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Viral and cellular oncogenes in papillomavirus-associated cancers

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Summary Papillomaviruses are one of several factors implicated in the aetiology of squamous cell carcinomas both in man and in animals. Their potential for malignant transformation is fully expressed when co-operation takes place between viral functions, cellular functions and chemical or physical co-carcinogens.

This review presents a brief description of the viral transforming genes and of the cellular genes involved in transformation, and attempts to analyse how the co-operation between the two sets of genes is achieved.

Papillomaviruses are small DNA tumour viruses, and induce papillomas or warts in a variety of animals, including man. They infect basal epidermal cells, leading to cell transformation, proliferation and papillomatosis. It is well established that, whereas most papillomas are benign tumours which either regress or, more rarely, persist, some of them can undergo malignant progression to carcinomas, as a result of the synergism between the virus, physical or chemical carcinogens present in the environment and the genetic constitution of the host (Jarrett *et al.*, 1978; Kreider, 1980; Orth *et al.*, 1980; zur Hausen, 1982). It is therefore clear and widely accepted that virus infection alone, although responsible for the primary lesion, is not enough to promote its conversion to cancer. Moreover, some papillomavirus are associated with malignancies only, or with much higher frequency, in hosts that are not their natural ones (Pfister, 1984). This argues for a complex interaction between the virus and its host, with cellular and immunological components either controlling and eventually defeating viral infection, or failing to do so, depending on a number of variables.

The purpose of this article is to analyse the contribution of viral, chemical, cellular and immunological factors to cell transformation and malignant progression, in an attempt to both dissect a complex system and consider it in its entirety.

Papillomaviruses and cancer

Papillomaviruses are highly heterogeneous, and have been classified in groups, types and subtypes, on the basis of both pathological and molecular criteria (Pfister, 1984; Jarrett *et al.*, 1984*a*).

Despite their multiplicity, only few types are associated with naturally occurring cancers. Thus, only the genome of human papillomavirus (HPV) type 5 and 8, and more rarely type 14 and 17, is found in carcinomas derived from epidermodysplasia verruciformis (EV) lesions (Smith & Campo, 1985), and the DNA of HPV-16 and 18, and occasionally 33, is found more often in genital carcinomas than that of HPV-6 or 11 (Smith & Campo, 1985; Beaudenon *et al.*, 1986). Likewise, among the BPVs, only BPV-4 is associated with carcinomas of the upper alimentary canal (Campo *et al.*, 1980), despite abortive infection of the oesophagus by BPV-2 (Jarrett *et al.*, 1984*b*).

The more frequent association of some papillomavirus types with cancer has led to the hypothesis that these viruses have a 'more malignant' potential than others. However, no obvious differences have been found in the genomic organization of the 'more malignant' virus types (Giri & Danos, 1986) and it is not yet known whether the apparent greater 'malignancy' of these viruses is due to subtle genetic differences, or to a greater propensity of their target cell to neoplastic transformation, or to a combination of both. The observation that BPV-2 is strongly associated with neoplastic transformation in the urinary bladder (Campo & Jarrett, 1986) but not in the skin or in the oesophagus, where it causes benign proliferation only (Campo *et al.*, 1981; Jarrett *et al.*, 1984*b*), argues for the importance of the cellular environment for the full expression of the virus oncogenic potential.

Organization and function of the viral genome

The vegetative cycle of papillomavirus is tightly linked to the differentiation of epithelial cells (Orth *et al.*, 1971) and is therefore confined to warts. No *in vitro* cell culture system is yet available for virus propagation, and the analysis of the viral genome and its functions relies heavily on recombinant DNA technology and on heterologous *in vitro* transformation systems.

Molecular cloning and DNA sequencing have shown that all papillomaviruses share a similar genetic plan (Giri & Danos, 1986) as exemplified by the genomic structure of BPV-1 (Figure 1; Chen *et al.*, 1982). Large overlapping open reading frames (ORFs) are present only in one strand. By analogy with other viruses, the ORFs involved in cell transformation and DNA replication are called 'early' or E, and those coding for virion structural proteins are called 'late' or L (Figure 1). Between the E and L ORFs, there is a non coding region, ~1,000 bp long, which contains regulatory elements.

