in transfected cells, facilitating immunofluorescent characterization of the replication complexes in transiently transfected cells.

We first demonstrated via GFP-E1dm that the HPV-11 ori replication complex is indeed observable in primary human keratinocytes, the native host cells for HPV infection. The large GFP-E1dm foci only formed in the presence of E2 and ori plasmid. These foci colocalized with RPA as well as with cyclin A, a regulatory subunit of cdk2 and cdk1, necessary for maintaining E1 in the nucleus (44). Next, we performed localization studies of GFP-E1dm-positive replication foci to ascertain if any of the components of the MRN complex were recruited to the viral replication centers.

Our results show that both Mre11 and Nbs1, but not Rad50, colocalize to the replication centers and that this recruitment is dependent on active DNA replication. Cotransfection of an E2 expression vector and p11URR-GFP-E1dmL465P, which harbors the HPV ori and encodes a replication-incompetent E1 mutation, did not form replication foci, nor did we detect altered localization of Mre11 or Nbs1. Thus, the recruitment of these two host proteins is not mediated by the E2 protein.

Furthermore, we identified a sequence motif in the E1 helicase domain which is conserved among E1 proteins of many papillomavirus genotypes. This motif is responsible for binding of Mre11. The wild type, but not the mutation W440A in this motif, was able to co-immunoprecipitate Mre11. Of note, Nbs1 was not detected in the immunoprecipitates, suggesting that it is brought to the replication centers via binding to

Mre11. In contrast, the SV40 T-antigen, which functions as the origin binding protein and replicative helicase during viral DNA replication, associates with Nbs1 (156).

There are additional major differences between our observation and those of SV40. First, we observed no reduction in Mre11 or Nbs1 protein levels under HPV ori replication conditions. Second and more importantly, our data suggest the recruitment of Mre11 and Nbs1 is not necessary for HPV-11 ori replication, as the HPV-11 E1W440A mutation, which was unable to bind Mre11 (as assayed by coIP) nor to recruit of Mre11 and Nbs1 to the replication centers, was nevertheless fully competent for ori replication. Third, recruitment of Mre11 and Nbs 1 to the papillomavirus ori replication centers is not accompanied by an activation of ATM at these foci. Although phosphorylation of H2AX to γH2AX increased significantly upon the expression of GFP-E1dm, the replication centers did not colocalize with γH2AX. All these observations are completely contrary to SV40 replication for which the activity of ATM is necessary during which γH2AX colocalizes with the replication centers (156).

Why then would E1 recruit Mre11 and Nbs1 to the replication centers? It is a possibility that the functional significance for Mre11 or Nbs1 will only be manifested in the context of the whole viral genome in differentiated epithelia. Our lab has recently developed a novel system in which the HPV-18 genomic plasmid is generated by Cre-loxP mediated recombination in vivo (Wang et al., in press). The excised HPV-18 genome then amplifies to high copy number and high-titer infectious virus can be produced for the first time. It will be most interesting to test an HPV-18 genome with the Mre-binding mutation in the E1 protein in this culture system to determine whether this mutation impairs viral DNA amplification.

In conclusion, detailed microscopic studies and molecular evaluations of the E2 protein and the viral DNA replication complex have added considerably to our understanding of the viral life cycle, particularly in viral persistence and viral genome amplification. Prophylactic HPV vaccines such as Gardasil by Merck, Cervarix by GlaxoSmithKline (GSK), as well as newer generations of effective and inexpensive vaccines, will be increasingly effective preventive measures against new HPV infections. Excellent molecular diagnostic tests for HPV DNA and RNA are providing the tools for sensitive and accurate early detection of existing viral infections. Presently missing are pharmaceutical capabilities in the form of small molecule antiviral agents or therapeutic vaccines to treat diagnosed lesions. A thorough knowledge about how papillomaviruses replicate will be centrally instrumental in discovery of drugs to treat HPV lesions while they are still benign and well before they have progressed to high-grade squamous intraepithelial lesions. Within our life times, HPV-associated diseases worldwide will be significantly reduced.

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