From “Papillomavirus Biology: from Natural History to Vaccine and Beyond” Ed.

MS Campo, Caister Academic Press, England, 2006

Chapter 23

Bovine Papillomavirus: old system, new lessons?

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Abstract

Bovine papillomavirus (BPV) is perhaps the most extensively studied animal papillomavirus. BPVs induce exophytic papillomas of cutaneous or mucosal epithelia in cattle. The papillomas are benign tumours and generally regress without eliciting any serious clinical problems in the host, but occasionally persist and provide the focus for malignant transformation to squamous cell carcinoma, particularly in the presence of environmental cofactors. This has been experimentally demonstrated for cancer of the urinary bladder and cancer of the upper alimentary canal in cattle feeding on bracken fern.

Six BPV types (BPV-1-6) have been characterised associated with specific, lesions with different histopathological characteristics. Recently the biology of BPV-5 has been reassessed, and novel types have been found, the biological characterisation of which is awaited with great interest.

BPV has been studied both as an infectious agent in its own right and as a model in which to investigate the interaction of papillomavirus with its natural host and with environmental cofactors. It has also provided a powerful model for vaccination against human papillomavirus (PV). It continues to provide information applicable to HPV: new functions recently discovered for BPV proteins have been confirmed for HPV proteins.

Introduction - BPV, an old system

Papillomaviruses (PVs) are strictly species-specific and, even in experimental conditions, do not infect any other host than their natural one. The only known case of cross-species infection is the infection of horses and other equids by BPV-1 and BPV-2 (see Chapter 24). Given the apparently insurmountable problem of species-specificity, no animal model of HPV infection exists and papillomavirologists have had to rely for direct experimentation on animal papillomavirus systems.

BPV has been invaluable in the investigation of virus biology, the direct link between virus infection and neoplasia, the relationship between virus and host and environment, the host immune response to the virus, and in the development of the first anti-papillomavirus vaccines. Additionally, and equally importantly, BPV is an agent of disease in farm animals and therefore of considerable veterinary and agricultural importance (Campo, 2002).

Heterogeneity of BPV

The six well characterised BPVs were originally classified into two subgroups, based on their genome structure and recognised pathology: subgroup A and subgroup B. Subgroup A comprised BPV-1, BPV-2 and BPV-5. These BPVs are commonly defined as fibropapillomaviruses, that is, viruses that infect both the epithelium and the underlying derma, giving rise to fibropapillomas (Figure 1, upper panel A). Subgroup B comprised BPV-3, BPV-4 and BPV-6, purely epitheliotropic BPVs which infect only the epithelium and induce true papillomas (Figure 1, upper panel B). Papillomaviruses have recently been re-classified (de Villiers et al., 2004) (Chapter 2) following the Greek letter nomenclature used for other virus families. According to the new nomenclature, which will be followed from now on, the epitheliotropic BPV-3, BPV-4 and BPV-6 are defined as Xi-papillomaviruses and BPV-1 and BPV-2 as Delta-papillomaviruses. The genome of BPV-5 appears to share homology with both Xi-PVs and Delta-PVs (Bloch and Breen, 1997) and interestingly BPV-5 appears to have a dual pathology causing both fibropapillomas and epithelial papillomas (Bloch et al., 1994). These two observations have lead to the re-classification of BPV-5 as the single member of the Epsilon-PV genus (de Villiers et al., 2004). It is clear that this virus deserves further investigation.

Thirteen additional novel BPVs have been reported recently (Ogawa et al., 2004), more than trebling the heterogeneity of BPVs. It is likely that further investigations will reveal the existence of even more BPVs and it will be very interesting to establish whether their pathology follows Delta-, Xi- or Epsilon-PVs.

The virion, the viral genome and viral transcripts

The virion. Independently from the site or type of lesion, the BPV virion has a constant morphology and structure. Like other PV virions, the BPV virion is a non-enveloped icosahedral structure of 55-60 nm diameter, which forms paracrystalline arrays in the nucleus of infected cells; it contains the double stranded covalently closed circular DNA complexed with cellular histones. The virion is composed of the major L1 and minor L2 capsid proteins. The detailed structure of the BPV virion has been determined recently and an atomic model has been generated which shows that

the C-terminus of L1 is exposed on the surface of the virion and is thus likely to have a role in infection and immunogenicity (Modis et al., 2002).

The viral genome. The genome of BPV-1, BPV-2 and BPV-5 is a double stranded closed circle of approximately 8000 nucleotides (nt), but only approximately 7300 nt in BPV-3, BPV-4 and BPV-6. The genome is divided into 3 canonical regions: a long control region (LCR, containing the necessary cis-regulatory elements for the replication and transcription of the viral DNA), the region containing the early genes (encoding non-structural proteins) and the region containing the late genes (encoding the capsid proteins) (Figure 2). The genomic plan of the BPV-1, BPV-2 and BPV-5 is similar to that of most other PVs, whereas BPV-3, BPV-4 and BPV-6 lack the E6 gene, which has been replaced by the E5 gene (Figure 2). The E5 gene and its encoded protein were originally defined as E8. The realisation that most (although not all) of the functions of this protein are shared with BPV-1 E5, prompted its redefinition as E5 (Morgan and Campo 2000).

Transcriptional control. Another difference between BPV-1, -2 and -5 and BPV-3, -4 and -6 is the organisation of the LCR. The LCR of the former group of viruses contains 12 E2 DNA binding sites (BS), whereas the LCR of the latter group contains only 4 E2BS, with an arrangement very similar to that of the LCR of genital HPVs (see Chapter 4 for a diagrammatic representation of the LCR of genital HPVs).

E2 is the viral transcription regulator, and its binding to the LCR activates or represses transcription of the viral genes (see Chapter 6 for a detailed description of E2 functions). In the BPV-4 system, E2 has different affinities for its cognate binding sites and binding to the different sites results in different outcomes. E2 binding to the TATA box-distal sites 3 and 4 results in transcriptional activation, whereas E2 binding to the TATA-proximal sites 1 and 2 leads to transcriptional repression in an epithelial specific manner (Jackson and Campo, 1995; Morgan et al., 1998).

The LCR of BPV-4 contains also positive and negative E2-independent regulatory elements both in the promoter and in the enhancer regions (Jackson and Campo, 1991; Morgan et al, 1999; Vance et al., 1999; Vance et al., 2001), which interact with a number of cellular transcription factors (McCaffery and Jackson, 1994; Jackson and Campo, 1995). As is the case in HPVs, regulation of BPV-4 gene transcription is a very finely tuned circuit where positive and negative, viral and cellular controlling

elements all contribute to the spatially and temporally regulated expression of the viral genes. If this delicate balance is subverted, the outcome is often cell transformation to the neoplastic state.

Viral transcripts. Expression of the viral genes takes place through a complex pattern of RNA splicing events which process mature mRNA from polycistronic precursor RNA both in Delta- and in Xi-BPVs (Yang et al., 1985; Campo et al., 1994a). As a general rule, the late transcripts encoding the structural proteins are found only in the more differentiated layers of warts and other productive lesions (see Chapter 15), while the RNAs encoding the early proteins are found both in warts and in transformed cells.

