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Topography of bovine papillomavirus E2 protein on the viral genome during the cell cycle

Suzanne M. Melanson [1], Elliot J. Androphy *

Department of Medicine, University of Massachusetts Medical School, 364 Plantation Street LRB 328, Worcester, MA 01605-2324, USA

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ABSTRACT

The multifunctional papillomavirus E2 protein serves important roles in transcriptional activation and genome maintenance and cooperates with the viral E1 helicase for the initiation of viral DNA replication. The bovine papillomavirus genome contains seventeen E2 binding sites, largely concentrated within the long control region, and a single E1 binding site at the origin of viral replication. Using chromatin immunoprecipitation (ChIP) followed by restriction enzyme digestion and PCR, we show that BPV E1 was present only in the region of an active origin of replication and that BPV E2 remained attached to definable segments of the viral genome at specific stages of the cell cycle.

Introduction

Papillomaviruses (PV) are responsible for a variety of benign and malignant epithelial lesions including carcinoma of the cervix (Zur Hausen, 2002). Papillomavirus genomes consist of approximately 8 kilobases of double stranded DNA that, upon infection, are stably maintained as low copy episomes in the basal layer of epithelial cells until induced by cellular differentiation to initiate programmed viral genome amplification. Bovine papillomavirus type 1 (BPV-1) has been historically utilized as a model for the study of PV genome replication. BPV-1 genomic or sub-genomic DNAs that are transfected into murine C127 and NIH3T3 cell lines can replicate autonomously, displaying a stable copy number and expressing the essential early viral proteins (Law et al., 1981). The murine C127 cell line is also transformed by BPV-1 without integration of viral DNA into the host genome. The mechanism of cell transformation by BPV-1 is still not fully understood, however both the BPV-1 E5 and E6 proteins efficiently perform this task (Bergman et al., 1988; Schiller et al., 1984).

The major function of E2 is regulation of BPV-1 transcription. The N-terminal two-thirds of E2 encodes a potent transcriptional activation domain (TAD) that stimulates expression from E2 binding site containing reporters in mammalian and yeast cells (Haugen et al., 1987; Morrissey et al., 1989; Spalholz et al., 1985; Stanway et al., 1989). E2, through its C-terminal domain, binds to a specific palindromic sequence found in all PVs (Androphy et al., 1987; McBride et al., 1988; Sedman and Stenlund, 1995). There are 17 potential E2 binding sites of variable affinities within the BPV-1 genome (Li et al., 1989), the majority of which are clustered in the long control region (LCR), which includes the major promoter elements and the viral origin (Fig. 1). Several E2 binding sites appear adjacent to promoters within the E1 and E2 genes.

In addition to its function in transcriptional activation, the E2 protein is necessary for both transient replication and persistence of replicated viral episomes. BPV and HPV genomes have been shown to associate with cellular chromatin during mitosis. This is thought to retain viral episomes following dissolution of the nuclear envelope in order to ensure equal partitioning of newly replicated PV into emerging daughter cells (Ilves et al., 1999; Lehman and Botchan, 1998). The E2 C-terminal DNA binding domain identifies the viral genome, while cellular factors that interact with the N-terminal transactivation domain (TAD) facilitate genomic tethering to mitotic chromosomes (Abroi et al., 2004). The cis-acting sites involved in mitotic segregation of the viral genome were defined by deletional studies and overlap the cluster of E2 binding sites in the 5′ BPV-1 LCR. This region has been labeled as the minichromosome maintenance element (MME) (Abroi et al., 2004; Lehman and Botchan, 1998; Pirsoo et al., 1996). The *trans* factors that connect E2 to mitotic chromosomes have been intensively studied. Our lab reported that the interaction of E2 with ChlR1, a DNA helicase involved in establishment of proper chromatid cohesion, is necessary for maintenance of viral episomes (Parish et al., 2006). While it was proposed that the bromodomain protein Brd4 tethers E2 proteins to mitotic chromosomes more recent investigations indicate that Brd4 is also necessary for the transcriptional functions of E2 (Ilves et al., 2006; McPhillips et al., 2006; Schweiger et al., 2007; Wu et al., 2006; You et al., 2004).

