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MINIREVIEW

Papillomavirus DNA Replication

PAUL F. LAMBERT

McArdle Laboratory for Cancer Research, Department of Oncology, University of Wisconsin School of Medicine, 1400 University Avenue, Madison, Wisconsin 53706

The capacity for bovine papillomavirus type 1 (BPV-1) to transform rodent cells in tissue culture has provided a system for studying regulated replication of a small DNA virus. In these cells, the double-stranded, circular, viral DNA genome stably persists as a multicopy nuclear plasmid (see reference 13 for a review). Both *trans*- and *cis*-acting viral elements are required for plasmid replication. During the past year, many important contributions have been made which have enhanced and changed our outlook on many aspects of papillomavirus replication. This review presents a synopsis of these new findings in the context of earlier studies.

trans ELEMENTS

Papillomaviruses encode proteins necessary for their plasmid DNA replication. In BPV-1, these viral gene products are encoded both by the E1 and E2 translational open reading frames (ORFs). Recent studies indicate that E1 and E2 proteins may directly interact with one another. Viral gene products encoded from other translational ORFs also have been implicated in replication, though their possible roles are uncertain. Our knowledge of the BPV-1 E1 and E2 ORFs is summarized below.

E1 PROTEINS

BPV-1 mutants disrupted in any portion of the E1 ORF are defective for plasmid replication, be it in a transient or stable replication assay (11, 24, 33). These data indicate that the full-length E1 gene product is a positive replication factor. A full-length E1 gene product has been detected in BPV-1-transformed rodent cells and has an apparent molecular size of 68 to 72 kDa (27, 31). E1 is a nuclear protein (4, 27, 31), is phosphorylated both at its N terminus and its C terminus (19, 27, 32), and appears to be short-lived (27).

How does the E1 protein function in replication? E1 and simian virus 40 (SV40) large T antigen (Tag) share regions of amino acid similarity (8) (Fig. 1B). In Tag, these regions contain signals for nuclear localization, sites of amino acid phosphorylation, domains for ATP binding, ATPase, and DNA helicase activities—all properties implicated in Tag's role in viral DNA replication. Supporting the hypothesis that E1 possesses a subset of the Tag activities associated with viral replication, E1 protein can bind ATP (27, 31). This activity can be abrogated by an amino acid substitution at P_{434}; a similar mutation eliminates ATP binding (P_{427}) in Tag (31). BPV-1 containing this P_{434} mutation does not replicate (19, 31). Additional replication-defective E1 mutants have been isolated that are altered at amino acid positions analogous to positions altered in replication-defective Tag mutants, lending further support to the similarity between these two proteins (19).

Unlike SV40 Tag, E1 protein may not possess sequence-specific DNA binding activity or its DNA binding capacity may be altered or augmented by other viral proteins. While several studies on E1 protein overexpressed from recombinant baculovirus vectors have demonstrated that E1 protein alone binds DNA but with no apparent site specificity (4, 27), another recent study suggested that bacterially produced E1 protein binds DNA specifically in or around the unique *Hpa*I site at BPV-1 nucleotide (nt) 1 (36). Sequence-specific binding of E1 to viral DNA may be determined by E1 protein's capacity to complex in vitro with the full-length viral E2 protein, the E2 transcriptional transactivator E2TA (21). The E2TA protein is a sequence-specific DNA binding protein (see reference 13 for a review); correspondingly, the E1-E2TA complex is capable of specifically binding viral DNA fragments containing E2 DNA binding sites (21). A subsequent study indicates that the E1-E2TA complex's affinity for viral DNA fragments may be altered from that of E2TA alone (19). The C terminus of E1 (19) and the N terminus of E2TA (21) are required for this protein-protein complex to form.

In addition to the 68- to 72-kDa full-length E1 ORF gene product, a 23-kDa E1 ORF gene product has been detected in BPV-1-transformed mouse cells (32). It is a nuclear phosphoprotein that is translated from a spliced mRNA species that fuses the 5′ third of the E1 ORF at BPV-1 nt 1235 to a small 13-amino-acid exon at nt 3225 (Fig. 1B). To date no function has been ascribed to this 23-kDa E1 protein. A BPV-1 mutant, predicted to be disrupted in the expression of this protein, was replication competent both in transient (33) and in stable (14) assays. Nevertheless, this small E1 protein is postulated to play a role in replication since it shares a domain with the full-length E1 replication protein.