Transcription is unidirectional and the viral RNAs are generated by complex splicing mechanisms (Figure 1; Yang *et al.*, 1985; Baker & Howley, 1987), which juxtapose non-contiguous ORFs giving rise in some cases to fusion proteins.

Genetic manipulation of the viral genome has led to the identification and mapping of several *cis* and *trans* regulatory elements (Figure 1). Thus, transcriptional enhancer and promoter sequences have been identified (Campo *et al.*, 1983; Lusky *et al.*, 1983; Androphy *et al.*, 1987; Spalholz *et al.*, 1987); the origin of DNA replication has been mapped (Waldeck *et al.*, 1984); and sequences responsible for the control of DNA replication and for the maintenance of the DNA as a multicopy plasmid have been located (Lusky & Botchan, 1984, 1986).

The 'early' genes of papillomavirus and cell transformation

The observation that BPV-1 or its DNA could transform mouse fibroblasts in culture (Dvoretsky *et al.*, 1980) opened up an entire new field of investigation in papillomavirus research. A subgenomic fragment comprising 69% of the BPV-1 DNA was shown to encode the necessary *in vitro* transforming functions (Lowy *et al.*, 1980) and, later, sequence analysis (Chen *et al.*, 1982) revealed that this fragment contains all the E ORFs and the non coding regulatory region (Figure 1).

The L ORFs, coding for the capsid proteins (Table I; Engel *et al.*, 1983), will not be further discussed, and this section will deal in some details only with the early functions.

Genetics and biochemical analysis has made possible the assignment of particular functions to individual E ORFs (Table I). These features of the virus cycle have been elucidated for BPV-1 in experimental tissue culture systems