BPV proteins in papillomas and transformed cells

Early proteins. Delta-BPVs encode three oncoproteins, E5, E6 and E7, whereas the Xi-BPVs lack the E6 gene and only encode E5 and E7. The relative importance of each protein in cell transformation varies between different BPVs. E5 is the major oncoprotein of BPV-1 (see Chapter 8), followed by E6, with a more modest role played by E7. The transforming proteins of BPV-4 are E5 and E7, with E5 promoting the acquisition of anchorage independent growth and E7 inducing growth advantage and extended life span (Pennie *et al.*, 1993).

E6. BPV-1 E6 is itself a transcriptional activator; it interacts with and inhibit the transcription co-activator CBP/p300 and in so doing inhibits also the function of p53 (Zimmerman et al., 2000), thus achieving the same result as the E6 proteins of Alpha-HPVs, namely the inactivation of the apoptotic and cell cycle arrest functions of p53 (see Chapter 9). E6 binds the focal adhesion protein paxillin and blocks the interaction between paxillin and several other focal adhesion proteins, including vinculin and focal adhesion kinase (Tong et al., 1997). Disruption of focal adhesions thus likely underlies the observed anchorage-independent growth and disruption of the actin cytoskeleton induced by E6. Furthermore, E6 interacts with AP-1, the trans-Golgi network-specific clathrin adaptor complex and this interaction is likely to affect cellular processes involving clathrin-mediated trafficking pathway (Tong et al., 1998).

E7. BPV-1 E7 cooperates with E5 as well as E6 in cell transformation, as judged by anchorage-independent growth of mouse cells (Bohl et al., 2001). It is not yet clear how BPV-1 E7 contributes to cell transformation as it lacks the canonical p105Rb-binding domain LXCXE (see below; Narechania et al., 2004). On the contrary, like most other E7 proteins, BPV-4 E7 contains two CXXC motifs and the p105Rb-binding domain LHCEE. Mutations that disrupt the CXXC motifs, or the p105Rb-binding domain, drastically reduce or abolish its transforming activity. However, in common with CRPV, HPV-5 and HPV-8 E7 proteins, BPV-4 E7 does not possess a CKII phosphorylation site (Campo, 1997a).

E5. The E5 proteins of BPV-1 and BPV-4 are probably the best studied oncoproteins of these viruses. They will be discussed only briefly here as they are described in great depth and detail in Chapter 8. Both E5 proteins are very hydrophobic with a high leucine content and similar hydrophobicity profiles; they are both localised in the cell endomembrane compartments, particularly the Golgi apparatus, and both bind the 16k ductin/subunit c of the vacuolar H^+ ATPase. This interaction results in the inhibition of gap junction intercellular communication and in the lack acidification of endosomes and Golgi apparatus.

Gap junctions are channels for small molecular weight secondary messengers, important in the homeostatic control in a tissue; if a transformed cell is released from the control of the surrounding normal cells, it can proliferate freely and give rise to an expanding transformed clone. Accordingly, the lack of gap junction communication in papillomavirus transformed cells is probably an early event in transformation and, by isolating the newly infected cell from the surrounding normal sisters, allows other transforming events to become established.

The inhibition of acidification of endosomes and Golgi apparatus leads to disruption of cellular protein processing and sorting (see below and Chapter 8), resulting in the retention and re-cycling of undegraded activated growth factor receptors from endosomal compartments. Additionally BPV-1 E5 interacts directly with and activates the PDGF receptor. Therefore, the mitogenic response is potentiated, directly, by E5 interacting with the PDGF receptor, and, indirectly, by E5 interacting with the 16k protein, and in so doing inhibiting receptor down regulation. Moreover, E5 activates several protein kinases involved in the control of cell cycle, thus causing a general dysregulation of the normally tightly controlled programme of cell proliferation

(Chapter 8). For instance, the activation of the G1-S cyclin A-cdk2 complex underlies the ability of BPV-4 E5 to induce anchorage-independent growth both in established murine cells and in primary bovine PalF cells (O'Brien and Campo, 1998; O'Brien et al., 1999; Zago et al., 2004).

Consequences of the lack of E6 in Xi-BPVs. While BPV-1 can transform primary foetal bovine cells (PalF) by itself and thus encodes all the required genetic information for cell transformation (our unpublished observations), BPV-4 alone is insufficient to induce transformation of PalF cells. However, the complete E5 and E7 ORFs of BPV-4 co-operate with an activated *ras* oncogene in the morphological transformation of PalF cells, although the cells are neither immortal nor tumorigenic (Jaggar *et al.*, 1990). Xi-BPVs are unique among the papillomaviruses in not possessing an E6 ORF (Jackson *et al.*, 1991). Thus for these viruses an E6 protein is not required for a successful infection cycle, or for papilloma progression to carcinoma in the case of BPV-4 (see below). This raises the question of whether E6 functions are not required by BPV-4, or whether these functions are supplied by another viral or host protein. The demonstration that HPV-16 E6 confers immortality to PalF cells transformed by BPV-4 E7 and *ras*, whether E5 is present or not, shows that HPV-16 E6 does indeed provide functions which are not supplied by the BPV-4 proteins (Pennie *et al.*, 1993). Even after immortalisation by HPV-16 E6, transformed PalF cells are not tumorigenic. Tumorigenicity is achieved by the further introduction into PalF cells of a mutant p53 gene (Scobie et al., 1997), thus indicating that dysfunction of p53 is required for the ultimate step in cell transformation. The impact of the lack of an E6 gene on the abrogation of p53 functions necessary for cell transformation is highlighted by the ability of BPV-4 DNA to transform p53-null fibroblasts by itself. Further addition of HPV-16 E6 increases the number of transformed colonies (Scobie et al., 1997). Therefore, in the absence of E6-like functions, the controlling activity of p53 must be either bypassed or abrogated by cofactors during papilloma development and neoplastic progression to carcinoma. This is confirmed by the observation that the p53 gene is mutated in at least some papillomas and carcinomas (Scobie et al., 1997).

In vivo protein expression. The distribution of BPV-1 and BPV-4 E5 and E7 proteins in bovine warts is similar but not identical (Anderson et al., 1997; Bohl et al., 2001; Araibi et al., 2004). E5 of both viruses is expressed in the cytoplasm of both basal and superficial differentiating keratinocytes. BPV-4 E5 is present in papillomas at early stages of development but its expression is extinguished as the papilloma ages (Anderson et al., 1997); this temporally regulated expression, together with the established functions of E5 (see above), supports the hypothesis that BPV-4 E5 is needed during the early stages of viral infection, while its presence in the more differentiated layers suggests an involvement of E5 in the late stages of the virus life cycle (see Chapter 13 for a discussion of E5 as a "late" protein).

BPV-1 E7 is only observed in the cytoplasm and nucleoli of cells in the basal and lower spinous layers (Bohl et al., 2001), whereas BPV-4 E7 is expressed in all layers of papillomas at all stages (consistent with its necessary role in cell transformation) and its cellular localisation changes from nuclear in the basal and suprabasal layers, to cytoplasmic in the more differentiated upper layers (Anderson *et al.*, 1997). The significance of the cytoplasmic localization of BPV-4 E7 in the differentiated layers is unclear, but it has been suggested that E7's frequent co-expression with E5 in the lower layers serves to modulate the cellular response of basal epithelial cells to E5 (Bohl et al., 2001).