While most current evidence indicates that the full-length E1 protein has a positive replication function, this activity was localized to the 3′ two-thirds of the E1 ORF in an earlier study (18). This C-terminal-specific E1 gene was termed E1-R (R for replicator). At the genetic level, these results are incompatible with that from other laboratories mapping this function to the full-length E1 ORF (11, 24, 33); at the biochemical level, no E1-R mRNA or protein has been detected. The same studies that led to the definition of the E1-R gene also mapped a nonoverlapping gene to the 5′ third of the E1 ORF (2, 18) which is the same region that encodes the 23-kDa E1 protein (32). In these studies there was put forth the hypothesis that the 5′ third of the E1 ORF exclusively encoded a replication modulator, E1-M (18). As with the E1-R data, the data that led to this conclusion are incompatible with those from a more recent study (33).

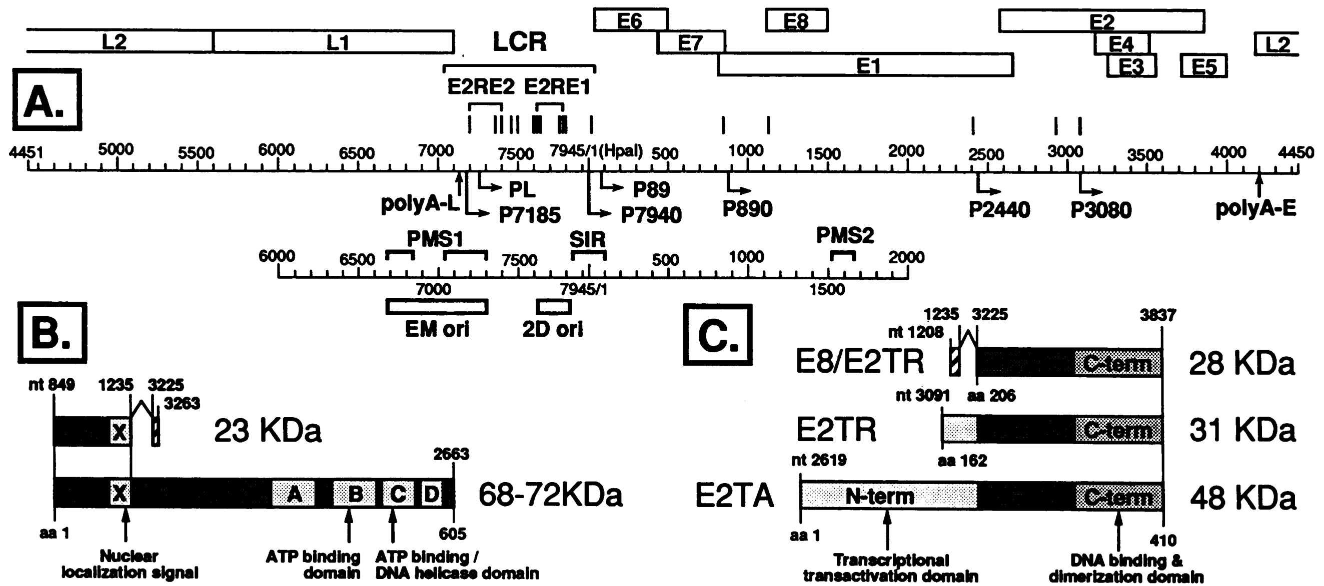

FIG. 1. (A) Illustration of the circular double-stranded DNA 7,945-bp BPV-1 genome linearized at the unique *Bam*HI site at nt 4450. Boxes above the line drawing indicate the positions of the translational ORFs. LCR indicates the position of the long control region (also referred to as the URR and the NCR). Vertical lines above the line drawing indicate positions of the multiple E2 DNA binding sites. The E2-responsive elements E2RE1 and E2RE2 are indicated by brackets; multiple viral promoters are indicated by horizontal arrows; and positions of the early and late mRNA polyadenylation sites are indicated by vertical arrows. The positions of the replication signals discussed in the text are at the bottom. (B) Illustration of the two E1 ORF proteins expressed in BPV-1-transformed rodent cells. Labeled boxes indicate regions of similarity between E1 and Tag as identified by Clertant and Seif (8). (C) Illustration of the three E2 ORF proteins expressed in BPV-1-transformed rodent cells.

E2 PROTEINS

The BPV-1 E2 ORF encodes three nuclear proteins (Fig. 1C) that have been well studied and demonstrated to be regulators of viral transcription (for a review, see reference 13). The full-length E2 protein is a transcriptional transactivator that activates multiple viral promoters through E2-responsive enhancer elements, E2REs. In addition to E2TA, there are two N-terminally truncated E2 ORF gene products, the E2 transcriptional repressor E2TR and the E8/E2 transcriptional repressor E8/E2TR. E2TR and E8/E2TR both share the C-terminal region of E2TA responsible for DNA binding and dimerization (Fig. 1C). They repress the activity of E2TA either through competitive DNA binding at the E2 DNA binding sites or by forming functionally attenuated heterodimers with the E2TA.

