Lack of the canonical pRB-binding domain in the E7 ORF of artiodactyl papillomaviruses is associated with the development of fibropapillomas

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INTRODUCTION

Papillomaviruses (PVs) are highly species-specific pathogens. They form a heterogeneous group of closed-circular, double-stranded DNA viruses measuring about 8 kb in size (Sundberg et al., 1996). Most PVs target the basal cells of dermal or mucosal epithelia (Cheville & Olson, 1964), and infection has been implicated in both benign and malignant lesions of epithelia in a broad range of animals (Sundberg & O'Banion, 1989). Though almost all known human PVs (HPVs) infect dermal or mucosal surfaces, in some animal PVs, most notably in the artiodactyl ruminant PVs, fibroblasts appear to be the primary target cells (Sundberg et al., 1985). However, regardless of species, infection generally manifests as a papilloma or fibropapilloma, and follows a cytopathic mechanism of cell proliferation (Sundberg et al., 1996).

Historically, PVs had been classified according to their tissue tropism, and this grouping was supported by later phylogenetic analysis of PV sequence data (Chan et al., 1995; de Villiers, 2001; Myers, 1994). PV phylogenies typically subdivide into mucosal/genital HPVs, cutaneous EV HPVs and three main animal PV clades: the artiodactyl ruminant PVs, the distant avian PV group and a group containing canine, feline, rabbit and rodent PV types. However, sequence analysis has highlighted some significant exceptions. BPV-3, BPV-4 and BPV-6 do not group with the artiodactyl PVs, but instead form an isolated taxon (Jarrett et al., 1984), and HPV-1, HPV-41 and HPV-63 are most closely related to the canine and feline PVs, sharing little similarity to HPVs in either the mucosal or the cutaneous groups (Egawa et al., 1993).

Although most work in the area has focused on HPVs, an ever-increasing number of animal PV isolates have been sequenced. These animal PVs share key clinical features of...
PV infection with their HPV counterparts, making them valuable models of HPV infection. Comparative analysis of a wide variety of PV genomes can contribute to understanding the molecular basis of PV evolution and pathogenicity.

To study the molecular evolution of PVs, we determined the complete nucleotide sequences of bovine PV type 3 and type 5, equine PV and reindeer (Rangifer tarandus) PV, and compared their sequences with the genomes of 34 other animal and human PVs.

METHODS

Sequencing. BPV-3 was isolated from a cutaneous wart of an Australian cow (Pfister et al., 1979). Its genome was cloned into the EcoRI site of the pAT153 vector (Campo & Coggins, 1982; Gissmann et al., 1982; Twigg & Sherratt, 1980). BPV-5 was isolated from a 'rare grain' papilloma of a cow and cloned into the BamHI site of pAT153 (Campo et al., 1981). EQPV was isolated from a naturally occurring cutaneous lesion in a horse. Its DNA was cloned into the BamHI site of pBR322 (O’Banion et al., 1986). RPV was isolated from the epithelial layer of a cutaneous fibropapilloma on a Swedish reindeer and cloned into the BamHI site of pML2 (Moreno et al., 1987). The cloned PV genomes were kindly provided by Dr John Sundberg (The Jackson Laboratory, Bar Harbor, ME 04609).

Plasmid DNAs were purified according to the manufacturer’s instructions (Qiagen plasmid mini kit, Qiagen). Each plasmid was directly sequenced, first with primers selected from the vector sequence and later with additional primers designed from sequence walking (Delius & Hofmann, 1994). Sequencing was performed in the Einstein DNA sequencing core facility, and the overlapped sequences were assembled manually. Several additional primers were designed and used to clarify sequence ambiguities.

Sequence analysis. Using GenBank’s taxonomy browser, 4011 protein sequences and 1983 nucleotide sequences (some redundant) of papillomaviruses were identified with a batch entrez query on the keywords Papillomavirus and Papillomaviridae (Wheeler et al., 2003). These sequences were downloaded and indexed as local BLAST databases of PV protein and nucleotide sequences. A set of human papillomavirus genomes representative of the various HPV genera was selected for inclusion with all available animal PV genomes. The GI numbers and names of the specific PV genomes used for this study can be found in the supplementary information.