The E4 protein of BPV-4 is expressed only in the cytoplasm of keratinocytes that support the productive phase of viral DNA replication (Anderson *et al.*, 1997), implicating it in the late stages of the virus life cycle, in agreement with the E4 proteins of other papillomavirus (see Chapter 7 for a full description of the function of E4).

The structural proteins L1 and L2 are expressed in the nucleus of the differentiated keratinocytes (Anderson *et al.*, 1997). During virion morphogenesis L2 binds to viral DNA, favouring its encapsidation (Fay et al., 2004 and references herein). L1 and possibly also L2 mediate virus attachment to the cell receptor (see Chapters 12 and 18 for further details). Both proteins encode virus neutralising epitopes and, in the case of BPV-2 L2, tumour rejection epitopes; both proteins have been successfully used as vaccines (see below; Campo, 1997b).

BPV and benign disease

The natural history of BPV infection has been extensively studied both in the field and in experimental conditions (Jarrett, 1985). The stages of experimental disease are indistinguishable from those encountered in the field.

Infection by Delta-PVs (fibropapillomaviruses) leads to an initial transformation of the subepithelial fibroblasts followed by epithelial plexiform acanthosis and then papillomatosis, while infection by (epitheliotropic) Xi-PVs induces epithelial papillomas without fibroblast involvement (Figure 2, upper panel B; Jarrett, 1985). A large survey of bovine papillomatosis in Scotland established that BPV-1 causes teat and penile fibropapillomas; BPV-2, common cutaneous warts and oesophageal fibropapillomas; BPV-4, papillomas of the upper gastrointestinal (GI) tract; BPV-6, frond papillomas of the teats, and BPV-5, rice grain fibropapillomas of the udder. Workers in Australia have found that BPV-5 has a dual pathology (Bloch et al., 1994) and thus epithelial papillomas should be added to the spectrum of lesions induced by this virus. More work should be done on BPV-5 to establish if genetic differences between the Scottish and Australian isolates may be responsible for differences in the observed pathogenesis. BPV-3 was isolated in Australia from an epithelial papilloma of the skin (Pfister et al., 1979), and very little is known about its natural history. The tumours induced by BPV-3 are characterised by the absence of papillomatous fronds which are typical of tumours induced by BPV-4 and BPV-6. It should be noted that infection of the oesophagus by BPV-2 is abortive and a dead-end process in which cell transformation is achieved but no infectious virus progeny is produced, despite the presence and replication of multiple episomal copies of the viral genome.

Normally, the papillomas regress, but some animals are unable to reject the infection and succumb to widespread cutaneous or mucosal involvement. These forms of papillomatosis are problematic and of economical significance.

Papilloma of the teats and udder

The teats and udders of cows are subjected to infection by three different types of BPVs: BPV-1, BPV-5 and BPV-6 (Campo, 2002). This disease, especially if caused by BPV-6, is not only a health problem but has also economic consequences. One characteristic of BPV-6 papillomas is their secondary and even tertiary spread around

the primary tumours. This spread is clinically significant, as cows with teat papillomas cannot be milked, young calves cannot suckle, and often the peduncolated papillomas snap off, the sites become infected and mastitis may ensue with distortion of the milk canals. Occasionally, entire herds have to be culled if the papillomatosis does not regress. The reasons why BPV infection of the teats is more prolonged and less prone to resolution than BPV infection of the skin are not known and more work is needed to clarify them. Regression of BPV-6 papillomas has not been observed in experimental situations.

Fibropapilloma of the penis

These lesions, caused by BPV-1, occur both in the penile and preputial epithelia and can spread along the perineum and up toward the back of the bull; they can become necrotic and cause loss of reproductive functions. Although the penile fibropapilloma goes through the stages delineated for the fibropapillomas of the teats and udder (Jarrett, 1985), its life span is longer, especially if the tumour is large. The reasons for this persistence are not known and the tumour causes relevant economic damage.

Florid papillomatosis of the upper GI tract

Papillomas of the upper GI tract are induced by BPV-4. All sites from the tongue to the rumen can be affected. In healthy cattle the papillomas are few and normally regress after approximately twelve months. In chronically immunosuppressed animals regression does not take place and the papillomas spread and persist (Figure 2, lower panel). Chronic immunosuppression in cattle commonly results from exposure to immunosuppressive chemicals present in bracken fern (*Pteridium aquilinum*), on which the animals graze in the marginal lands of North-West Scotland and in several other countries (Borzacchiello et al., 2003a, Campo, 2002), but can be due to other causes, including infection with bovine viral diarrhoea virus (Tsirimonaki et al., 2003). Animals that develop such extensive papillomatosis have difficulty in eating and breathing and have to be culled. If the papillomatosis is not too severe, the animals survive but are at great risk of developing squamous cell carcinomas (see below).

BPV and cancer

Persistent PV-induced lesions are at risk of progression to cancer. Cancer, including PV-associated cancer, is a multi-factorial disease and several steps are required before full neoplastic transformation is achieved. Bracken fern has been identified as a major environmental co-factor in BPV-induced carcinogenesis in cattle. Bracken fern contains immunosuppressants and a number of mutagens.

Bracken-induced immunosuppression is associated with two marked haematological changes. The first of these is a dramatic fall in polymorph nuclear leucocytes. If unchecked, this leads to severe acute immunosuppression with invasion of the bloodstream by alimentary bacteria and death from septicaemia. This is the well described veterinary syndrome of Acute Bracken Poisoning (ABP). The second effect of bracken feeding is a chronic drop in circulating lymphocytes. Even during periods of bracken withdrawal the lymphocyte count remains very low.

Field cases of upper and lower GI tract cancer are found to occur at a high frequency in areas such as the Nasampolai Valley of Kenya and the Western Highlands of Scotland where cattle graze on bracken-infested land (Plowright et al., 1971; Jarrett et al., 1978). In addition, bracken-eating cattle develop chronic enzootic haematuria, urinary bladder cancers and chromosomal abnormalities (Borzacchiello et al., 2001a, Borzacchiello et al., 2003b, Jarrett et al., 1978, Lioi et al., 2004, Moura et al., 1988, Stocco dos Santos et al., 1998).

Cancer of the upper GI tract. In cattle feeding on bracken fern, the BPV-4-induced papillomas of the upper GI tract are at a high risk of progressing to cancer. The contribution of viral, immunological and chemical factors in progression of papillomas to carcinomas was first established in the field (Jarrett et al., 1978) and then in experimental conditions (Campo et al, 1994b). In both circumstances, healthy cattle infected with BPV-4, grazing on grass or kept on a diet of hay, reject their papillomas after approximately one year from infection. In contrast, in bracken-eating immunosuppressed cattle the papillomas spread throughout the oro- and nasopharynx, often coalescing in numerous large clumps (Figure 2, lower panel B), do not undergo regression and progress to cancer (Figure 2, lower panel C). The animals maintain their tumours for the rest of their life: in experimental conditions, one animal died 13

years after infection and still had papillomas which had spread from the mouth to the lower oesophagus and the rumen. In the same experiment, two animals infected with BPV-4 and fed with bracken developed foci of carcinoma in the oesophagus, which infiltrated the subjacent tissue, and multiple polyps, adenomas and adenocarcinomas in the duodenum, jejunum and colon (Campo et al., 1994c).