E1 and E2 are the only viral proteins required for transient replication of the genome (Mohr et al., 1990; Sedman and Stenlund, 1995; Ustav and Stenlund, 1991). The E1 protein is an ATP dependent

* Corresponding author. Fax: +1 508 856 6797.
E-mail address: elliot.androphy@umassmed.edu (E.J. Androphy).
[1] Current address: Department of Molecular Genetics and Microbiology, Cancer Research Facility University of New Mexico Health Sciences Center, Albuquerque NM 87131.

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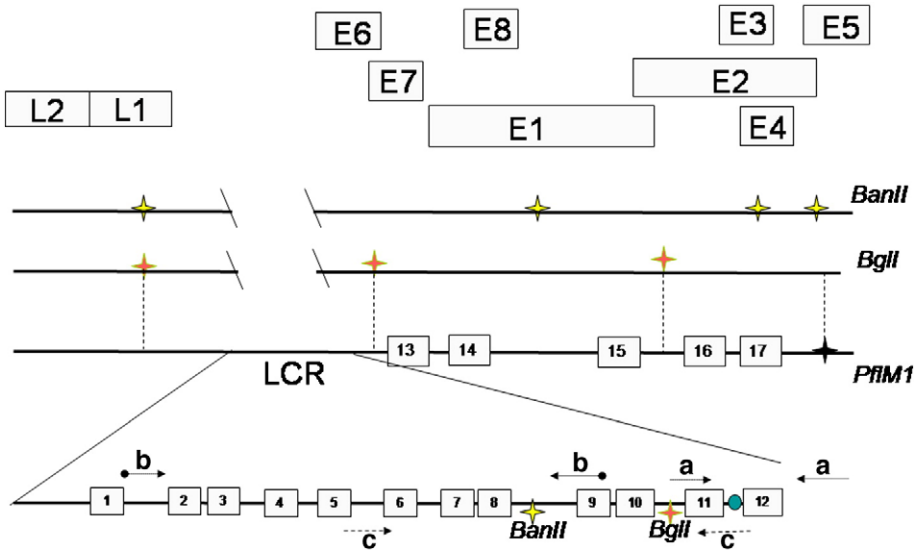

Fig. 1. Schematic of the BPV-1 genome showing E1 and E2 binding sites. E2 binding sites are shown as numbered boxes 1–17. Symbols on the upper line represent BanII restriction sites outside the viral LCR. The viral origin is depicted between E2 sites 11 and 12. The LCR sense and antisense primers which flank the BglI and BanII cut sites are represented by dashed arrows; the primers for the origin are shown as simple arrows, with the primers for the upstream region of the LCR which flank only the BanII cut site represented by ball and arrow. The letters A, B and C represent primer pairs listed in Materials and methods.

replicative helicase that binds DNA with low specificity (Sedman and Stenlund, 1995; Ustav et al., 1993). In a cell free system, E1 can initiate DNA replication in the absence of the viral E2 protein (Grossel et al., 1996; Lusky et al., 1993). The role of the E2 binding sites in replication *in vivo* has been characterized by transfection of engineered plasmids with mutant LCR constructs along with E1 and E2 expression plasmids, and in cell lines stably expressing E1 and E2 (Liang and Botchan, 1990; Lusky et al., 1993, 1994; Mohr et al., 1990; Sedman and Stenlund, 1995; Ustav et al., 1993; Ustav and Stenlund, 1991). *In vitro* and *in vivo* data demonstrated that a functional origin for transient replication consisted of E2 binding site 12 (BS12) flanking an E1 specific binding sequence (Lusky et al., 1993; Sedman and Stenlund, 1995; Ustav et al., 1993). The prevailing model is that E2 directly binds and targets E1 to the origin (Mohr et al., 1990). *In vitro* and structural data indicate that E2 must dissociate from E1 to allow for assembly of E1 double hexamers and initiation of DNA replication (Abbate et al., 2004; Lusky et al., 1994; Sedman and Stenlund, 1996, 1998; Yang et al., 1993). The experiments presented herein provide physical evidence to support this model as we detected the presence of both E1 and E2 at the origin *in vivo* during G1/S.