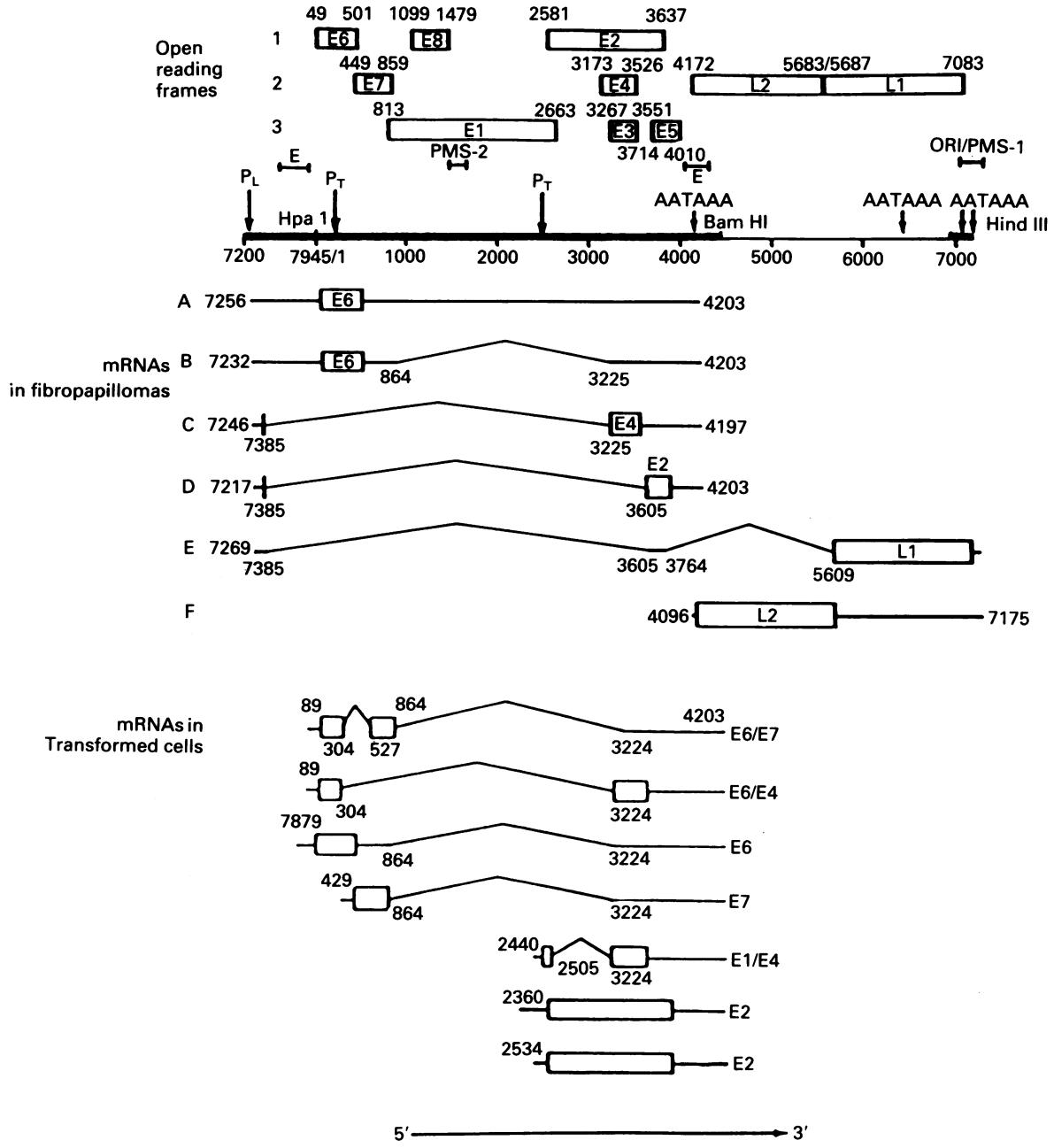

Figure 1 Genomic and transcriptional organization of BPV-1. The linearized viral genome is represented by the solid line, and the region encoding the early transforming functions by the thick line. The numbered boxes represent the early (E) and the late (L) open reading frames (ORFs). The numbers indicate nucleotide positions. E = transcriptional enhancer; PL = late promoter; PT = early promoter; AATAA = polyadenylation site; ORI = origin of DNA replication; PMS = plasmid maintenance sequence. The open boxes represent the coding sequences of the mRNAs and the solid lines their untranslated sequences. The slanted lines represent intervening sequences. (Adapted from Giri & Danos, 1986; Yang *et al.*, 1985; Baker & Howley, 1987).

Table I Functions of BPV-1 genes

ORF	*Function*
E1	DNA replication and plasmid maintenance (Lusky & Botchan, 1984, 1986)
E2	Trans-acting transcription factors (Spalholz *et al.*, 1985; Lambert *et al.*, 1987)
E3	Unknown
E4	Virion maturation[a] (Doorbar *et al.*, 1986)
E5	Cell transformation (Shiller *et al.*, 1986)
E6	Cell transformation (Yang *et al.*, 1985)
E7	Regulation of copy number (Lusky & Botchan, 1985)
L1	Major capsid protein (Engel *et al.*, 1983)
L2	Minor capsid protein (Engel *et al.*, 1983)

[a]Determined for HPV-1.

but, within these limitations, it is assumed that the same, or similar mechanisms are operational *in vivo*, and applicable to other papillomaviruses. There are however pitfalls in assuming that individual genes code for the same functions in all papillomaviruses, and some of these will be pointed out below.

The E1 and E2 ORFs code for both positive and negative regulatory elements which control *in trans* DNA replication and transcription respectively (Lusky & Botchan, 1986; Berg *et al.*, 1986; Roberts & Weintraub, 1986; Spalholz *et al.*, 1985; Lambert *et al.*, 1987). The E2 ORF of several HPVs also encodes *trans*-acting factors (Hirochika *et al.*, 1987), and these can be complemented by the corresponding BPV-1 functions (Phelps & Howley, 1987).

The E7 ORF is involved in the maintenance of BPV-1 DNA as a multicopy episome in mouse fibroblasts (Lusky & Botchan, 1985). However, both the 15 kiloDaltons (kD) E7 protein and the 70 kD E1 protein have been found in CaSki and HeLa cervical carcinoma cell lines (Smotkin & Wettstein, 1986, 1987; Seerdof *et al.*, 1987), where the HPV DNA is integrated, and it is therefore unlikely that the E7 and E1 proteins will function in these cells as they do in BPV-1-transformed mouse fibroblasts.