Protein (BLASTP) and nucleotide (BLASTN) homology searches were performed for all translated open reading frames in the newly sequenced BPV-3, BPV-5, EQPV and RPV (Altschul et al., 1997). BLASTP scores an amino acid sequence against a standard protein database and identifies similar sequences, but is limited in that it restricts searches to a translated set of ORFs, some of which may be frame-shifted due to sequencing error. ORFs are also occasionally misidentified or unrecognized in the annotated record. A BLASTN search clarifies these uncertainties by querying a protein against all six potential reading frame translations in a nucleotide database.

Standard BLAST parameters were used for most analyses, including filters for non-informative sequence (seg), composition based statistics, a word size of three and the BLOSUM62 scoring matrix. For small ORFs (15–30 amino acids in length), the BLAST searches were modified by removing the filter, turning off composition-based statistics, using a word size of two and employing the PAM30 scoring matrix. A 1e−5 significance cut off was chosen for all queries. The searches were implemented locally using PERL scripted queries to the local PV databases. The analysis relied heavily on the open-source BioPerl (Stajich et al., 2002) modules available at www.bioperl.org.

Multiple sequence alignments of PV clusters were performed using CLUSTALW with the gap cost 10.0 and the GONNET cost matrix (Higgins & Sharp, 1988). Concatenated E6, E7, E1, E2, L2 and L1 translated open reading frames constitute an alignment of largely conserved PV elements. E4, E5, E6 and E7 protein alignments were also individually generated for those PVs from Fig. 1 that contain them. Genealogical relationships were reconstructed using equal weighted characters. To ensure adequate searches in the tree’s space, 100 random addition heuristic searches and TBR (tree bisection and reconnection) swapping were employed in PAUP* version 4.10 (Swofford, 1998). Alignment gaps were coded as missing before parsimony and neighbour-joining trees were reconstructed. To assess robustness, 100 bootstrap and 100 jackknife replicates were performed for both the parsimony and distance analyses. The resampling approaches yielded essentially identical results, so only bootstrap results are reported here. Bayesian trees were constructed with the Markov Chain Monte Carlo technique in MRBAYES (Huelsenbeck & Ronquist, 2001) over 10 000 generations with sampling every 100 generations.

Correlated changes tests to examine patterns of change in sequence binding motifs, presence or absence of oncogenic ORFs and lesion phenotypes (papilloma versus fibropapilloma) were performed in MACCLADE (Maddison & Maddison, 1993) as described by Maddison (1991) using 1000 random simulations in heuristic searches.

RESULTS AND DISCUSSION

After assembly, the genomes of BPV-3, BPV-5, EQPV and RPV measured 7276, 7841, 7613 and 8090 bp in length, respectively. The full genomic sequences are available from GenBank under the accession numbers AF486184 (BPV-3), AF457465 (BPV-5), AF394740 (EQPV) and AF443292 (RPV). The predicted ORFs are shown in Fig. 1.

BPV-3, BPV-5, EQPV and RPV all have the typical complement of E1, E2, L2 and L1 genes comparable in size and position to other PVs. The canonical E6–E7 ORFs are also evident in the newly sequenced PVs except BPV-3, which instead contains an E8 ORF with homology to E8 in BPV-4 (69 % amino acid identity) and BPV-6 (Jackson et al., 1991) (66 % amino acid identity). The E8 ORF of BPV-4 has been shown to share functional similarity with BPV-1 E5, although sequence comparisons indicate these are not homologous ORFs. It has been suggested that the functionally similar BPV-4 E8 should be considered an E5 protein (Morgan & Campo, 2000). Since the ORFs are not homologues we have continued with the E8 nomenclature. Other small, putative ORFs include BPV-3 L3 and RPV E9, homologous to BPV-4 L3 (Patel et al., 1987) (41 % amino acid identity) and EEPV E9 (Eriksson et al., 1994) (45 % amino acid identity), respectively. The atypical and rare ORFs (e.g. BPV-3 E8 and RPV E9) identified here are difficult to confirm or discount using sequence analysis alone. If an unusual ORF such as RPV E9 exhibits little similarity to any other PV nucleotide or protein sequence, it may be either spurious or novel, and will require identification of the protein in vivo for validation. Even
convincing homology to a putative related PV is not definitive evidence that an ORF is actually expressed.

