# 3. Molecular Mechanisms of Carcinogenesis

## 3.1 Experimental data supporting the carcinogenicity of specific HPV genotypes and analysing the mechanism of HPV-linked carcinogenesis

### *3.1.1 Low-risk and high-risk HPV infections*

Shortly after the isolation and characterization of the most prevalent HPV types found in genital warts (HPV-6 and -11) (Gissmann & zur Hausen, 1980; Gissmann *et al.*, 1982) and cervical cancer (HPV-16 and -18) (Dürst *et al.*, 1983; Boshart *et al.*, 1984), the concept of low-risk and high-risk HPV infections for malignant conversion was introduced (zur Hausen, 1985). Initially it was based exclusively on the high prevalence of type 16/18 viruses in malignant tumours and the rarity of cancer biopsies containing HPV-6 or -11.

During subsequent years, functional differences between both subgroups of papillomaviruses became apparent, which will be described below. Increased chromosomal abnormalities and concomitant aneuploidy were observed in high-risk HPVs selectively (Fu *et al.*, 1988; Claas *et al.*, 1992). In analogy to several other DNA tumour virus systems, oncoproteins of high-risk HPVs showed specific interactions with cellular proteins engaged in the regulation of proliferation.

The terminology of high-risk HPV types in this section is restricted to those genital virus types that perturb normal growth and genomic stability of primary human cells *in vitro*. It is likely, however, that a number of other types, found less frequently in anogenital cancers, also share these properties, although they have not yet been analysed experimentally to the same extent.

### *3.1.2 Chromosomal instability of high-risk HPV-infected cells*

The induction of chromosomal instability had been observed initially in cells infected with SV40, polyomavirus or oncogenic adenoviruses and could be attributed to functions of the T antigen or the adenovirus E1A oncoprotein (Caparossi & Bacchetti, 1990; Drews *et al.*, 1992; Woods *et al.*, 1994; Laurent *et al.*, 1995). Based on in-vitro data demonstrating the induction of chromosomal instability by the overexpression of high-risk HPV genes and in other oncogenic DNA virus systems, it was proposed that high-risk HPVs could act as solitary carcinogens (zur Hausen, 1991). The gradual accumulation of mutations within latently infected host cells and of chromosomal abnormalities resulting from the function of viral oncoproteins would lead to a selection of clones with specific cellular DNA modifications. In a detailed study, White *et al.* (1994) reported that the expression in human cells of HPV-16 *E6*, and, to a lesser extent and through a different mechanism, HPV-16 *E7*, results in an accumulation of genetic alterations within those cells carrying the respective gene (Hashida & Yasumoto, 1991). The expression of HPV-6 *E6* or *E7* genes did not lead to detectable modifications of host cell DNA.

These data may point to a critical mechanistic difference between low- and high-risk HPV infections. In high-risk infections, time may be a sufficient factor to permit the accumulation of mutational changes due to the function of viral oncoproteins, and may suffice for the gradual progression towards malignant conversion in the absence of other cofactors (zur Hausen, 1991). However, additional genotoxic cofactors may accelerate this process. Infections with low-risk viruses, in contrast, would lead to cancer only in exceptional circumstances where other factors contribute mechanistically to modifications of the host-cell genome. Interestingly, cancers containing high-risk HPVs are aneuploid and regularly progress to metastatic growth, whereas those containing low-risk HPV genotypes frequently reveal polyploidy, grow invasively and metastasize rarely (reviewed in zur Hausen, 1991).

### *3.1.3 Immortalization and transformation of cells by HPVs*

Immortalization of cells is defined as continuous in-vitro growth of primary cells, whereas transformation is defined as the ability to induce tumours in immunocompromised hosts.

Organotypic cultures initiated from immortalized cells reveal a histological pattern virtually indistinguishable from intraepithelial neoplastic lesions (reviewed in McDougall, 1994), indicating that HPV-immortalized keratinocytes are a useful model for the analysis of the role of HPVs in cervical neoplasia. Furthermore, immortalized cell lines containing high-risk HPVs have been established from genital intraepithelial neoplasms (Schneider-Maunoury *et al.*, 1987; Stanley *et al.*, 1989; Bedell *et al.*, 1991). Based on these studies, it has been suggested that HPV-immortalized cells correspond to the clinical pattern of intraepithelial neoplasia (zur Hausen & de Villiers, 1995).

#### *(a) Contribution of viral and cellular genes*

HPVs can induce immortalization in primary cultures of human and rodent cells. There is a good correlation between the clinical classification of high risk and low risk of a given HPV type and its immortalization potential *in vitro*. In human cells, immortalization is restricted to high-risk viruses, whereas in rodent cells, low-risk HPV types also possess weak immortalizing potential.

In the first published transformation assay for a high-risk HPV, NIH 3T3 cells were transfected with recombinant full length HPV-16 DNA and a selectable marker, and transformed foci were obtained after drug selection (Yasumoto *et al.*, 1986). Similar assays were subsequently developed with several other rodent cell lines including Rat-3Y1 and Rat-1 cells (Bedell *et al.*, 1987; Watanabe & Yoshiike, 1988). Mutational analyses revealed that the HPV *E7* gene codes for the major transforming function with *E6* contributing to the formation of soft agar clones (Phelps *et al.*, 1988; Vousden *et al.*, 1988; Sedman *et al.*, 1991).

Subsequently it was discovered that the HPV *E7* gene can cooperate with the *ras* oncogene to transform primary baby rat kidney cells (Matlashewski *et al.*, 1987; Phelps *et al.*, 1988; Storey *et al.*, 1988). The *E6* gene can cooperate with *ras* and immortalize baby mouse kidney cells (Storey & Banks, 1993).

Potentially more relevant to the biology of the human papillomaviruses was the finding that high-risk HPVs could induce immortalization of primary human cells, including primary human genital keratinocytes (Dürst *et al.*, 1987a; Pirisi *et al.*, 1987; Pirisi *et al.*, 1988; Kaur &

McDougall, 1988; Pirisi *et al.*, 1988; Schlegel *et al.*, 1988; Woodworth *et al.*, 1988). The immortalized cell lines developed a fully transformed phenotype only after prolonged passage in tissue culture (Hurlin *et al.*, 1991; Pecoraro *et al.*, 1991; Pei *et al.*, 1993) or transfection with an activated viral Ha-*ras* gene (DiPaolo *et al.*, 1989; Dürst *et al.*, 1989). Co-transfection of primary human foreskin keratinocytes with HPV-18 and v-*fos* gave rise to cell lines producing non-invasive tumours in immunocompromised mice (Pei *et al.*, 1993). In one study, HPV-immortalized human keratinocytes were induced to tumorigenic progression by treatment with *N*-methyl-*N*-nitrosourea (see IARC, 1987a) (Klingelhutz *et al.*, 1993). These cells showed chromosome 18q deletions involving the tumour suppressor gene *DCC*. The cells also showed aberrations of chromosome 5q, also detected in primary cervical cancers, but this was not associated with detectable alterations in the expression of the *APC* gene associated with the development of adenopolyposis coli. These results support the hypothesis that additional cellular mutations may be required for malignant progression (zur Hausen, 1986, 1994).