These epidemiological and molecular studies have allowed the determination of the events that take place during carcinogenesis. In addition to the action of the viral oncoproteins (see above), the number of the cellular receptors for epidermal growth factor increases, the ras gene is activated and the p53 gene is mutated (Campo, 2002). These transforming events, involving cellular genes, are ascribed to the bracken fern mutagens, but, although this has been proved *in vitro* (Beniston et al., 2001; Cairney and Campo, 1995; Connolly et al., 1998; Pennie and Campo, 1992), it remains to be established *in vivo*. In support of a role for bracken mutagens in carcinogenesis, two of the most potent mutagens of the plant (quercetin and ptaquiloside) are found in urine, serum and milk of bracken-eating cattle (Alonso-Amelot et al., 1996; Lioi et al., 2004).

Cancer of the urinary bladder. Cattle feeding on bracken fern are also affected by cancer of the urinary bladder (Campo, 1997a; Campo, 2002). Urinary bladder cancer comprises two main types, carcinoma of the urothelium, as seen in humans, and haemangioendotheliomas of the subjacent capillaries. Often the two types of tumour occur together in the same bladder. The involvement of bracken and BPV-1 or BPV-2 in bladder carcinogenesis has been recognised for a long time (Olson et al., 1959), recently the virus-bracken interaction has been reproduced experimentally (Campo et al., 1992) and a carcinogenic process, similar in outline to the one established for BPV-4 has been recognised. BPV-1/2 infects the epithelium of the urinary bladder (perhaps as a secondary infection deriving from infection of the paragenital area) and establishes an abortive infection, with no production of virus, as is the case for the fibropapillomas of the alimentary canal (see above). The viral DNA in the bladder lesions is still infectious and capable of initiating a replicative cycle in the permissive environment of the skin, as extracts from urinary bladder cancers could induce skin warts (Olson et al., 1965). The viral oncoprotein E5 is expressed (Borzacchiello et al., 2003b), the ras gene is activated (Campo, 2002, Shahin et al., 1998), and expression of the tumour suppressor *fragile histidine tetrads* (*FHIT*) locus is down-regulated

(Borzacchiello et al., 2001b). The immunosuppression induced by bracken fern prevents tumour rejection and the fern mutagens contribute to genome destabilisation. Interestingly fragile sites are often disrupted by integration of HPV DNA in cervical cancers (Butler et al., 2000) and alterations of *FHIT* expression has been observed in many cervical carcinomas (Takizawa et al., 2003).

Are BPV-induced, bracken-triggered cancers a model for human carcinogenesis?

Cancer of the oesophagus in humans. Carcinoma of the upper GI tract is one of the most frequent forms of human cancer: oesophageal cancer ranks sixth in developing countries and eighteenth in developed countries respectively (Globocan, 2001). Mortality is high in both developed and developing countries. In Latin America there is little or no difference between the incidence and death rates of oesophageal cancer (Day and Varghese, 1994). Although in the western world alcohol and tobacco are deemed to be the main causative agents of this type of cancer (Graham et al., 1990), these factors seem to play a lesser role in other countries (Demirer et al., 1990).

Several HPV types have also been found with variable prevalence in oral cancers (Syrjanen S., 2003) and oesophageal cancers and precancers (Syrjanen K., 2002; see Chapter 15 of this book). HPV is more prevalent in oesophageal cancer from the developing world than the developed world. The highly variable geographical incidence of oesophageal cancer and of HPV detection in the cancer suggests a multi-factorial aetiology and the involvement of environmental dietary co-factors (Syrjanen, 2002; Chapter 15).

In humans too, exposure to bracken fern has been linked to oesophageal cancer in several parts of the world (Alonso-Amelot and Avendano, 2001, Alonso-Amelot and Avendano, 2002, Alonso-Amelot et al., 1996, Hirayama, 1979, Marliere et al., 1999, Villalobos-Salazar et al., 1995). Bracken fern is eaten and used as a herbal remedy in South America, Japan, China, Korea and other eastern countries. Exposure to bracken fern directly through consumption or possibly inhalation, or indirectly through milk from bracken eating cattle has been epidemiologically linked to human oesophageal and gastric cancer in Western Europe, South America and Japan. The relative risk reported for bracken exposure and oesophageal cancer ranges from 2.6 in Wales (Galpin et al., 1990), 3.7 in Japan (Hirayama, 1979) to 8.12 in Brazil (Marliere et al.,

2002) independent of other factors such as age, gender, smoking and tobacco consumption. Chromosomal abnormalities have been found both in bracken-grazing cattle (Lioi et al., 2004, Moura et al., 1988) and in bracken-eating people (Recouso et al., 2003). There is a geographical overlap between consumption of bracken fern, infection of the alimentary canal mucosa by HPV and oesophageal cancer in the Minas Gerais region of Brazil (our unpublished observations). Bracken, milk from bracken fed cows and bracken spores have all been shown to be carcinogenic in experimental animals (Santos et al., 1987, Santos et al., 1992) and DNA adducts have been found in the upper GI tissue of mice fed bracken fern (Povey et al., 1996).

These findings suggest that some cases of cancer of the upper GI tract in humans may have the same aetiology as in cattle, i.e. papillomavirus and bracken, and open up the possibility that the molecular mechanisms elucidated for cell transformation and cancer in cattle operate similarly in humans. If this is the case, the bovine system acquires even greater relevance to human papillomavirus infection and cancer.

Cancer of the urinary bladder in humans. The involvement of HPV in cancer of the urinary bladder in humans is still an unresolved issue. HPV DNA has been found in a number of cases of urinary tract cancer, sometime ascribed to secondary spread from penile or vulval condylomas. However, the involvement of HPV in this type of human cancer has been disputed (Oliver et al., 1998). Recent evidence supports the idea that the virus is instrumental only in a proportion, perhaps a minority, of urinary tract cancer in selected populations, in combination with other infections such as Bilharzia (Khaled et al., 2001, Khaled et al., 2003). This would explain the contradictory results and leave the door open for a role of HPV in human bladder cancer, perhaps as a co-factor.

BPV-4 E7 AND QUERCETIN

Given the important role played by bracken fern in carcinogenesis in cattle, it is of great interest to identify the factors involved and their cellular targets. Several mutagenic and/or carcinogenic substances have been isolated from bracken, including ptaquiloside and quercetin (Alonso-Amelot and Avendano, 2002). Ptaquiloside, a norsesquiterpene glucoside, induces tumours and haematuria in rats and produces chromosomal aberrations in Chinese hamster cells; breakdown products of

ptaquiloside, detected in milk of bracken-eating cows (Alonso-Amelot et al., 1996, Lioi et al., 2004), exhibit mutagenic activity. The flavonoid quercetin (3,3',4',5,7-pentahydroxyflavone) is mutagenic in both prokaryotic and eukaryotic cells, can act as an initiator in a two-stage transfomation assay *in vitro* and induces chromosomal aberrations in mammalian cells. In addition, quercetin activates or inhibits protein kinases and phosphatases thus interfering with critical steps in signal transduction .