A direct role of E2TA in viral plasmid replication has been recently demonstrated. Expression in *trans* of both the full-length E1 protein and the E2TA protein was sufficient for transient replication of a minimal BPV-1 replicon (33). Neither E1 nor E2TA protein alone was sufficient to support this plasmid replication. As mentioned previously, the DNA binding protein E2TA, through its association with E1 protein, may act to localize E1 on the viral DNA (19, 21). Alternatively, E2TA, in binding to DNA sites in and around the replication origin, may alter the chromatin structure, providing cellular replication proteins greater access to the viral DNA template.

Most genetic studies have supported a role of E2TA in replication (9, 11, 24). However, a direct function for E2 in BPV DNA replication was previously discounted because mutant BPV-1 genomes containing deletions in the E2 ORF replicated as plasmids in rodent cells (15, 16). The E2 repressor proteins may also play a role in viral plasmid replication in that a mutation which specifically disrupts the major E2 repressor gene E2TR results in a 20-fold greater plasmid copy number (14, 25). Given that the E2 proteins are known to regulate viral transcriptional promoters, it has been predicted that they could indirectly control replication through the modulation of E1 protein expression; however, no proof exists for this indirect role.

OTHER BPV-1 ORFs

Other than the full-length E1 protein and the E2TA protein, no other proteins are required for BPV-1 plasmid DNA replication in transient assays (33). However, a number of viral ORFs, E5, E6, and E7, that encode transforming proteins (for a review, see reference 13) have been previously implicated in stable assays. A BPV-1 mutant defective in the E5 transforming gene was found to sometimes integrate into the host chromosome (24) and in an extended study BPV-1 mutants disrupted in either the E6 or E7 ORF have been found to be defective in maintaining a wild-type plasmid copy number (2, 3, 16). A more recent study, however, did not find a low-copy-number phenotype for these or other E6 and E7 mutants (22). That these genes are known to cause cellular transformation raises the question whether the proliferative status of the cell might indirectly affect the capacity of the cell to support viral replication. Such indirect effects may only be manifest under certain assay conditions.

In summary, an absolute requirement in replication has been demonstrated for the full-length E1 and E2TA proteins. The E2TR and E8/E2TR are likely to play a regulatory role, as does, perhaps, the 23-kDa N-terminal E1 protein. Other viral proteins, such as the E5, E6, and E7 transforming gene products, may indirectly affect replication.

cis ELEMENTS

A recent study maps the *cis* elements necessary for BPV-1 replication to a 105-bp region centered around nt 1 on the BPV-1 map (34), the same DNA region that bacterially synthesized E1 protein can bind (36). Furthermore, linker insertions in this region (referred to as SIR in Fig. 1A) disrupt both transient and stable replication (34). This mapping data contrasts with earlier studies that defined sequences in the BPV-1 genome which could confer on a bacterial plasmid vector the capacity to stably replicate in BPV-1-transformed cells. Two such plasmid maintenance sequences (PMS) were identified (15, 17, 35) on the BPV-1 genome at sites that are separate from that of the SIR element (Fig. 1A). The need for these PMS1 and PMS2 sequences in the context of the entire viral genome, however, was not demonstrated.

Electron microscopic analysis of replication intermediates present in BPV-1 DNA preparations isolated from virally transformed cells led to the first mapping of the replication origin to nt 6958, with a published error value of 5% of the genome size (35) (Fig. 1A, EM ori). Analysis of replicative intermediates by two-dimensional (2D) agarose gel electrophoresis has been recently employed by two laboratories to map independently the site of replication initiation (28, 37). One of these 2D gel analyses (37) claimed to precisely map the site of initiation to nts 7630 to 7830 (Fig. 1A, 2D ori). This region does not overlap the positions of EM ori, PMS1, PMS2, or SIR. The requirement of E2 protein for replication (33) implicates the multiple E2 DNA binding sites present on the viral genome (Fig. 1A) as potential *cis* elements for viral replication, in addition to their roles in transcription. The major E2-responsive transcription enhancer element, $E2RE_1$, that contains multiple high-affinity E2 DNA binding sites maps to nts 7608 to 7805; this overlaps the 2D ori (37). It is also interesting to point out that the SIR region (34) contains, in addition to the E1 protein binding site (36), two E2 DNA binding sites though they are not part of $E2RE_1$.