Mutational analyses showed that both *E6* and *E7* were required for efficient immortalization of primary human genital tract keratinocytes (Hawley-Nelson *et al.*, 1989; Münger *et al.*, 1989a; Hudson *et al.*, 1990). In organotypic cultures of keratinocytes, *E6/E7* expression caused alterations in terminal differentiation resulting in histopathological abnormalities that are similar to those seen in preneoplastic lesions *in vivo* (McCance *et al.*, 1988; Hudson *et al.*, 1990; reviewed by McDougall, 1994).

Epithelial cells derived from different anatomical regions were immortalized by HPVs and in most cases *E6* and *E7* together were required for efficient immortalization although, under certain conditions, the HPV *E6* or *E7* genes alone were found to have a low immortalization potential (Halbert *et al.*, 1991). One exception is mammary epithelial cells, in which early passage cells could be immortalized by the *E7* gene alone (Wazer *et al.*, 1995) and relatively late passage cultures were immortalized by the HPV *E6* gene alone (Band *et al.*, 1990, 1991).

The requirement for viral oncoprotein expression to maintain the immortalized state was initially demonstrated in tissue culture cells immortalized by temperature-sensitive mutants of SV40 and polyomaviruses (Brugge & Butel, 1975; Martin & Chou, 1975; Randa *et al.*, 1989; Strauss *et al.*, 1990). The additional involvement of cellular genes was suggested by the low efficiency of immortalization induced by these viruses and the fact that the majority of infected cells, although expressing viral oncoproteins, continued to have a finite lifespan (Whitaker *et al.*, 1992). In addition, senescing somatic cell hybrids continued to transcribe HPV-18 *E6/E7* RNAs (Chen *et al.*, 1993b). The existence of at least four complementation groups for senescence in cells immortalized either by SV40 or high-risk HPV genes, respectively (Pereira-Smith & Smith, 1981; Whitaker *et al.*, 1992; Chen *et al.*, 1993b; Duncan *et al.*, 1993), underlines the importance of loss of specific cellular gene functions in the process of immortalization (for review, see zur Hausen & de Villiers, 1995). The specific host cell genes involved in this process have not yet been identified.

*(b) Chromosomal abnormalities in HPV-immortalized cells*

Chromosomal abnormalities and alterations in ploidy are regularly detected in HPV-immortalized cell lines, with some evidence for additional specific changes during oncogenic progression. Cells immortalized following the introduction of HPV sequences frequently show

alterations involving chromosome 1 (Smith *et al.*, 1989), which may contribute to the escape from normal differentiation pathways and the acquisition of a growth advantage. HPV-immortalized cells also frequently show abnormalities of chromosome 3 (Smith *et al.*, 1989), non-random loss of chromosomes 11, 19 (Popescu & DiPaolo, 1990), 5, 7, 8, 12, 16 and 22 (Smith *et al.*, 1989) and abnormalities in chromosome 10 (Crook *et al.*, 1990; Pei *et al.*, 1991). Cytogenetic analysis of HPV-18-immortalized foreskin keratinocytes showed that loss of chromosomes 21 and Y and alterations in chromosome 5q correlated with the spontaneous progression to tumorigenicity following continuous propagation in culture (Hurlin *et al.*, 1991). Treatment of the same cell line with *N*-methyl-*N*-nitrosourea also induced tumorigenic progression, which was associated with 5q+, 18q– and loss of Y (Klingelhutz *et al.*, 1993).

Chromosomal alterations occurring in tumours are described later.

*(c) Transcriptional modulation by HPV oncoproteins*

Neither HPV E6 nor E7 are DNA-binding proteins, yet they both can influence transcription from certain promoters. The HPV-16 E7 protein was first shown to be able to activate transcriptionally the adenovirus *E2* promoter (Phelps *et al.*, 1988). In the murine fibroblast cell line NIH 3T3, the E6 protein can also activate this promoter efficiently (Sedman *et al.*, 1991). Promoter mapping studies showed that binding sites for the E2F transcription factors were required for the E7-mediated transcriptional activation of the adenovirus *E2* promoter, thereby establishing a functional similarity between E7 and the 12S mRNA-encoded adenovirus E1A protein (Phelps *et al.*, 1991). These studies provided the first evidence that E7 can interfere with the regulation of viral and cellular genes mediated by members of the E2F family of transcription factors.

Conversely, the E6 protein was found to be able to modulate the expression of several basic promoters that contain a TATA box only (Lamberti *et al.*, 1990; Desaintes *et al.*, 1992). In addition, the HPV E6 protein can also interfere with the transcriptional activation and repression functions of the p53 tumour suppressor protein/transcription factor (Lechner *et al.*, 1992; Mietz *et al.*, 1992). p53-independent transcriptional repression by HPV-16 E6 also has been reported (Etscheid *et al.*, 1994). The modulation of cyclin A expression by viral oncoproteins has been reported recently (Oshima *et al.*, 1993; Spitkowsky *et al.*, 1994).

*(d) Positive and negative transforming functions of HPV*

The *E2*, *E5*, *E6* and *E7* open-reading frames of several HPV genotypes contribute to the immortalization/transformation function of the viruses. The expression of *E6* and *E7*, but not *E2* or *E5*, is regularly maintained after integration of the viral genome during carcinogenic progression (Schwartz *et al.*, 1985). Expression of *E6* and *E7* appears to be necessary for the induction as well as the maintenance of the transformed state (von Knebel-Doeberitz *et al.*, 1988; Crook *et al.*, 1989; von Knebel-Doeberitz *et al.*, 1992, 1994).

*(i) HPV E5*

The *E5* genes from both low-risk and high-risk HPVs encode short hydrophobic peptides (Bubb *et al.*, 1988; Halbert & Galloway,1988) that have mitogenic activity in a number of cell types including primary human foreskin keratinocytes and synergize with the epidermal growth factor (EGF) (Leechanachai *et al.*, 1992; Pim *et al.*, 1992; Straight *et al.*, 1993; Bouvard *et al.*,

1994). The number of EGF receptors was found to be increased two- to five-fold and enhanced receptor recycling was also detected in HPV *E5*-expressing human keratinocytes. Similarly, EGF-induced receptor phosphorylation was significantly increased in *E5*-expressing cells (Straight *et al.*, 1993). Like BPV-1 E5 proteins (Burkhardt *et al.*, 1989; Goldstein & Schlegel, 1990; Goldstein *et al.*, 1991), HPV E5 proteins were detected in the Golgi apparatus, the endoplasmatic reticulum, as well as in nuclear membranes, and they are able to form complexes with the 16 kDa subunit of a vacuolar ATPase (Conrad *et al.*, 1993). The E5 oncoprotein of HPV-16 inhibited the acidification of endosomes in human keratinocytes (Straight *et* al., 1995). Therefore, the HPV E5 proteins, like the BPV-1 E5 (Martin *et al.*, 1989; Petti *et al.*, 1991), appear to interfere with growth-factor-mediated signal transduction. According to this model, the E5 protein may endow HPV-infected cells with an initial growth advantage by interfering with the integration of positive and negative extracellular growth signals. This might lead to an enhanced responsiveness to positive extracellular signals of HPV-infected cells.