**PV core ORFs: E1, E2, L2 and L1**

To probe evolutionary relationships between BPV-3, BPV-5, EQPV, RPV and other animal PV genomes, the core ORFs (E1, E2, L1 and L2), common to all PVs, and the E6 and E7 oncogenes (where available) were compiled and aligned with the oncogenic and core ORFs of 34 representative PV genomes. The major (L1) and minor (L2) structural proteins contribute to the formation of the viral capsid, and the replication proteins (E1 and E2) interact with cellular polymerases and primases, stimulating viral genome replication (Scheffner et al., 1994). The proteins encoded by these core ORFs are essential to the structural integrity and biochemical viability of every PV sequenced to date. The resulting phylogenetic tree (Fig. 2) subdivides into five major clades.

To various degrees, the animal PVs sort into all the major clades. Avian PVs (FPV and PePV, clade 5) comprise an outgroup, reflecting an early point of divergence between mammalian and avian viruses (Terai et al., 2002). The cancerous and non-cancerous mucosal HPVs of clade 1 cluster with a number of non-human primate PVs, and the members of this clade along with clades 2, 3 and 5 lead predominantly to papilloma formation (Sundberg et al., 1996). Both RPV and BPV-5 sort into clade 4, a group dominated by artiodactyl ruminant PVs. This group is notable in that PV infection largely results in the development of fibropapillomas (Bernard & Chan, 1997), indicating that the pathogenic mechanism of the viruses in clade 4 appears to be unique among papillomaviruses. The phylogenetic division associated with lesion type suggests an early evolutionary divergence, accompanied and possibly driven by a pathological split. Interestingly, bovine species exhibit both types of pathology (Jarrett et al., 1984). BPV-3 and BPV-4 group into clade 3 and cause papillomas in their hosts, whereas BPV-1 and BPV-2 sort into clade 4 and lead to fibropapillomas. In the case of BPV-5, a single PV appears capable of inducing both pathologies. Bloch & Breen (1997) reported that the 3' end of BPV-5 E1 demonstrates homology to BPV-1 and BPV-2 (clade 4), but that DNA at its 5' end hybridizes with BPV-3 (clade 3). This observation, coupled with BPV-5's dual pathology (Bloch et al., 1994), suggests that BPV-5 deserves intermediary classification, and that its pathogenic mechanism is probably novel, or perhaps combines elements from clades 3 and 4.

Fig. 1. ORF map. Location of the predicted ORFs of (a) BPV-3, (b) BPV-5, (c) EQPV and (d) RPV. Each ORF is represented as a rectangle occupying either the first, second, or third reading frames. Numbers show the nucleotide positions of the start and stop codons.
Though the topology of the tree is at least partially host-species driven, the fact that bovine species are found in two independent clades, defined most clearly by their differences in pathology, demonstrates that any discussion of PV evolution should also consider disease phenotype. This is especially important in the case of BPV-5, where two disease phenotypes, normally tied to deep monophyletic groups, result from infection by a single PV.

**E4 and E5**

E4 and E5, unlike the core ORFs, are not essential to PV function, but E5 in particular has been established as one of the main factors in host-cell transformation (Petti & Ray, 2000). E4 has also been shown to effect transformation by modulating the cell division cycle (Nakahara et al., 2002), but its full biological activities are still being explored. The
mechanisms of E5 driven transformation in rodent and human fibroblasts have, however, been largely elucidated. E5 activates platelet-derived growth factor (PDGF) β-receptor tyrosine kinase in a ligand-independent fashion. BPV-1 E5 proteins have been shown to bridge two molecules of transmembrane PDGF receptors, resulting in receptor dimerization, activation and recruitment of cell signalling and proliferative proteins (DiMaio & Mattoon, 2001). In addition, BPV-1 E5 binds to the 16 kDa transmembrane subunit of vacular H⁺-ATPase (Goldstein et al., 1991), impairing the acidification of the Golgi apparatus and other intracellular organelles. A number of growth regulatory proteins, including PDGF β, are processed in the Golgi, suggesting that the ability of BPV E5 to influence intracellular pHe may be a factor in transformation. The mucosal HPVs also encode a small, hydrophobic protein superficially resembling the artiodactyl PV E5, but the E5 of these two PV lineages are not homologues and are probably the result of convergent evolution.