Another example of the danger of extrapolating from one system to another is provided by the E4 product. This has been shown to be associated with virion maturation in HPV-1 warts (Table I; Doorbar *et al.*, 1986; Breitburd *et al.*, 1987), but a 10 kD E4 protein has been found in the CaSki cervical carcinoma cell line, totally unproductive for virus (Seerdof *et al.*, 1987).

The E5 and E6 ORFs have been identified as the transforming genes of BPV-1 (Table I; Yang *et al.*, 1985; Schiller *et al.*, 1986). Both the E5 and E6 proteins have been found in transformed cells (Androphy *et al.*, 1985; Schlegel *et al.*, 1986). The 15.5 kD E6 protein is localized in both the nuclear and membrane fractions, whilst the 7 kD E5 protein is only found associated with membranes. Both proteins contain the repeat cys-x-x-cys (Giri & Danos, 1986), which is an important domain of DNA-binding proteins (Berg, 1986). The nuclear localization of E6 is consistent with the hypothesis that E6 may be a DNA binding protein. The role, if any, of the cys motif in E5 still requires clarification. Interestingly, in BPV-4 the cys repeat is found also in E8 (Patel *et al.*, 1987). The putative E8 protein is hydrophobic (S. Campo, unpublished observations) and this suggests a membrane localization.

Although these studies have furthered our understanding of *in vitro* cell transformation by BPV-1, several considerations have to be kept in mind. First, whereas the E6 and E7 proteins have indeed been identified in naturally-occurring cancer and their cell lines (Banks *et al.*, 1987; Smotkin & Wettstein, 1987; Seerdof *et al.*, 1987), no E5 protein has been found so far outside the BPV-1/mouse fibroblasts system. Second, although BPV-1 does transform fibroblasts *in vitro*, bovine fibroblasts explanted from BPV-1 fibropapillomas do not exhibit in culture the traits of morphological transformation (Pfister, 1984). Third, BPV-1 is not associated with malignancies in its natural host; it induces malignant tumours only when it jumps species, as in the cases of horses, hamsters or other experimental animals (Pfister, 1984). Moreover, which viral gene(s) induces the benign proliferation of both fibroblasts and keratinocytes observed in bovine warts is still a matter of speculation.

Although it is beyond doubt that papillomaviruses are involved in cell transformation, opinions are divided on whether the continuous expression of viral functions is indeed a prerequisite for progression to full malignancy or for maintenance of the neoplastic state. The finding of HPV RNA and proteins in cervical carcinoma cell lines (see above) contrasts with the absence of viral transcripts in a number of primary carcinomas of the cervix, despite the presence of integrated HPV DNA (Lehn *et al.*, 1985). The presence of multicopy episomal genomes of BPV-1 in equine and hamster sarcomas (Pfister, 1984) is in marked contrast with the absence of BPV-4 DNA in the overwhelming majority of alimentary carcinomas in cattle (Campo *et al.*, 1985).

Moreover, again contrary to the BPV-1 system, transformation of cultured fibroblasts by BPV-4 requires several cooperating factors, including tumour promoters (Smith *et al.*, 1987; Smith & Campo, 1988), and, although HPV-16 DNA can transform established NIH 3T3 cells (Tsunokawa *et al.*, 1986), transformation of primary cells requires the cooperation of an activated *ras* gene (Matlashewski *et al.*, 1987).

A conservative interpretation of these conflicting results would suggest that, if the molecular basis of transformation is the same for all papillomaviruses in all systems studied, then viral functions are needed for some but not all stages of malignant progression, and that at later stages cellular (onco)genes take over, irrespective to whether the viral genome is present or not, expressed or not.

Cellular (onco)genes in papillomavirus-transformed cells

Since the discovery that in the majority of the cervical cancers HPV DNA is integrated into the host genome (Durst *et al.*, 1985; Schwarz *et al.*, 1985), several groups have investigated the possibility that, at least in human genital malignancies, cell transformation occurs by insertional mutagenesis, with the consequent activation of cellular oncogenes. This mechanism of transformation is well established in the case of retrovirus-induced transformation. The insertion of a foreign genetic element near or adjacent to cellular genes controlling growth would unbalance their expression and lead ultimately to uncontrolled cell division.