*(ii) HPV E6*

The HPV *E6* open-reading frames encode zinc-binding nuclear proteins of approximately 150 amino acids in length with a rather basic isoelectric point. They contain two pairs of Cys–X–X–Cys-sequence motives with a characteristic spacing of 29 amino acids (Cole & Danos, 1987). These cysteine-rich sequences have been implicated in zinc binding (Barbosa *et al.*, 1989). One biochemical function for the E6 proteins was provided by the discovery that, like the SV40 T antigen and the Ad5 E1B 55 kDa protein (Lane & Crawford, 1979; Linzer & Levine, 1979), high-risk HPV E6 proteins can interact specifically with the p53 tumour suppressor protein (Werness *et al.*, 1990; Crook *et al.*, 1991b).

It was observed that the levels of p53 in HPV-positive cells were very low (Matlashewski *et al.*, 1986b). This led to the suggestion that, unlike SV40 T antigen and the E1B 55 kDa protein, which inactivate p53 by sequestering it in stable complexes, the E6 proteins may inactivate p53 through degradation (Werness *et al.*, 1990). This hypothesis was supported by in-vitro studies that showed that the interaction between p53 and a high-risk HPV E6 protein resulted in the rapid degradation of p53 through the ubiquitin-mediated proteolysis pathway (Scheffner *et al.*, 1990; Crook *et al.*, 1991b). Biochemical studies showed that a 100 kDa host cellular protein designated E6-AP mediates the interaction between E6 and p53 (Huibregtse *et al.*, 1991, 1993). The E6/E6-AP complex acts as a ubiquitin ligase and therefore plays a direct enzymatic role in the ubiquitination reaction of p53 (Scheffner *et al.*, 1993). The E6/E6-AP functional inactivation of p53 is therefore mediated by a ubiquitin cascade with the E6/E6-AP complex directly conjugating ubiquitin to p53 (Scheffner *et al.*, 1995). As a consequence, the protein levels and the metabolic half-life of p53 in high-risk HPV E6-expressing cells are decreased (Hubbert *et al.*, 1992; Lechner *et al.*, 1992; Band *et al.*, 1993).

The interaction between the HPV E6 protein and p53 causes the functional inactivation of p53. The p53 tumour-suppressor protein has the properties of a sequence specific transcriptional activator (Kern *et al.*, 1991; El-Deiry *et al.*, 1992; Funk *et al.*, 1992; Pietenpol *et al.*, 1994) and this activity plays a pivotal role in p53-mediated tumour suppression. It has been shown that the high-risk HPV E6 proteins, like SV40 T antigen, are able to abrogate the transcriptional activation and repression potential of p53 (Lechner *et al.*, 1992; Mietz *et al.*, 1992; Hoppe-Seyler & Butz, 1993), which supports the notion that the regulatory function of p53 is disrupted

in high-risk HPV-infected cells. The functional importance of the interaction between high-risk HPV E6 and p53 was further corroborated by studies that analysed a p53-controlled G1/S cell cycle checkpoint. The intracellular p53 levels rapidly increase in response to DNA damage, resulting either in cell-cycle arrest or apoptosis (reviewed by Lane, 1992). The augmented levels of p53 induce the expression of negative growth regulators including the cyclin-dependent kinase inhibitor $p21^{cip1}$ leading to a growth arrest at the G1/S boundary (El-Deiry *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993). The integrity of this checkpoint appears to contribute critically to the ability of a normal cell either to repair the damaged DNA prior to initiation of DNA replication or undergo apoptosis. This checkpoint is not functional in cells transfected transiently with high-risk HPV E6 (Kessis *et al.*, 1993a; Foster *et al.*, 1994; Gu *et al.*, 1994; Canman *et al.*, 1995), which is manifested by genomic instability (Livingstone *et al.*, 1992). However, in HPV-positive cervical carcinoma cell lines, functional p53 protein has been detected (Butz *et al.*, 1995).

Since the ability of HPV E6 to bind and/or degrade p53 does not seem to account completely for the biological activities of E6 (Sedmann *et al.*, 1992; Ishiwatari *et al.*, 1994), several laboratories are in the process of defining additional cellular targets of the HPV E6 protein using various biochemical and genetic screening methods. These studies have been hampered somewhat by the absence of definitive structure–function studies. Cellular proteins associated with E6 have been identified, but with the exception of the E6-AP protein (Huibregtse *et al.*, 1993), their identity and biological importance are unknown (Keen *et al.*, 1994).

*(iii) HPV E7*

HPV *E7* genes encode zinc-binding nuclear phosphoproteins, of approximately 100 amino acids in size, that have an acidic isoelectric point. The amino terminal domain of the HPV E7 protein is strikingly similar to regions of the Ad E1A proteins and the SV40 T antigen (Figge *et al.*, 1988; Phelps *et al.*, 1988). The conserved regions of the three DNA tumour virus onco-proteins participate in the binding of the pocket proteins, a family of structurally related cellular proteins that includes the retinoblastoma tumour-suppressor protein (pRB), p107 and p130 (DeCaprio *et al.*, 1988; Whyte *et al.*, 1988; Dyson et al., 1989). The pocket protein binding site was mapped to a short peptide sequence located in the amino terminal domain of E7 (Münger *et al.*, 1989b; Barbosa *et al.*, 1990; Jones *et al.*, 1990b). Co-precipitation experiments showed that HPV-16 E7, like SV40 T antigen, binds preferentially to the hypophosphorylated form of pRB *in vivo* (Imai *et al.*, 1991; Dyson *et al.*, 1992). Cell cycle analyses have revealed that the state of phosphorylation of pRB is regulated in a cell-cycle-dependent manner. In the G0 and G1 phases of the cell cycle, when pRB is active as a growth suppressor, it is in a hypophosphorylated form. Upon cell cycle progression, pRB becomes phosphorylated at multiple serine residues by one or more cyclin-dependent kinases at the G1/S boundary. Consequently, pRB is present in a hyperphosphorylated form during S, G2 and early M phases, and is dephosphorylated in late M phase (reviewed by Weinberg, 1995). Complex formation between E7 and the pocket proteins results in their functional inactivation thereby dysregulating progression of the cell cycle into S phase. The ability to form complexes with the pocket proteins may therefore contribute to the ability of the HPV E7 protein to induce DNA synthesis (Sato *et al.*, 1989b; Banks *et al.*, 1990).

Members of the E2F family of transcription factors are regarded as modulators of pocket protein function (reviewed by Nevins, 1992). When bound to the G0/G1 specific hypophosphorylated form of pRB, E2F acts as a transcriptional repressor. Upon G1/S-specific phosphorylation of pRB, the E2F/pRB complex is disrupted and E2F has transcriptional activation function (Weintraub *et al.*, 1992). E2F/pocket protein complexes are detected by electrophoretic mobility shift assays using nuclear protein extracts and an E2F binding site oligonucleotide as a probe. In high-risk HPV E7-expressing cell lines, the E2F/pocket protein complexes are functionally compromised (Chellappan *et al.*, 1992; Pagano *et al.*, 1992) — the HPV E7 protein can disrupt pRB/E2F complex and interact with the cyclin A/E2F complex without efficiently disrupting it (Arroyo *et al.*, 1993). The interaction of E7 with the cyclin A/E2F complex leads to its functional inactivation resulting in the transcriptional activation of responsive promoters (Lam *et al.*, 1994).