In this report we used a modified chromatin immunoprecipitation (ChIP) assay to define the segments of the BPV-1 episome that interact with E2 and E1 during cell cycle in monolayer cultures of transformed mouse cells. Published data would suggest that E2 is released from the E1 complex that assembles on the origin during G1/S phase; however the question remains what happens to E2 between G1/S and mitosis? Our ChIP protocol was modified to include restriction enzyme digestion, following immunoprecipitation to release segments of the viral genome that were not cross-linked to the E2 complexes. Bound regions of the DNA were differentiated using specific primers and amplification by polymerase chain reaction (PCR). Since sonication stochastically disrupts DNA, this modified protocol allows for finer resolution of binding site occupancy. With this assay we show binding of E2 to specific sites in different phases of the cell cycle and place E1 at the origin at initiation of viral replication.

Results

To investigate E2 binding site occupancy *in vivo*, we used the C127 cell lines ID13 and A3 that stably replicate and maintain BPV-1. The A3 genome contains three serine-to-alanine mutations in the hinge region of the E2 protein at amino acids 290, 298 and 301. These mutations stabilize the E2 protein and result in increased average genome copy number (Lehman and Botchan, 1998; Lehman et al., 1997; McBride and Howley, 1991). To initiate the ChIP studies, it was first necessary to test the efficacy of the available antibodies to the BPV-1 proteins. A rabbit antibody to E2 (II-1) was used in immunoprecipitations from ID13 and A3 cells along with the parental C127 cell line. Detection on an immunoblot was achieved with the monoclonal E2 antibody B201 (Fig. 2 top panel). It is interesting to note that the C-terminal repressor form of E2 (E2R) (Hubbert et al., 1988; Lambert et al., 1987) was not detected in the A3 cell lysates. The BPV-1 E1 protein is expressed at low levels in cells that stably replicate viral episomes. Prior to this study detection of E1 has been largely restricted to overexpression systems and natural expression has been rarely visualized (Sun et al., 1990). Here the BPV-1 E1

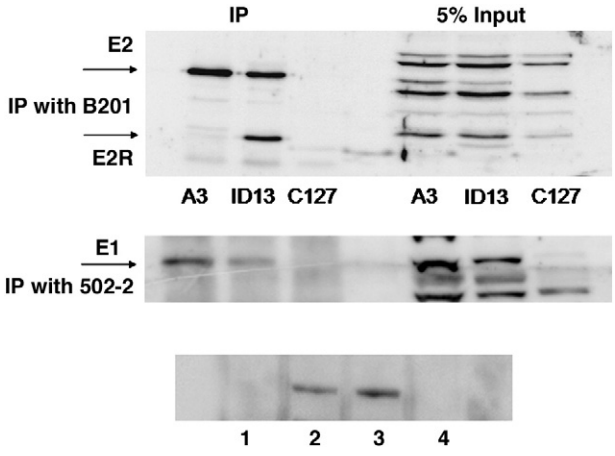

Fig. 2. A3 cells, ID13 cells and the parental cell line C127, were lysed and immunoprecipitated with monoclonal antibody to BPV-1 E2 (B201). The proteins were immunoblotted using the rabbit antibody II-1 and visualized with the Thermo-scientific dura ECL detection kit (top panel). A3, ID13 and C127 cells were blocked overnight in 5 mM thymidine and BPV-1 E1 was immunoprecipitated using the 502-2 rabbit antibody. E1 was then detected with 502-2 (center panel). A3 cells were blocked in thymidine (lane 2), uncycled (lane 3) or blocked overnight with nocodazole (lane 4) and immunoprecipitated and blotted with the 502-2 rabbit antibody. Lane 1 shows thymidine treated C127 cells (bottom panel).