The contribution of quercetin to neoplastic cell transformation has been established in primary foetal bovine cells (PalF). In addition to the oncogenes of BPV-4, these cells need an activated ras gene and, as BPV-4 does not possess an E6 gene, the E6 oncogene of HPV-16 to be established as immortal transformed clones. Addition of a mutated p53 gene, leads to the formation of tumorigenic clones (see above). In this cell system, quercetin can substitute for more than one oncogene (Pennie and Campo, 1992). In the presence of quercetin, E7 is the only BPV-4 oncogene required for full transformation, as quercetin substitutes for E5 in conferring anchorage-independence, for E6 in inducing immortalisation and for mutant p53 in inducing tumorigenicity (Cairney and Campo, 1995). The potent effects of quercetin upon primary cells in tissue culture strongly suggest that quercetin is, if not *the* active, one of the most active components of bracken in carcinogenesis of the upper GI tract.

The effect of quercetin on cell transformation is underpinned by transcriptional activation of the BPV-4 LCR; a *cis*-acting element has been mapped in the LCR which responds to quercetin (Connolly et al., 1998). LCR activation, in turn, stimulates expression of the E7 oncoprotein. E7 inhibits one of the negative regulators of the cell cycle, $p27^{kip1}$, and in so doing allows quercetin-damaged cells to proliferate, rather than arresting in G1, and thus spread and maintain any genetic damage caused by quercetin (Beniston et al., 2001).

Ptaquiloside, another potent BF mutagen, has been implicated in neoplasia but its role in cell transformation and its relationship with papillomavirus are unknown .

BPV latency

Like many viruses, BPVs can establish a latent infection. The viral genome can be often found in normal epithelia with no clinical sign of disease (Ogawa et al., 2004) both in tumour-bearing and in clinically normal hosts. Normal epithelia are the accepted site of latent infection, and indeed the reactivation of BPV at sites of trauma

suggests that viral DNA is present in these sites in latent form, and that damage of the epithelium, possibly through production of inflammatory cytokines and stimulation of cell proliferation, induces expression of viral genes leading to papilloma formation (Campo et al., 1994b). However, epithelia may not be the only site of latent papillomavirus. BPV DNA is present in episomal form in circulating lymphocytes of cattle (Campo et al., 1994b; F. Roperto, University of Naples, personal communication) and latent BPV infection of lymphocytes has been established in experimental cattle (Stocco dos Santos et al., 1998). The detection of BPV DNA in cattle lymphocytes by three independent groups in three different, geographically separated countries is a strong indication that the presence of viral DNA in these cells is not accidental but likely to have biological implications. HPV DNA has been found in blood cells and lymph nodes of women with urogenital HPV infection and cervical cancer (Kedzia et al., 1992, Pao et al., 1997, Pao et al., 1991; Payne et al., 1993, Tseng et al., 1999) but the significance of these observations is not clear.

Intriguingly, papillomavirus virus-like particles have been shown *in vitro* to bind strongly to a variety of immuno cells, including dendritic cells, B cells, monocytes, and macrophages, and it has been hypothesised that this interaction is likely to be important in the immune response to PV capsids (Da Silva et al., 2001; Chapter 12).

These findings present the very attractive, but still purely speculative, possibilities that blood cells represent another site of latent virus and that, additionally, the relationship between PV and immune cells can modulate the host immune response to virus. Clearly more work needs to be done in this respect.

Immune response to BPV

Viruses and their hosts are in a delicately balanced relationship. Viruses must be able to overcome the host immune response to replicate and produce infectious progeny. Nevertheless, despite the virus ability to evade immune surveillance, eventually the host mounts an effective immune response and virus and virus-infected cells are eliminated.

The immune response of cattle to BPV is surprisingly poor (Campo, 1998). Animals may carry massive tumours, actively producing virus in large quantities, but cattle do not respond easily to BPV antigens during the course of infection and anti-BPV antibodies are seldom detected. This is the case for all the BPV types investigated,

ruling out either the virus type or the site of infection for the poor humoral response. The failure of the immune system to recognise either incoming virus or progeny virus is due to fact that all the virus life cycle is restricted to the epithelium and therefore is not in contact with the immune system (O'Brien and Campo, 2002; Tindle, 2002). This interpretation is supported by the fact that field animals with ulcerated and bleeding tumours do have high titres of natural anti-BPV antibodies and good antibody responses can be obtained after intramuscular inoculation with purified virus or viral proteins, confirming that only when the papilloma is damaged, or a threshold of unknown nature is reached via immunisation, viral antigens come into contact with immune cells (Campo, 1998). Weak T- and B-cell responses to capsid proteins or to the transforming protein E7 can observed in some animals at later stages of infection and appear to be associated with papilloma rejection (Campo, 1998).

During rejection of BPV-4 papillomas, large masses of activated lymphocytes accumulate in the derma underlying the papilloma. In these clusters $CD4^+$ lymphocytes are the predominant subtype, followed by γδ T-cells and $CD8^+$ lymphocytes. In contrast, γδ and $CD8^+$ lymphocytes predominate in the basal layer and among the keratinocytes (Knowles et al., 1996; Campo, 1998). The contribution of the individual lymphocyte subtypes to papilloma regression remains to be established but a similar differential distribution of $CD4^+$ and $CD8^+$ lymphocytes has been reported for regressing genital warts in humans and regressing papillomas in rabbits (Chapters 19 and 25 respectively). The overall temporal and topological similarity between regressing lesions in different papillomavirus systems confirms the generality of the observations.

The poor immune response to BPV is likely to be the main reasons for the persistence of infection: even in immunocompetent hosts, the papillomas persist for many months before regression takes place. It has recently become clear that, in addition to the passive immunoescape due to the viral life cycle being confined to the epithelium, papillomaviruses have evolved active ways of hiding from the host immune system (O'Brien and Campo, 2002). Among these there is down-regulation of the Major Histocompatibility Complex class I (MHC I) (see below).

BPV vaccines

One of the major goals of papillomavirus research is the development of vaccines which will either prevent viral infection (prophylactic vaccines) or accelerate rejection of papillomas (therapeutic vaccines) thus leading to a decreased incidence of human anogenital cancer, in which HPV infection plays a pivotal role (Chapter 21).

The observation that, although the immune response to BPV infection is poor, a prompt and prolonged immune response is elicited when cattle are immunised with BPV proteins has lead to the successful development of anti-BPV vaccines.

Prophylactic vaccination was achieved with BPV-1 virions and virus neutralising antibodies were elicited by vaccination with BPV-1 L1 protein (Campo, 1997b). Following these earlier studies, BPV-2 and BPV-4 were chosen as emblematic for skin and mucosal papillomavirus, respectively, and because, as described above, both are implicated in cancer. The first series of vaccines consisted of intramuscular vaccination with virus. These "classical" vaccines result in the production of neutralizing antibodies and complete protection from subsequent challenge. They are however type-specific conferring protection only against the homologous virus (Campo, 1997b). This serotype specificity suggests that for an HPV vaccine to be widely protective it must incorporate antigens from several of the commonest infecting high risk HPV types.