The regulation of the transcriptional activity of E2F by the pocket proteins may be an important step for ordered G1/S transition and the disruption of this regulatory circuit by the E7 protein in HPV-infected cells is thought to contribute critically to carcinogenic progression. In agreement with this model, it has been shown that overexpression of E2F leads to cell cycle progression and morphological changes characteristic of cellular transformation (Johnson *et al.*, 1994; Singh *et al.*, 1994). *E7* expression can abrogate at least some of the growth-suppression functions of p53 (reviewed in Farthing & Vousden, 1994), although wild-type *p53* expression can inhibit transformation by E7 (Crook *et al.*, 1991c, 1994). In cells with an intact p53 pathway, overexpression of E2F induces apoptosis (Qin *et al.*, 1994; Wu & Levine, 1994) and it seems likely that *E6* expression is important in the abrogation of this function.

Like Ad E1A, HPV-16 E7 is able to bind cyclin A as well as a histone H1 kinase (Dyson *et al.*, 1992; Davies *et al.*, 1993; Tommasino *et al.*, 1993). Interestingly, the pocket protein p107 can serve as a substrate for this protein kinase activity (Davies *et al.*, 1993). Whether this is a direct interaction or is mediated by an adaptor protein is unclear at present.

Several mutagenic analyses of *E7* have helped define the molecular determinants for the multiple biological and biochemical properties of E7. The ability of E7 to interact efficiently with the pocket proteins was found to be critical for many of the biological and biochemical properties of E7 (Edmonds & Vousden, 1989; Storey *et al.*, 1990; Watanabe *et al.*, 1990; Phelps *et al.*, 1992). This was illustrated clearly by studies of chimeric high-risk/low-risk HPV E7 proteins, which showed that the differences in transformation efficiencies in rodent cells correlated directly with the relative binding efficiency to retinoblastoma protein. Sequence comparisons revealed a single consistent amino acid sequence difference in the pRB binding sites between the high-risk and the low-risk E7 proteins (Asp 21 in HPV-16 E7 corresponding to Gly 22 in HPV-6 E7). Mutation of the corresponding residues in E7 showed that this difference is largely responsible for the differential transforming capacity of the low-risk and the high-risk E7 proteins (Heck *et al.*, 1992; Sang & Barbosa, 1992a). The relevance of these findings, however, may be limited to E7 proteins encoded by HPVs that are associated with anogenital tract lesions since the E7 protein encoded by HPV-1, an HPV type associated with benign plantar warts, also efficiently interacts with pRB (Ciccolini *et al.*, 1994; Schmitt *et al.*, 1994).

Several studies have provided evidence for the presence of additional regions that, independent of or in addition to pRB binding, are important for E7 function in cellular immorta-

lization and transformation. Mutations in the amino terminal CR1 homology domain of E7 interfere with its transformation potential independent of pRB binding (Edmonds & Vousden, 1989; Phelps *et al.*, 1992; Brokaw *et al.*, 1994) and pRB-binding-deficient E7 mutants, in the context of the whole genome, are able to induce cellular immortalization of primary human genital epithelial cells (Jewers *et al.*, 1992). It has also been shown in the CRPV system that CRPV mutations in the pRB binding site did not result in the prevention of papillomas (Defeo-Jones *et al.*, 1993). The pRB binding function of E7 is necessary for the immortalization of human cells, when only E6 and E7 proteins are expressed (Melillo *et al.*, 1994).

The specific contributions of the cysteine-rich carboxyl-terminus of E7 are poorly understood. Mutations in the carboxyl-terminal half of E7 that interfere with any of the functions of E7 generally also affect the intracellular stability of the protein, suggesting that the integrity of the carboxyl-terminus is important for its intracellular stability (Phelps *et al.*, 1992; McIntyre *et al.*, 1993). There are several studies that suggest specific contributions of the carboxyl-terminus to E7 functions. Binding of E7 to pRB abrogates the nonspecific DNA-binding properties of pRB and studies with truncated E7 polypeptides suggest that, in addition to the pRB binding site, carboxyl-terminal sequences are also necessary for this activity (Stirdivant *et al.*, 1992). Similarly, the ability of E7 to disrupt the complex between the cellular transcription factor E2F and pRB requires additional sequences in the carboxyl-terminus of E7 (Hwang *et al.*, 1993; Wu *et al.*, 1993). It has been suggested that this region of E7 constitutes an independent low-affinity binding site for pRB (Patrick *et al.*, 1994). The only mutation that rendered E7 incompetent for immortalization was located in the carboxyl-terminus (Jewers *et al.*, 1992). The HPV E7 protein can form dimeric or multimeric complexes through carboxyl-terminal sequences. The biological implications of this property of E7 are not clear (Roth *et al.*, 1992; McIntyre *et al.*, 1993).

*(iv) HPV E2*

Expression of the HPV *E2* gene is frequently disrupted as a result of the integration of the viral genome during carcinogenic progression. HPV *E2* gene encodes DNA-binding proteins that can repress transcription from the HPV promoter(s) that govern expression of the *E6* and *E7* oncogenes (Thierry & Yaniv, 1987; Romanczuk *et al.*, 1991; Thierry & Howley, 1991). HPV genomes in which *E2* expression is disrupted have higher levels of *E6/E7* expression (Sang & Barbosa, 1992b) and an enhanced immortalization activity as compared to a wild type genome (Romanczuk & Howley, 1992). In accord with these results, the re-establishment of *E2* expression in cervical carcinoma cell lines results in growth suppression, which is at least in part mediated via the repression of the HPV *E6/E7* promoter (Thierry & Yaniv, 1987; Hwang *et al.*, 1993).

*(e) HPV-targeted cellular proteins in cervical carcinogenesis*

*(i) p53*

The most intensively studied genetic alterations in cervical cancers are those affecting the tumour suppressor gene *p53*. Alteration in *p53* is a common event in the development of most major carcinomas, suggesting that loss of normal p53 function is an important step during carcinogenesis (Hollstein *et al.*, 1991). The observation that one of the HPV-encoded oncoproteins, E6, can interact with, and functionally inactivate, the p53 protein (see above) suggested that somatic alteration within the *p53* gene may not be a necessary step during the development