Though E4 and E5 are not ubiquitous among animal PVs, among the artiodactyl PVs (clade 4), both ORFs are largely conserved. An alignment of the artiodactyl PV E5 ORFs shows very close similarity, indicating that the E5–PDGF β-receptor interaction and H⁺-ATPase binding are probably shared mechanisms of transformation among the PVs of clade 4. Since the pathological hallmark of this clade is the development of fibropapillomas, the underlying genetic basis of fibroblast transformation is thought to involve the E5 transforming factor (Munger & Howley, 2002). Once again, however, BPV-5 is a glaring exception. As the clade’s outlier, it lacks observable E4 and E5 ORFs, but still retains the clade’s overall pathology, albeit in somewhat mixed fashion (Bloch & Breen, 1997). Consequently, the mechanism of fibropapilloma development is probably reinforced by the E5 ORF, but not solely contingent on its presence.

**Fig. 2.** Phylogeny. A phylogenetic tree was inferred from maximum-parsimony (MP), neighbour-joining (NJ) and Bayesian methods. The tree shown was generated from the MP analysis. Numbers on or near branches indicate support indices from the three methods in the following order: parsimony bootstrap percentage, Bayesian credibility value and neighbour-joining bootstrap percentage. N reflects disagreement between a given method and the reference MP tree at a particular node. All component trees were based on the alignment of the amino acid sequences of compiled ORFs (E6, E7, E1, E2, L2 and L1) of the indicated PV genomes. The alignment used to construct the compiled trees contained 2734 aligned characters. Gaps were removed for the parsimony and distance analyses. The resulting parsimony tree was 27671 steps long with a consistency index (CI) of 0.57 and a retention index (RI) of 0.62. The neighbour-joining tree had an ME-score of 8.35. The Bayesian consensus tree was constructed from 50 largely stationary trees after discarding 50 samples in the ‘burn in’ region. Lesion abbreviations: P, Papilloma; FP, Fibropapilloma; unk, unknown. pRB-binding characters: (+) indicates the presence of the L-X-C-X-E motif, (-) indicates its absence. E5 characters: (+) indicates the presence of E5, (-) indicates the absence of E5 and (+/-) indicates that an intact E5 ORF exists but lacks a start codon. Virus abbreviations: PsPV, *Phocoena spinipinnis* (seal) PV; ChPV, common chimpanzee PV; PCPV, pygmy chimpanzee PV; RhPV, rhesus monkey PV; COPV, canine oral PV; FdPV, feline PV; CRPV, cottontail rabbit PV; ROPV, rabbit oral PV; MnPV, multimammate rat PV; EQPV, equine PV; RPV, reindeer PV; DPV, deer PV; EEVP, European elk PV; FPV, finch PV; PePV, *Paittacus erithacus timneh* (African grey parrot) PV; OvPV-1, OvPV-2, ovine PVs types 1 and 2; BPV-1, BPV-2, BPV-3, BPV-4, BPV-5, bovine PVs 1, 2, 3, 4, and 5; HPV-6, HPV-7, HPV-32, HPV-16, HPV-34, HPV-51, HPV-56, HPV-18, HPV-2, HPV-90, HPV-61, HPV-84, HPV-77, HPV-1, HPV-5, HPV-23, human PVs 6, 7, 32, 16, 34, 51, 56, 18, 2, 90, 61, 84, 77, 1, 5 and 23.

**E6 and E7**

E6 can be identified in every PV genome except BPV-3, BPV-4 and the two avian PVs, FPV and PePV. Instead, the bovine species contain E8 (Jackson et al., 1991), and the avian species contain a novel ORF with no significant homology to the rest of the PV proteome (Tera et al., 2002). The remaining PV genomes exhibit E6 sequence homology and share four distinct C-X-X-C motifs, conserved residues that seem to be essential structures in the formation of a multimerized complex. With four cysteine sulphur ligands, two C-X-X-C motifs can sequester a zinc ion in a tetrahedral configuration (Grossman & Laimins, 1989). E6 proteins complex the tumour suppressor protein, p53, a key factor in flagging cell growth in differentiated or damaged cells. E6 therefore has anti-apoptotic activity, and interferes with the anti-proliferative signalling system of differentiated cells. In PVs that lack E6, like BPV-3, an E8 transforming protein induces the anchorage independent growth of the infected cells and suppresses contact inhibition (O’Brien et al., 1999), but does so independent of a p53 binding mechanism and appears to have functional similarity to BPV-1 E5, as indicated above (Morgan & Campo, 2000).