BPV-2. As for BPV-1, a subunit vaccine based on the L1 protein of BPV-2 elicits neutralising antibodies and confers protection from infection. This is in accordance with the structure of the BPV virion, in which the C-terminus of L1 is displayed on the virion surface (Modis et al., 2002). On the contrary, a BPV-2 L2 vaccine does not afford protection from viral challenge but induces early regression of warts; antibodies against the antigen are generated in the vaccinated animals, but they are not neutralizing. Large infiltrates of lymphocytes and macrophages are observed in the regressing warts, indicative of a cell-mediated immune response (Campo, 1997b). L2-induced wart regression challenges the old, and perhaps naïve, belief that viral structural protein vaccines would only provide protection from infection.

BPV-4. A more extensive analysis of vaccination and immune response has been conducted in the BPV-4 system (Campo, 1997b).

Prophylactic vaccination. Vaccination with BPV-4 L2 induces protection from challenge by BPV-4 (Campo *et al.*, 1993). Similar results have been obtained following vaccination of rabbits with CRPV L2 (see Chapter 25) indicating that L2-induced protection may be a general phenomenon. Immunity induced by BPV-4 L2 is

long lasting and the vaccinated animals are refractory to a second virus challenge more than a year after vaccination. Immunity is conferred by the N-terminus of L2 (Chandrachud et al., 1995) and the vaccinated calves develop virus neutralising antibodies (Gaukroger *et al.*, 1996). Three immunodominant B-cell epitopes have been identified in the N-terminus of L2 (Knowles et al., 1997), and a single 20 amino acid long peptide (residue 131-151) corresponding to one of the epitopes is sufficient for full protection from infection (Campo et al., 1997). These results are consistent with the observation that the N-terminus of L2 is exposed on the surface of the BPV-1 virion (Liu et al., 1997) and therefore accessible by the immune system. Interestingly, the BPV-4 L2 epitopes are homologous to and cross-react with epitopes identified in the L2 protein of several genital HPVs (Campo et al., 1997) leading to the prediction that vaccines based on HPV L2 proteins may elicit cross-type protective immunity in humans (Roden et al., 2000).

Papillomavirus capsid proteins can self-assemble in empty virus-like particles (VLPs) when expressed in eukaryotic cells. VLPs are both structurally and antigenically similar to virus, and present conformational neutralising epitopes (Chapter 18). BPV-4 VLPs containing either the L1 and L2 proteins or only the L1 protein are very potent prophylactic vaccines (Kirnbauer *et al.*, 1996), while their status as therapeutic vaccines is less certain. Protective immunity against BPV-4 can therefore be achieved either with L2 alone or with L1 alone.

However, although vaccination with either L1 or L2 induces the production of neutralising antibodies, not all virus neutralisation events occur at the cell surface. BPV-4 is neutralised by anti-L2 sera after cell entry (Gaukroger et al., 1996; Sibbet et al., 2000), but the mechanisms of this intracellular neutralisation are not known. Therefore, although BPV-4 L2 provides a powerful prophylactic vaccine, it protects not against infection but against disease.

Therapeutic vaccination. Vaccination of calves with the BPV-4 E7 protein does not prevent infection. However in the vaccinated animals papilloma development is slower and the duration of papillomatosis shorter than in control animals. In vaccinated calves papillomas do not reach the final stage of development and start regressing at an earlier stage (Campo et al., 1993). The vaccinated calves produce high titre antibodies directed to three immunodominant B-cell epitopes (Chandrachud et al., 1994) and develop a strong cellular immune response to two T-cell epitopes (McGarvie et al., 1995).

Naturally regressing papillomas are infiltrated by large numbers of activated lymphocytes (see above) but it is not known against which viral antigens the lymphocytes are directed, or whether the cellular immune response in vaccinated calves is similar to the one that mediates natural papilloma rejection.

BPV, new lessons

Most, if not all, of the functions of papillomavirus proteins have been elucidated in BPV first and then confirmed for HPV. This continues to be the case, as exemplified below.

E5 and MHC class I down-regulation. MHC I plays a critical role in immune surveillance as it is responsible for the presentation of antigenic peptides to effector T-cells. Once the heavy chain (HC) of MHC I associates with β2-microglobulin and peptides, the complex is transported from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane for recognition by T-cells (Cresswell, 2000). E5 is the major transforming protein of BPV (see Chapter 8 for a full description of E5's cell transforming activities). A recently discovered function of BPV E5 is the down-regulation of MHC I (Ashrafi et al., 2002, Marchetti et al., 2002). Down-regulation of MHC I by BPV E5 takes place at multiple levels: transcription of the MHC I HC gene is reduced, the MHC I HC peptide is degraded (Ashrafi et al., 2002) and the MHC I complex is sequestered in the Golgi cisternae and is prevented from reaching the cell surface (Marchetti et al., 2002). Retention of MHC I in the Golgi cisternae is due, at least in part, to the E5-induced alkalinisation of the Golgi apparatus as a similar reduction of surface MHC I is observed in cell treated with ionophores that prevent the acidification of endomembranes (Marchetti et al., 2002). Importantly, BPV E5 down-regulation of MHC I is not observed only in cultured cells but also in bovine papillomas (Araibi et al., 2004). This strongly supports the notion that BPV E5 helps the establishment of a successful infection not only through cell transformation but also by down-regulating MHC I, and thus allowing the infected cells to evade host immunosurveillance.

These new facets of BPV E5 have prompted similar investigations on HPV E5. Indeed E5 proteins of both mucosal (HPV-16 and HPV-6) (Ashrafi et al., 2005) and

cutaneous viruses (HPV-2) (Cartin and Alonso, 2003) prevent the transport of MHC I to the cell surface. However, down-regulation of MHC I by HPV E5 is less severe that that brought about by BPV E5, as neither transcription of HC gene nor stability of HC peptide are affected (Ashrafi et al., 2005). Thus, although down-regulation of surface MHC I is a common function of E5 proteins, it appears that there is a correlation between their transforming "strength" and the extent to which they interfere with MHC I (O'Brien and Campo, 2003).

E2 and mitotic chromosomes. BPV-1 E2 associates with mitotic chromosomes (Abroi et al., 2004, Bastien and McBride, 2000, Ilves et al., 1999) (see Chapter 6) via its amino terminal portion allowing its DNA binding domain to remain associated with viral genomes. In so doing, BPV E2 mediates the association of the viral genomes with the mitotic chromosomes resulting in efficient distribution of the BPV genomes into the daughter cells. The E2 proteins of HPV-11, -16, and -18 associate with the mitotic spindle during mitosis and HPV-11 E2 also associates with centrosomes (Van Tine et al., 2004). Although the mechanism underlying partition of the viral genomes between daughter cells may be different between BPV E2 and HPV E2 (Van Tine et al., 2004), it is clear that the E2 protein is pivotal in ensuring that the viral genomes are properly segregating during mitosis.

E2, L2 and ND10. A further role for E2 in the regulation of the viral life cycle has come from the observation that BPV-1 E2 can enhance the packaging of plasmid DNA into pseudovirions (Zhao et al., 2000) and appears to be directed to ND10 domains by the minor capsid protein L2 (Day et al., 1998, Heino et al., 2000). ND10 are nuclear domains associated with virus replication, and localisation of E2 to these structures is suggestive of its role in promotion of virion assembly.