In summary, there remains considerable confusion over the exact location of the origin of replication and the DNA sequences required in *cis* for replication initiation in BPV-1-transformed cells. Yet there is still general agreement on their location within or close to the long control region in BPV-1. Given the newly predicted role in viral replication of E2 DNA binding sites, it is conceivable that the confusion over the location of *cis* elements results from the redundancy of the E2 DNA binding sites on the BPV-1 genome.

REGULATION OF VIRAL DNA REPLICATION

BPV-1 can stably replicate as a multicopy nuclear plasmid in virally transformed rodent cells, strongly suggesting that viral replication can be tightly controlled. In the past, several studies have attempted to address the mechanism of replication control, with varying and often contradictory conclusions. One study presented evidence that BPV-1 plasmids replicated on average once per cell cycle, analogous to the random replication seen with bacterial high-copy-number ColE1 plasmids (10); however, a second study concluded that each BPV-1 plasmid copy replicated only once per cell cycle (5). The latter conclusion was supported by the studies on chimeric SV40/BPV-1 replicons in which an E1 protein was found to suppress Tag-dependent DNA amplification (26). At this time there is no consensus in the literature as to which model for BPV-1 plasmid replication regulation is correct.

NEW VIEW OF BPV-1 REPLICATION

Given the growing wealth of information on BPV-1, a new view of papillomavirus replication initiation can be proposed on the basis of the following conclusions: (i) that the viral E1 and E2TA proteins are necessary and sufficient for transient plasmid replication in cells (33); (ii) that the full-length E1 protein and the E2TA proteins associate (21); (iii) that the E1-E2TA complex binds specifically to viral DNA (19, 21); (iv) that the DNA sequences required in *cis* for replication initiation potentially include sites for both E1 and E2 protein binding (34, 36, 37); and (v) that the E1 protein likely possesses an ATPase-dependent DNA helicase activity that unwinds the DNA at the origin as does SV40 Tag, facilitating the assembly of the cellular replication machinery on the viral genome and resulting in bidirectional replication (8, 19, 27, 31).

Initiation is thought to be the rate-limiting event in DNA replication. For BPV-1, likely points of regulation are either the association of E1 with E2TA, the capacity of this complex to bind to viral DNA, or the activity of the E1 protein itself. Possible mechanisms for controlling E1-E2TA complex formation include (i) the phosphorylation state of E2TA (E1 protein only binds to underphosphorylated forms of E2TA [19], a property that was predicted on the basis of the high plasmid copy number phenotype of a BPV-1 E2 phosphorylation mutant [20]), (ii) the abundance of the E2TR and E8/E2TR proteins (these proteins cannot bind E1 protein [21], but can form heterodimers with E2TA that may be unable to bind E1 protein), and (iii) the limiting amounts of E1 and E2TA proteins (their levels are very low in virally transformed cells, and induction of BPV-1 DNA amplification correlates with large increases in E2 protein levels [6, 7]). Possible mechanisms for controlling the binding of the E1-E2TA complex to the DNA include (i) the presence of E2TR and E8/E2TR (these proteins are in large abundance over the E2TA protein and could modulate E1-E2TA function through competitive binding at the multiple E2 DNA binding sites) and (ii) multimeric complexes between E2TA protein (E2TA has been shown to induce DNA looping through self-association [12]). A model for the regulation of bacterial plasmid replication has been discussed in which DNA looping, induced by the cooperative association of DNA binding proteins at multiple DNA binding sites, results in the tagging of molecules that have already replicated (23). Also, by analogy to SV40 Tag, the cdc2 kinase could regulate the activity of the E1 protein in either of the two steps discussed above or in subsequent steps in replication. Further studies are needed to identify which if any of these possible mechanisms plays a role in controlling replication initiation in BPV-1.

PERSPECTIVES

As described above there has been a recent though not complete clarification of the *cis* and *trans* requirements for BPV-1 replication. This new understanding of BPV-1 replication genetics is likely to assist in the development of an in vitro system for BPV-1 replication and in the study of human papillomavirus (HPV) replication. Stable replication of HPV plasmids has recently been demonstrated in tissue culture cell lines derived from cervical intraepithelial neoplasia I lesions (1, 29), and it has also been possible to reproduce certain characteristics of the productive infection state, including HPV DNA amplification (1, 30). These studies may

lead to the establishment of a tissue culture system for studying the genetics and biochemistry of HPV replication.

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