of HPV-associated cancers. A large number of studies have attempted to address this question by analysing the status of the *p53* gene in HPV-positive and HPV-negative cancers and cancer cell lines. Results from the cancer cell lines were very striking and reproducible between different groups. No *p53* mutations were detected in a total of 11 independently derived HPV-positive cell lines, whilst three separate HPV-negative cervical carcinoma cell lines showed evidence of point mutation at a hot spot region within the *p53* gene, two at codon 273 and one at codon 245 (Crook *et al.*, 1991d; Scheffner *et al.*, 1991; Iwasaka *et al.*, 1993). This inverse relationship between *p53* mutation and HPV infection was interpreted as evidence supporting the importance of loss of p53 function in the development of these tumours and the functional significance of the E6/p53 interaction. Subsequent studies in primary cancer biopsies have been less clear. In 16 independent analyses of HPV-associated genital cancers, eight tumours with evidence of *p53* mutation were detected in a total of 375 cancers (2%) (Crook *et al.*, 1991d, 1992; Fujita *et al.*, 1992; Lo *et al.*, 1992; Tsuda & Hirohashi, 1992; Chen *et al.*, 1993c; Choo & Chong, 1993; Helland *et al.*, 1993; Paquette *et al.*, 1993; Busby-Earle *et al.*, 1994; Chen *et al.*, 1994a; Jiko *et al.*, 1994; Kurvinen *et al.*, 1994; Lee *et al.*, 1994; Pao *et al.*, 1994b; Park *et al.*, 1994). The observation that *p53* mutations are extremely rare in HPV-positive cancers is not in dispute. The detection of *p53* point mutations in apparently HPV-negative cancers has been less consistent. Some studies reported a relatively high incidence of *p53* mutations in these tumors, although most found a low incidence of mutation, regardless of the HPV type. Overall, 146 HPV-negative tumours were analysed and *p53* mutations identified in 24 (16%). Four studies investigating head and neck tumours identified a total of six *p53* mutations in 27 HPV-positive cancers (22%) and 32 *p53* point mutations in 81 HPV-negative cancers (40%) (Brachman *et al.*, 1992; Lee *et al.*, 1993; Chang *et al.*, 1994; Snijders *et al.*, 1994b).

Analysis of p53 protein levels, as an indication of *p53* abnormalities, also fails to provide a good consensus; some studies showed a correlation between absence of HPV sequences and p53 protein detection (Tervahauta *et al.*, 1993), which is not detected by others (Cooper *et al.*, 1993). These studies are more difficult to interpret than those describing direct sequencing of the *p53* gene within the tumour cells, since the correlation between p53 levels and abnormalities within the protein is not perfect. One study in which both protein levels and *p53* sequence was determined revealed a point mutation in only one of eight cervical cancers with immunohistochemical p53 staining (Busby-Earle *et al.*, 1994). Loss of heterozygosity on chromosome 17p is associated with HPV-negative cancers (Kaelbling *et al.*, 1992), although the importance of this may be related to the loss of another gene in this region of chromosome 17, rather than *p53* itself.

The identification of tumours apparently lacking both HPV and *p53* mutations suggests that other pathways or different genetic lesions are involved in the development of these cancers (Park *et al.*, 1994). Studies of p53 function have shown that the activity of the p53 protein can be regulated by interaction with cell proteins. One of these, termed MDM2, is transcriptionally activated by p53 itself and binds to p53 within the activation domain, inhibiting p53 activity and providing a feedback mechanism for the modulation of p53 function. Some tumours that do not show frequent evidence of somatic mutation within the p53 protein itself have been shown to carry amplifications of the *MDM2* gene, although one study of HPV-negative cervical cancers failed to detect these amplifications (Kessis *et al.*, 1993b).

Several tumours have also been identified that are positive for both *p53* mutations and HPV. Some studies show an association of *p53* mutations with more aggressive cervical cancers, although this is not always seen (Chen *et al.*, 1994a; Jiko *et al.*, 1994). A small study of metastases from HPV-positive primary cancers suggested the possibility that the acquisition of *p53* mutations may contribute to the further progression of an HPV-associated primary cancer. *p53* Mutations have been identified as a late-stage event in other tumour types and under experimental conditions certain forms of mutant *p53* display transforming activities that are independent of the loss of wild-type growth suppressor function. It is therefore postulated that the interaction of E6 with the wild-type p53 protein in HPV-infected cervical cells results in the loss of protein function, whereas expression of a mutant p53 protein might provide an additional oncogenic signal at later stages of progression (Crook & Vousden, 1992). Some other studies have failed to detect *p53* mutations in HPV-positive metastases (Stanley & Sarkar, 1994) and not enough tumours have been examined to determine whether this genetic event contributes significantly to metastatic progression in these cancers. Transfection of HPV-16-immortalized and HPV-18-immortalized human cervical cell lines with a dominantly transforming mouse mutant p53 resulted in enhanced growth rates of the human cell lines and an increase in the efficiency of anchorage and independent colony formation; however, cells expressing the mutant p53 protein did not become tumorigenic (Chen *et al.*, 1993c).

*(ii) pRB*

Like p53, pRB is a tumour suppressor whose function is frequently lost during the development of several types of human cancer (Weinberg, 1995). The HPV-encoded oncoprotein E7 has been shown to interact with, and inactivate the function of pRB (see section above). Analysis of cervical carcinoma cells lines for alterations in the *pRB* gene revealed mutations only in the two HPV-negative lines studied, with wild-type present in the six HPV-positive lines (Scheffner *et al.*, 1991). This suggests an inverse correlation between HPV positivity and *pRB* mutation, similar to that postulated for HPV and *p53*. Further analyses of primary tumour material have been hampered because of the large size of the *pRB* gene, but one study of 12 HPV-negative small-cell cervical cancers failed to find any evidence of *pRB* mutation (Pao *et al.*, 1994b). Alterations of the cyclin dependent kinase inhibitor p16, which are frequently seen in other pRB-positive cell lines, are not detected in the HPV-positive cervical carcinoma lines, again supporting a functional significance of the E7/pRB interaction in these cells. Deletions of chromosome 9p21, containing the p16 locus, are also rarely seen in cervical cancer (reviewed in Stanley & Sarkar, 1994).

### *3.1.4 Experimental evidence for a role of high-risk HPVs in malignant conversion and in human cervical cancers*

#### *(a) Requirement for HPV gene expression for invasive growth and the malignant phenotype*

A low level of HPV *E6/E7* expression was noted in the proliferative layer of low-grade CIN in comparison with more advanced lesions (Dürst *et al.*, 1991; Iftner *et al.*, 1992; Stoler *et al.*, 1992).

Two sets of experimental data reveal the requirement of HPV *E6/E7* gene expression for the invasive phenotype of human cells after heterografting these cells into immunocompromised

mice. Cells immortalized by HPV-16 reduce E6/E7 transcription substantially upon heterografting (Dürst *et al.*, 1991). Similarly, non-malignant hybrid cells produced by cell fusion of HPV-18-containing HeLa cells and human fibroblasts or human keratinocytes, which expressed significant levels of viral oncoproteins when kept in tissue culture, revealed a substantial transcriptional repression of these genes within three days of transplantation into the nude mouse system (Bartsch *et al.*, 1992). Malignant revertants obtained from these lines or the parental HeLa cells fail to reveal this transcriptional down-regulation.

The requirement for high expression levels of viral oncoproteins for the malignant phenotype is further underlined by experiments using inducible *E6/E7* antisense constructs, in the human cervical carcinoma cell line C4-1 (von Knebel-Doeberitz *et al.*, 1988, 1992). By inducing the antisense transcription, cell growth was impaired and carcinogenicity abolished. In a specific cervical cancer cell line, SW 752, HPV-18 *E6/E7* transcription could be switched off by the addition of dexamethasone. These cells ceased to grow in culture and were no longer carcinogenic in nude mice receiving dexamethasone. Reconstitution of either *E6* or *E7* expression by an hormone-inducible promoter restored the carcinogenicity (von Knebel-Doeberitz *et al.*, 1994).