The localisation of L2 to ND10 has been confirmed for HPV-33 L2 (Florin et al., 2002). The translocation of L2 to ND10 causes their reorganisation, with dispersal of some, and the recruitment of other, of the ND10-associated proteins (Florin et al., 2002) suggesting that the ND10-associated proteins play an important part in the life cycle of the virus and in its relationship with the host cell.

The interaction between E2 and L2, established recently for HPV-16 too (Okoye et al., 2005) has functional consequences. L2 selectively inhibits the transcriptional activation function, but not the DNA replication function, of HPV16 E2 (Heino et al.,

2000, Okoye et al., 2005). The effect of L2 on E2 also has the potential to be crucial for regulation of the viral life cycle: L2-induced repression of E2 transcriptional functions would allow E2 to focus on DNA amplification and thus ultimately control virion production.

E7, pRb and fibropapillomas. The E7 protein interacts with, and inhibits, the pRb tumour suppressor protein through the canonical domain L-X-C-X-E (see Chapter 10). This domain is shared by all E7 proteins with the exception of the E7 proteins of artiodactyl fibropapillomaviruses (Narechania et al., 2004). It has been suggested that the absence of the pRb-binding domain contributes to the characteristic pathology of these viruses, that is, induction of fibropapillomas (Figure 1, upper panel) (Narechania et al., 2004). This is an interesting hypothesis that deserves exploration. It will be particularly interesting to understand how fibropapillomavirus E7 contributes to cell transformation and which cellular proteins it binds to. Although no HPV has been found so far with an E7 lacking the pRb-binding domain, the possibility remains that also among HPVs there are members which do not interact with pRb.

HPV-18 and quercetin. Quercetin is a potent co-factor of BPV-4 E7 in cell transformation: it substitutes for E5 in inducing anchorage independence, for E6 in inducing immortalization and for mutant p53 in inducing tumorigenicity. This synergy is molecularly underpinned by the transcriptional transactivation of the BPV-4 LCR with increased expression of E7, which drives the cells through the cell cycle, thus fixing and expanding quercetin-induced mutations and chromosomal abnormalities (see above). A similar situation is found with keratinocytes transformed by HPV-18 E6 and E7. In these cells, quercetin activates the HPV-18 LCR via an initiator-like quercetin responsive element, homologous to the one found in the LCR of BPV-4, and increases transcription of the E7 gene. E7 inhibits the negative cell cycle regulator $p27^{kip1}$, preventing the normal quercetin-induced cell cycle arrest in G1 and driving the cells through the cell cycle (Beniston and Campo, 2005). Therefore the effect of quercetin on the LCR and the E7 protein of HPV-18 parallels that on the LCR and E7 protein of BPV-4, and supports the involvement of HPV and bracken fern in oesophageal carcinogenesis in humans (see above).

Conclusions

The BPV system has been ground breaking in the recognition of the oncogenic nature of the virus, the elucidation of the relationship between virus and co-factors and the development of anti-viral vaccines. The HPV vaccines currently in clinical trials are based on vaccines developed against BPV (and CRPV), including VLP-based vaccines (see Chapters 18, 19, 21) and E6/E7/L2-based prime-boost vaccines (Davidson et al., 2004). Although not all the facets of the bovine model system can be applied to the human situation, comparative pathology studies between BPV and HPV-induced diseases have contributed in no small measure to the understanding of HPV-induced cancer in humans. The latest insights in BPV E5, E2 and E7 functions show that BPV can still lead the way and contribute significantly to our understanding of virus biology. There are still many things to be learned from BPV. It is not an exaggeration to say that the study of BPV has blurred the dividing line between veterinary and human medicine.

Acknowledgements

The author would like to acknowledge support from the Medical Research Council, the Association for International Cancer Research, the World Cancer Research Funds and Cancer Research UK. The author is a Life Fellow of CR UK.

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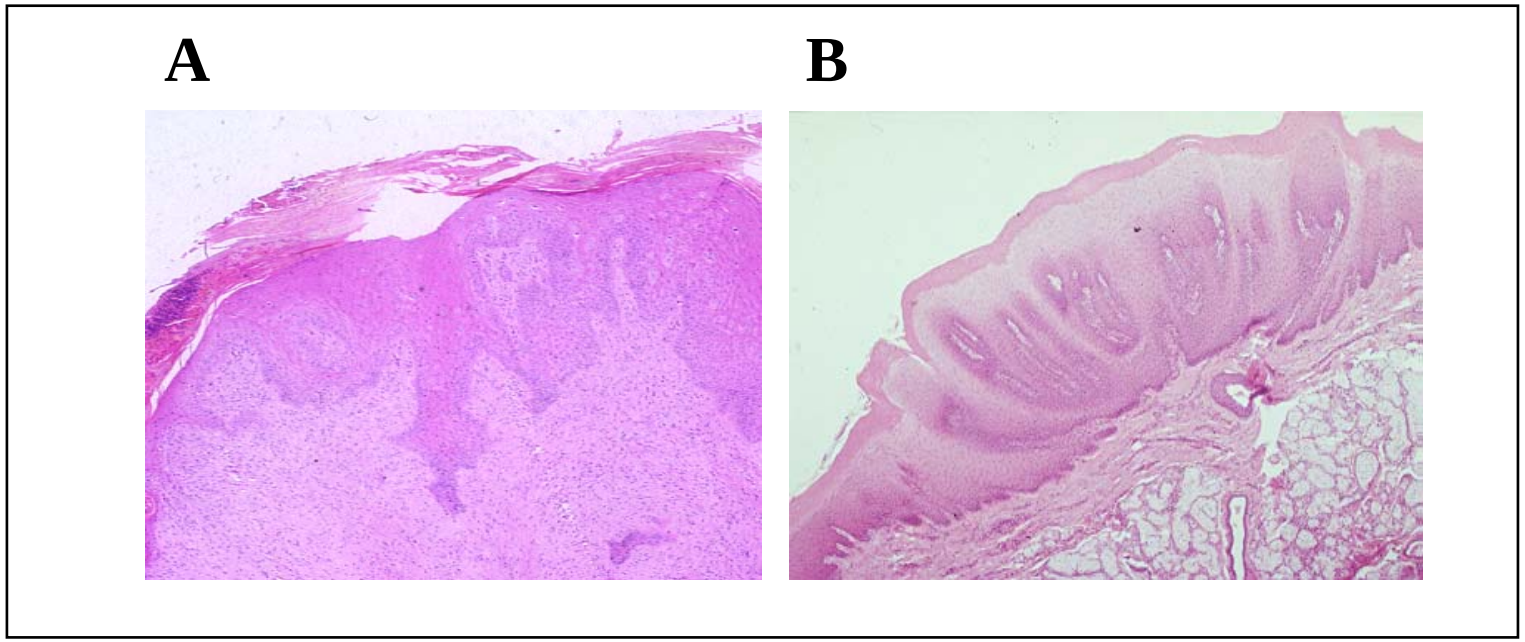
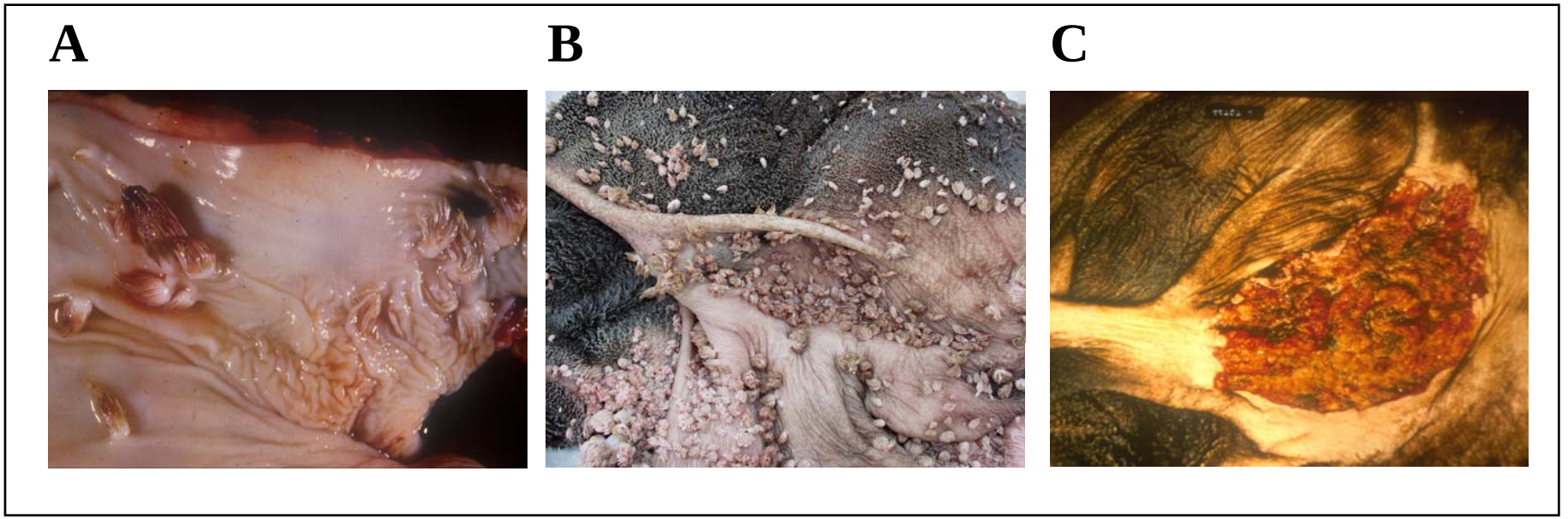
Figure Legends