The E7 oncogene also contributes to a PV’s interference with the host cell-cycle and cellular differentiation. But where E6 binds p53, the canonical E7 binds pRB, the retinoblastoma protein, preventing its interaction with transcription factor E2F-1, resulting in activation of E2F responsive genes, such as those involved in cell replication (Munger et al., 2001). The hallmark of the pRB-binding domain is an invariable L-X-C-X-E motif shared by most E7 ORFs, including members of clades 1, 2, 3 and 5 (Chan et al., 2001; Dahiya et al., 2000; Dick & Dyson, 2002). An alignment emphasizing pRB-binding conservation among representative PVs is shown in Fig. 3(a). Note that in addition to the pRB-binding domain, these ORFs all contain two conserved C-X-X-C motifs separated by 28–30
amino acids. Clade 4 also exhibits similar dicysteine motifs, separated by 29 amino acids, but the alignment is peculiar in that there is no evidence for the pRB-binding signature. Instead, a novel set of amino acids with a pattern of conserved proline and leucine residues is retained in all these genomes (Fig. 3b). Since both bird PV genomes (PePV, FPV) contain evidence for a pRB-binding domain (Terai et al., 2002), the existence of the novel E7 motif in clade 4 appears to have arisen in the common ancestor of clade 4's members, an early divergence from a pRB-binding progenitor. Remarkably, the distribution of papillomas and fibropapillomas almost exactly mirrors the distribution of pRB-binding motif loss. Every animal PV known to cause fibropapillomas also lacks the pRB-binding motif, including the distant EQPV, whose lesion types, like BPV-5, tend to be somewhat mixed (Hamada et al., 1990). This correlation is not an artefact of gaps in the character matrix underlying the phylogeny in Fig. 2, because gaps fixed into the CLUSTAL alignments are coded as missing and are therefore neutral with respect to the phylogenetic analysis.
A few HPVs also lack the pRB-binding motif. The cutaneous PVs HPV-4, HPV-60, HPV-48, HPV-50 and the EV-associated PVs, HPV-3, HPV-10 and HPV-28 seem the only HPVs without the potential for pRB-binding functionality. Despite their lack of the pRB-binding domain, HPV-48 and HPV-10 E7 proteins induce cell proliferation, indicating that transformation can operate through a pRB-independent pathogenic mechanism (Caldeira et al., 2000).

Still, the correlation between the lack of pRB-binding and development of fibropapillomas is a finding, thus far, restricted to animals, and is most conspicuous among the artiodactyl PVs in clade 4. As demonstrated, E4 and E5 are well conserved in the clade, but because BPV-5 lacks these ORFs, we conclude that the motif shift in E7 sequences of the artiodactyl papillomaviruses may be partially responsible for their unique pathology on infection. Pairwise correlated changes tests examining the association between pRB, E5 and fibropapilloma lesions in Fig. 2 reveal significant correlation between the three characters (pRB–FP, \(P < 0.001\); pRB–E5, \(P < 0.05\); E5–FP, \(P < 0.05\)). This correlation extends beyond the phylogenetic topology and pathology. In BPV-1 fibropapillomas, it has been shown that E5 and E7 co-localize within the cytoplasm of undifferentiated basal epithelial cells, and that this co-expression is the basis of cooperative transformation between E5 and an E7 lacking the pRB-binding motif (Bohl et al., 2001).

In the absence of E5, however, fibroblast transformation may be mediated by cooperation between E6 and E7 ORFs (Neary & DiMaio, 1989). Nevertheless, why a lack of E5 in genomes without E7 pRB-binding activity correlates with dual papilloma/fibropapilloma pathology remains enigmatic. The basal position of BPV-5 in clade 4 reflects the retention of ancestral character states in its protein sequences. BPV-5 may be considered a viral ‘missing link’ between papilloma-causing animal PVs, and the more derived fibropapilloma-causing clade 4 viruses that contain E5, but have lost the pRB-binding motif. Along with BPV-5, EQPV shares FP/P heterogeneity, lack of pRB-binding, and lack of E5, supporting the idea that the ancestral state shared these characteristics. We expect that other, as yet undiscovered, artiodactyl PVs will have proteins that also display the transitional nature of BPV-5 and EQPV.

Nevertheless, only when the presence of E5 is coupled to the absence of an E7 pRB-binding domain, does PV infection trigger the exclusive development of fibropapillomas. We suggest that the manifestation of fibropapillomas is not attributable to E5 alone, and that an adaptive shift in a single E7 motif that evolved early in the PV phylogeny is also instrumental, providing a distinct marker for risk of fibropapilloma development traced to the sequence level.

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**REFERENCES**