The selective down-regulation of HPV transcription in immortalized cells and not in malignant cells can be reproduced under tissue culture conditions by 5-azacytidine treatment or by the addition of human or murine macrophages (Rösl *et al.*, 1988, 1994; Kleine *et al.*, 1995). In the latter two studies it was demonstrated that the production of TNFα by macrophages was induced by a cytokine (MCP-1) selectively produced by non-malignant cells; TNFα mediates the down-regulation of HPV-16 and -18 transcription in these cells. This suggests that the transition from immortalization to malignant conversion involved the modification of genes engaged in the control of HPV transcription and which are activated by a paracrine regulatory pathway.

*(b) Integration of HPV sequences*

During the normal HPV life cycle, viral DNA is maintained episomally in the nucleus of the infected cell. The detection of integrated viral DNA sequences in cervical neoplasia (Schwartz *et al.*, 1985) is frequently associated with malignant progression, integration being more common in carcinomas than in cervical intraepithelial neoplasia (Cullen *et al.*, 1991), with some studies reporting integration in high-grade dysplasia (Lehn *et al.*, 1988; Fukushima *et al.*, 1990). The persistence of both episomal and integrated copies of the HPV genome in some cervical cancers has been reported (Kristiansen *et al.*, 1994b) and most studies identify at least some carcinomas with only episomal viral DNA (Das *et al.*, 1992b). Similar integration of HPV sequences is reported in HPV-associated vulval carcinomas (Venuti & Marcante, 1989). The consequences of integration and potential contribution to tumour progression may reflect perturbations in viral and host gene expression.

*(i) Effects of integration on viral gene expression*

Many studies agree that integration of viral sequences can result in partial loss of the viral genome and loss of expression of several viral open-reading frames (Wilczynski *et al.*, 1988b). The *E6* and *E7* genes, however, are almost always found to be expressed in HPV-associated cancers (van den Brule *et al.*, 1991b). Integration is postulated to deregulate *E6* and *E7* expression through loss of viral transcriptional regulators such as E2 and/or escape from intra-

cellular control through transcriptional initiation of these viral regions from flanking cellular promoters (Rösl *et al.*, 1991). However, a significant proportion of cervical tumours arise without evidence for integration of HPV sequences, and recent studies suggest that loss of the *E2* regulatory region is not the only mechanism to perturb *E6/E7* expression. A specific binding site for the transcription factor YY1 in the HPV-18 promoter was shown to be involved in the regulation of *E6/E7* gene transcription (Bauknecht *et al.*, 1992). Analysis of the viral promoters in malignant tumours harbouring episomal HPV-16 sequences only, showed that three of six cases carried a mutation in the YY1 binding site and that this resulted in enhanced activity of the *E6/E7* promoter (Dong *et al.*, 1994). Several papers examining individual tumours report evidence of modifications within the locus control region of both HPV-16-associated (Tidy *et al.*, 1989) and HPV-6-associated tumours (reviewed in Kitasato *et al.*, 1994) containing episomal viral DNA. Integration may also result in increased stability of the E6/E7 in mRNA (Jeon & Lambert, 1995), giving cells with integrated viral sequences a selective growth advantage (Jeon *et al.*, 1995).

*(ii) Effects of integration on cell gene expression*

Another potential oncogenic consequence of HPV integration is the transcriptional activation of cellular proto-oncogenes by viral promoters. Although several reports show integration of the HPV sequences close to proto-oncogenes such as *myc* at chromosome 8q24 (Dürst *et al.*, 1987b; Couturier *et al.*, 1991), there is no firm evidence for consistency in the integration site with respect to any one specific chromosomal location (Mincheva *et al.*, 1987). However, several studies have suggested that the integration may occur preferentially at fragile sites, frequently within the location of a proto-oncogene (Dürst *et al.*, 1987b; Popescu *et al.*, 1989).

Integration *per se* is not sufficient for malignant conversion. Somatic cell hybridizations have produced a non-malignant phenotype of hybrid cells in spite of the persistence of integrated viral DNA and the continued expression of viral oncoproteins (Saxon *et al.*, 1986; Koi *et al.*, 1989).

*(c) Chromosomal abnormalities in HPV-associated cancers*

*(i) Primary tumours*

Cytogenetic analyses of cervical carcinomas have demonstrated non-random alterations of several chromosomes. Chromosome 1 is commonly involved in structural or numerical alterations in cervical cancers (Atkin, 1986), with frequent loss of heterozygosity in the short arm of chromosome 1 at 1p36 (Wong *et al.*, 1993). These changes were found in both HPV-positive and HPV-negative cancers and were not related to the stage of the tumour, which suggests that they did not contribute to the progression of the established tumour. This is supported by the detection of chromosome 1 aberrations in early premalignant cervical lesions (Atkin *et al.*, 1983). Another common region of loss of heterozygosity is on the short arm of chromosome 3, identified as 3p14 and 3p25 (Chung *et al.*, 1992), 3p21-22 (Karlsen *et al.*, 1994) or 3p14-21 and 3p22-24.1 (Yokota *et al.*, 1989). As with the alterations on chromosome 1, loss of genetic material at these loci was seen in both HPV-positive and HPV-negative women and, although HPV integration has been reported occasionally in these regions (Mincheva *et al.*, 1987), there was no correlation between HPV infection and chromosome 3p deletion. Loss of heterozygosity on chromosome 3 was found in both invasive cancers and cervical premalignancies, suggesting

that like chromosome 1, these alterations play a role in tumour development. The detection of numerical and structural changes in chromosome 1 may provide a method to study premalignant stages in cervical smears (Segers *et al.*, 1994). A recent study has also described loss of heterozygosity for one or more markers on chromosome 11 in 14/32 patients, with evidence for the existence of a cervical cancer-related tumour suppressor gene at 11q22-11q24 (Hampton *et al.*, 1994). This correlates with the earlier observation that the tumorigenicity of the HPV-18-expressing cervical carcinoma cell line HeLa and of the HPV-16-expressing cell line SiHa can be suppressed by the addition of a normal human chromosome 11 in somatic cell hybrids (Saxon *et al.*, 1986; Koi *et al.*, 1989). A potential candidate for a tumour suppressor gene in this region has been reported (Lichy *et al.*, 1992). Interestingly, only human embryonic fibroblasts with a deletion on chromosome 11 were susceptible to HPV-16-mediated transformation. The region deleted in these cells was between 11p11.11-11p15.1, which may indicate the possible location of another tumour suppressor gene active in controlling malignant development of HPV-infected cells on this chromosome (Smits *et al.*, 1988). Cells with this chromosomal deletion reveal an upregulation of the regulatory subunit of the protein phosphatase 2A, which in turn leads to an upregulation of transfected E6/E7 transcription (Smits *et al.*, 1992b). Other regions sustaining loss of heterozygosity in cervical cancer include 9q, 10q and 17p (Jones *et al.*, 1994), 4q, 5p, 5q, 11p and 18p (Mitra *et al.*, 1994) and 1p, 1q, 2q, 3q, 5q, 6p, 6q, 9q, 10q, 11p, 11q, 17p and 17q (Sreekantaiah *et al.*, 1991).