Figure 1. Upper panel: BPV-induced tumours. A, fibropapillomas induced by BPV-1; B, epithelial papilloma induced by BPV-6.

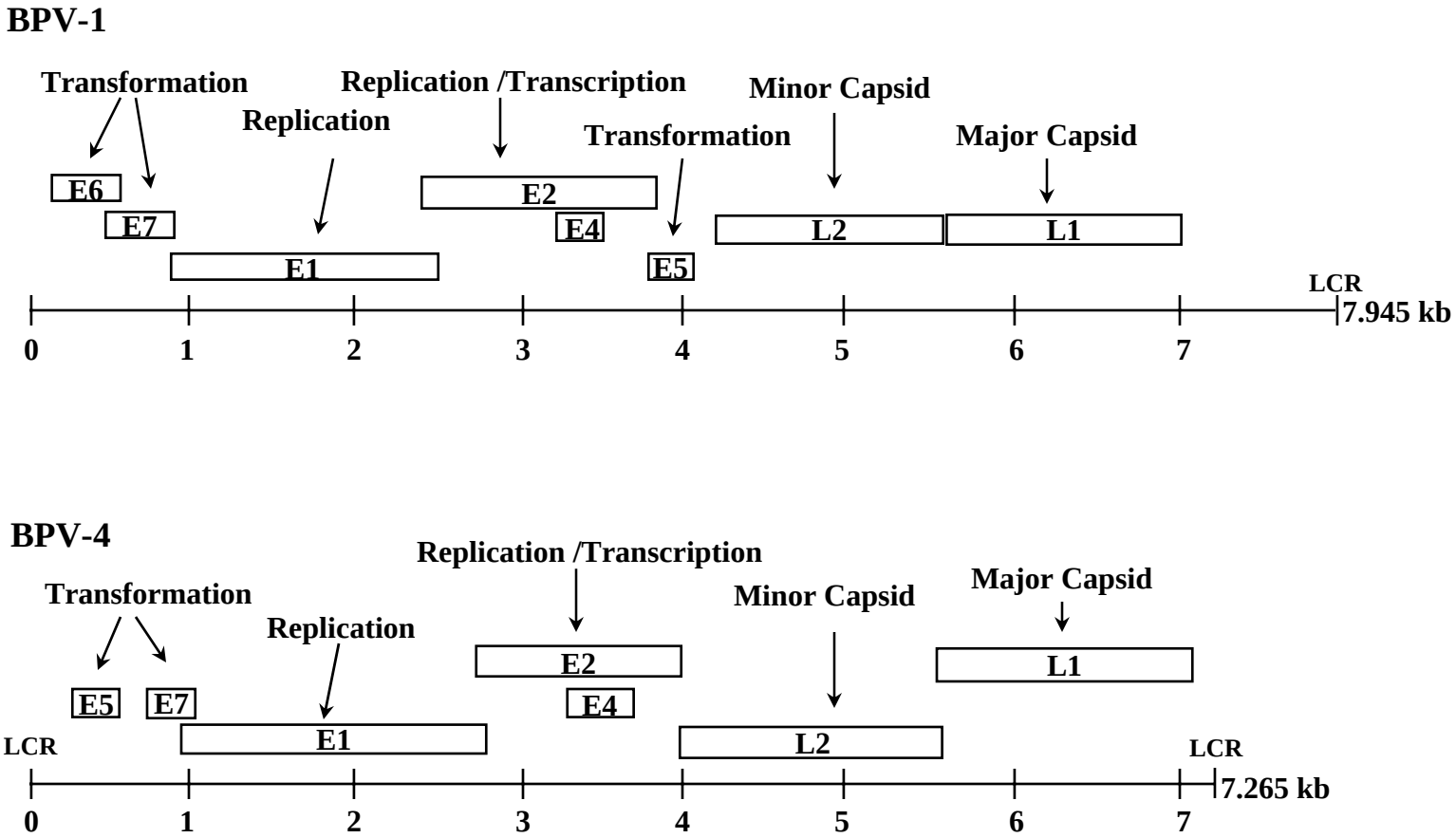
Lower panel: Neoplastic progression of BPV-4 papillomas. A, single papillomas of the oesophagus in a healthy immunocompetent animal; B, extensive florid papillomatosis of the rumen in an animal immunosuppressed by bracken fern; C, squamous cell carcinoma of the rumen in an animal immunosuppressed by bracken fern.

Figure 2. Diagrammatic genomic organisation of BPV-1 and BPV-4. The viral genomes are represented as linear, with the open reading frames (ORF) as rectangles. The function of the proteins encoded by the individual ORF is indicated.





Campo, Figure 1



Campo, Figure 2