In several carcinoma cell lines, the integrated HPV DNA sequences have been localized to chromosomal regions containing oncogenes such as c-*src*, c-*raf* and c-*myc* (Durst *et al.*, 1987), or to heritable fragile sites (Popescu *et al.*, 1987). However, integrated viral sequences have been found also in histologically normal cervical epithelium and in low grade neoplasias (Millan *et al.*, 1986; Schneider-Maunoury *et al.*, 1987) showing that integration can be an early event and is not sufficient by itself to confer a fully malignant phenotype. Amplification and rearrangements of the cellular oncogenes c-*myc* and/or c-Ha-*ras* have been observed in cervical cancers harbouring HPV genomes (Riou *et al.*, 1984) confirming the involvement of these genes in the neoplastic process, but whether or not their activation is due to direct viral action is still not clear.

The c-Ha-*ras* gene has been found amplified and rearranged in alimentary carcinomas of cattle, where no viral DNA can be detected, and also in some premalignant papillomas containing vast amounts of viral DNA. The carcinoma DNA induces focus formation in the NIH 3T3 assay and the *ras* gene is transferred into the transformed cells (Campo *et al.*, in preparation). This confirms yet again that the c-*ras* gene plays an extensive role in carcinogenesis, and suggests that its activation may be an early event. The co-operation of HPV and *ras* functions in the transformation of primary cells (Matlashewski *et al.*, 1987) would confirm that the *ras* gene is called into play at early stages.

Another set of cellular genes playing an important role in malignant growth is represented by the genes coding for growth factors and their receptors (Goustin *et al.*, 1986). We have recently found that the number of receptors for the epidermal growth factor (EGF) is elevated both in bovine cells transformed *in vitro* and harbouring multiple BPV genomes, and in *in vivo* transformed cells explanted from alimentary canal carcinoma containing no viral DNA (Smith *et al.*, 1987). These results suggest that the amplification of EGF receptors is related to the cell malignant phenotype. Work is in progress to establish whether the increased number of EGF receptors is a direct consequence of viral infection.

Recent results from our laboratory point yet again to the involvement of cellular functions in papillomavirus-mediated transformation. The great majority of cells transformed in culture by BPV-4 does not contain any viral DNA and BPV-4 sequences have been found rearranged in only a few cell lines (Smith *et al.*, 1987; Smith & Campo, 1988). Cellular DNA sequences have been identified which are greatly amplified in all the transformed cell lines, independently from the presence of viral DNA (Smith & Campo, submitted). The nature of these sequences is still unknown, but they appear to be amplified also in naturally occurring papillomas and carcinomas, this raising the hope that their further characterization will lead to a greater understanding of virus-induced cell transformation.

Conclusions

Papillomavirus infection does not by itself lead to neoplasia in a healthy host. For progression to malignancy to take place a number of other factors must intervene, and this points to the crucial role of immunological and cellular controls exerted by the host on the virus. Only when this dual control breaks down, the infected cell progresses along the pathway to transformation.

An hypothesis that takes into account the available experimental evidence would require that the virus is the executor of the initiation steps, and that progression to the transformed state and its maintenance are sustained by the anomalous expression of cellular genes.

Thus, genes such as c-*ras* or c-*myc* or the EGF receptor gene may be activated either by direct binding of the viral E6 protein, or by insertion of viral promoters, or *in trans* by the viral E2 protein. Constitutive activation of these genes would make further production of viral products unnecessary, and would push the cell into the transformed state. This process would be aided by co-factors (UV, chemical carcinogens, other viruses) and, particularly in an immunocompromized host, the transformed cells would be allowed to proliferate unhindered.

Whilst this scheme may be an oversimplification, or incorrect in some of its aspects, it is undisputable that the fully malignant phenotype is obtained through the synergism of several different elements, and only now we may begin to understand the complex interaction between viral and cellular functions.

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