*(d) Alterations of specific proto-oncogenes*

*(i) ras*

Analysis of cervical tumours for mutational activation of the Ha- or Ki-*ras* genes generally revealed no evidence that *ras* point mutation is an important step in the development of these cancers (Willis *et al.*, 1993), although one study reported mutations in Ha-*ras* in 7/29 advanced-stage tumours (Riou *et al.*, 1988). Anal cancers, which show an HPV association very similar to that seen with cervical cancers, and vulvar cancers also failed to display evidence of frequent *ras* mutation (Hiorns *et al.*, 1990; Tate *et al.*, 1994). Cancers at other sites which are less strongly linked to HPV infection, such as head and neck tumours or prostate cancers, show a significantly higher incidence of *ras* mutations, with some evidence of association with late-stage disease (Anwar *et al.*, 1992a, 1993).

*(ii) myc*

Reports of c-*myc* amplification or overexpression in cervical cancers are not consistent. Several studies report alterations in c-*myc* in a significant proportion of cervical cancers, some up to 90% (Riou *et al.*, 1984; Ocadiz *et al.*, 1987). However, other studies fail to find amplification of c-*myc* sequences in any of the tumours examined (Yokota *et al.*, 1989). The latter study examined only stage I and II tumours and this discrepancy may be related to the stage of the tumours under investigation. Overexpression of c-*myc* RNA has also been associated with aggressive disease, with c-*myc*-positive tumours showing a higher incidence of metastasis and recurrence (Riou *et al.*, 1992). c-*myc* Protein was detected in invasive carcinomas but not lower-grade cervical lesions in some studies (Devictor *et al.*, 1993), while other studies failed to find evidence for myc expressed-protein in cervical neoplasia (Hughes *et al.*, 1989).

Introduction of v-*myc* into HPV-immortalized human cervical epithelial cells did not result in the generation of tumorigenic lines (DiPaolo *et al.*, 1989). Elevated levels of c-*myc* expression have been detected in HPV-positive cervical carcinoma cell lines (Dürst *et al.*, 1987b) and amplification and overexpression of c-*myc* sequences in an HPV-16-containing cervical cell line was associated with an increased growth rate and resistance to differentiation, although in this case the potential acquisition of a tumorigenic phenotype was not examined (Crook *et al.*, 1990).

*(iii) Other alterations*

HPV-immortalized human keratinocytes, induced to tumorigenic progression following treatment with *N*-methyl-*N*-nitrosourea, showed chromosome 18q deletions and were subsequently shown to have undergone a deletion of the tumour-suppressor gene *DCC* (Klingelhutz *et al.*, 1993).

The role of other oncogenes in cervical cancers has been less extensively studied. Analysis of the c-*erb*B-2 gene provided some evidence that amplification and overexpression of this gene may be associated with advanced tumours with a poor prognosis (Kihana *et al.*, 1994). *LA-1* oncogene amplification may also play a role in cervical cancer development (Sharma *et al.*, 1994).

Analysis of the loss of heterozygosity frequently observed on chromosome 3p has implicated a role for the loss of the *raf-1* gene and the retinoic acid receptor, as in the development of cervical cancers (Yokota *et al.*, 1989; Chung *et al.*, 1992), although the significance of this to the biology of these tumours is not known.

A study of DNA hypomethylation in biopsy specimens from 41 patients with varying degrees of cervical abnormalities showed a progressive increase during progression through premalignant to malignant lesions (Kim *et al.*, 1994). The HPV status of the lesions examined in this study was not determined, so further studies of the relationship, if any, between HPV infection and DNA hypomethylation are warranted. At least one cervical cell line (CaSki) exists in which even HPV genomes are hypermethylated rather than hypomethylated (Rösl *et al.*, 1993).

### *3.1.5 Interactions between HPV and environmental agents*

Although there is compelling epidemiological and molecular evidence to link papillomaviruses to the etiology of squamous-cell carcinomas, additional factors contribute to this multistage process.

*(a) Interaction with other viruses*

Early suspicions of a role of herpes simplex virus (HSV) infections in cancer of the cervix were based on seroepidemiological studies (Rawls *et al.*, 1968; Nahmias *et al.*, 1970). Subsequently, in-vitro transformation studies of hamster cells by HSV-1 and HSV-2 have been reported (Duff & Rapp, 1971). In subsequent years, the value of these studies has been questioned (reviewed in zur Hausen, 1975, 1983), particularly after a large prospective study failed to provide support for the early suspicion (Vonka *et al.*, 1984).

The concept that HSV and HPV may act as syncarcinogens (zur Hausen, 1982) has not been supported by a case–control study conducted by Muñoz *et al.* (1995) in Spain and Colombia, yet

finds some support from in-vitro transformation studies. HPV-16-immortalized human foreskin keratinocytes transfected with a recombinant plasmid bearing the HSV-2 fragment Bg/II N yielded tumorigenic clones, whereas the parental HPV-immortalized cell lines were incapable of inducing tumours. Southern blot analysis of the viral sequences present in the transformed cell lines indicated that while HPV-16 genomes were maintained in an unchanged integrated state, a complete loss of HSV-2 sequences was observed in the tumour-derived cell lines (DiPaolo *et al.*, 1990). HSV-2 morphological transformation region III has been reported to induce rearrangements of HPV-18 DNA sequences in immortalized human keratinocytes and chromosomal changes in HPV-16-immortalized human cell lines (Dhanwada *et al.*, 1993). A previous report showed stimulation of HPV-18 transcription by HSV-1 DNA fragments (Gius & Laimins, 1989).

Significantly, herpes-group viruses, as well as vaccinia virus and adenoviruses, can induce amplification of papovavirus DNA contained in various cell lines, a process also observed in some of these lines after treatment with chemical or physical inhibitors (Lavi, 1982; Schlehofer *et al.*, 1983a; Matz *et al.*, 1985). HSV-1 infection of a BPV-1 transformed mouse cell line caused amplification of the BPV-1 DNA sequences (Schmitt *et al.*, 1989).

Papillomavirus expression *in vitro* can also be influenced by other viruses including human immunodeficiency virus (HIV) (see section 2.5.3) and human herpesvirus 6 (HHV-6). HHV-6, a T-lymphotropic virus, widely distributed in the general population, can transactivate the long terminal repeat (LTR) of HIV. Recently, human exocervical cells immortalized with HPV-16 and HPV-positive cervical carcinoma cell lines were successfully infected with HHV-6, as demonstrated by expression of the early–late antigens of HHV-6 and maintenance of the viral genome (Chen *et al.*, 1994b). HHV-6-infected cervical carcinoma cell lines were more tumorigenic in nude mice when compared to the parental counterparts; however, HPV-16-immortalized cells bearing HHV-6 episomes did not induce tumours in mice. The level of HPV *E6* and *E7* mRNAs was increased by HHV-6 in the infected carcinoma cell lines, possibly through the same mechanism by which HHV-6 transactivates the HIV-1 LTR. HHV-6 DNA was detected in six out of 72 cases of squamous-cell carcinoma and CIN in China, but it was absent from normal cervical tissues. HPV-16 was also found in four of the HHV-6-positive samples (two carcinomas, two CIN III) (Chen *et al.*, 1994b). The relevance of these interactions *in vivo* awaits further investigation.

Herpes-dependent and herpes-independent parvoviruses have been shown to interfere negatively with oncogenes (Toolan & Ledinko, 1968). In this context, the observed inhibition of DNA amplification by adeno-associated viruses (AAV) seems to be of relevance (Schlehofer *et al.*, 1983b). Recently, AAV-2 has been shown to inhibit HPV-16-induced oncogenic transformation '*in vitro*'. This inhibition is mediated by the AAV *Rep78* gene, possibly at the level of transcription of the viral oncogenes (Hermonat, 1994). This could explain the tumour suppressor properties of AAV and corroborate the seroepidemiological findings of higher titres of anti-adeno-associated virus antibodies in the normal population compared to cancer patients (Sprecher-Goldberger *et al.*, 1971; Mayor *et al.*, 1976; Georg-Fries *et al.*, 1984).

*(b) Hormones and antioestrogens*

Several studies show that hormones interact at the molecular level with papillomavirus genomes and modify their expression. The upstream regulatory region of papillomaviruses contained glucocorticoid responsive elements, as shown by the enhancement of HPV-16 transcription upon dexamethasone treatment of cells in culture (Chan *et al.*, 1988). This was also shown in transgenic mice containing the HPV-18 long terminal repeat linked to the *Escherichia coli* β-galactosidase gene (Cid *et al.*, 1993). Transformation of mouse primary cells by HPV-16 in combination with an activated oncogene has been shown to be dependent on glucocorticoids: cells became transformed and tumorigenic in the presence of dexamethasone (Crook *et al.*, 1988; Pater *et al.*, 1988). Furthermore, these experiments could be reproduced in the presence of progesterone and progestins; the latter are pharmacologically active components in oral contraceptives (Pater *et al.*, 1990). This effect could be inhibited by RU486, a synthetic antagonist of these hormones (Pater & Pater, 1991).

Dexamethasone has been shown to interfere differentially with the transcription of HPV *E6* and *E7* genes in HPV-18-positive cervical carcinoma cell lines as follows: enhanced gene transcription, associated with growth stimulation, was observed in the two cell lines C4-1 and C4-2; no effect was seen in HeLa cells, whereas a marked reduction in transcription, accompanied by growth retardation, was observed with SW 756 (von Knebel-Doeberitz *et al.*, 1991). These results may be explained by *cis* effects exerted by flanking host cell DNA sequences differing among the individual cell lines tested. Recently, the levels of the HPV-18-E7 protein in HeLa and C4-1 cell lines have been shown to be increased by hydrocortisone, while progesterone, oestrogen or testosterone had no effect (Selvey *et al.*, 1994). No progesterone or oestrogen receptors were detected in these carcinoma cell lines, but HPV-16 expression was markedly increased in human ectocervical cells exposed to glucocorticoid or progesterone (Mittal *et al.*, 1993a). This effect was inhibited by the anti-progestin RU486 and was shown to be dependent upon three hormone-responsive elements present in the viral regulatory region (Mittal *et al.*, 1993b).

Oestrogen and progesterone receptors were measured in normal cervical tissues and HPV-affected tissues from the cervix, vulva and penis (Monsonego *et al.*, 1991). While penile samples did not contain hormone receptors, cervical lesions had high levels, with high-grade lesions exhibiting the highest values, whereas low levels were detected in the vulvar tumours. Among the cervical tissues, squamous carcinomas had low levels of progesterone and an absence of oestrogen receptors. No association was found between levels of receptors and oral contraceptive use. Elevated progesterone receptor levels were more significantly correlated with HPV-16, -18-positive cervical lesions than to HPV-negative samples. Immunocytochemical localization of the receptors showed that they were evenly distributed in the connective tissue, but never detected in the epithelial cells. This is in contrast to what has been observed in cell culture and suggests an indirect effect of hormones on the HPV-infected cells. Additional studies are required to establish the relevance of the association between hormones and HPV during malignant transformation *in vivo*.

Oestrogen metabolism was measured in laryngeal papillomas, benign tumours that are known to be linked to HPV-6 and -11 infection, and in which oestrogen binding is known to be increased (Newfield *et al.*, 1993). Increased tumour risk is associated with increased 16-α-

hydroxylation, while reduced risk is related to 2-hydroxylation. Explant cultures of laryngeal papillomas were shown to have an increased 16-α-hydroxylation of oestradiol-17β (see IARC, 1987b) when compared to normal laryngeal cells. Oestradiol-17β and 16-α-hydroxyoestrone increased the proliferation of laryngeal papilloma cells; on the other hand, the alternative metabolite 2-hydroxyoestrone had an anti-proliferative effect on these cells. Since indole-3-carbinol is a potent inducer of 2-hydroxylation of oestradiol-17β, this compound was added to laryngeal cell cultures and shown to inhibit oestradiol-17β-induced cell proliferation, similar to the effect observed with 2-hydroxyoestrone. Further experiments have shown that dietary indole-3-carbinol reduced the development of tumours in infected laryngeal tissue xenografts in nude mice.

The chemotherapeutic agent tamoxifen has been shown to stimulate the proliferation of an HPV-16-positive cervical carcinoma cell line (Hwang *et al.*, 1992). At low concentrations, this drug was shown to increase both the HPV-16 mRNA and E7 levels, which may account for the higher proliferation rates observed. On the other hand, the growth of this HPV-16-positive cell line was inhibited by higher concentrations of tamoxifen, as seen in a wide variety of tissues and cell lines.

*(c) Chemicals*

The pioneering studies of Rous and his associates demonstrated that the tarring of skin or treatment of skin with other chemical carcinogens accelerates greatly the emergence of CRPV-induced papillomas and their malignant conversion (Rous & Kidd, 1938). Studies conducted by M.S. Campo and W.F. Jarrett and their colleagues revealed the interaction of BPV infection and exposure to bracken fern (see IARC, 1987c) in the induction of malignant tumours in bovines (see section 4). The contribution of bracken fern is due to both its carcinogenic and immunosuppressive properties (see section 4).

Mutagens and immunosuppressants, such as those present in bracken fern or derived from the constituents of tobacco smoke (see IARC, 1986), may co-operate with the papillomavirus in the induction of malignancies in different ways (Jackson *et al.*, 1993). Several reports describe the interaction between BPV and chemical cofactors in the induction of malignant tumours (see section 4). A recent observation of extensive papillomatosis of the hairy skin spreading from oral mucosa in an iatrogenically immunosuppressed dog provides further support for reactivation of latent papillomavirus infections (in this case, canine oral papillomavirus (COPV)) and expansion in the tissue tropism of the virus (Sundberg *et al.*, 1994).

Treatment of a mouse BPV-1-transformed cell line with *N*-methyl-*N*′-nitro-*N*-nitroso-guanidine (MNNG) (see IARC, 1987d) led to the amplification of both episomal and integrated sequences of BPV-1 DNA (Schmitt *et al.*, 1989). 12-*O*-Tetradecanoylphorbol 13-acetate (TPA), on the other hand, was ineffective, either alone or in combination with MNNG. Amplification of BPV DNA by initiating agents may be an important event in the co-operative effects between viral genomes and other carcinogens.

Constituents of tobacco smoke and its derived nitroso compounds have been shown to be potent carcinogens in different experimental systems (see IARC, 1986). Higher levels of these compounds were detected in cervical secretions of smokers compared with nonsmokers (Sasson *et al.*, 1985; Schiffman *et al.*, 1987). Mutagenic activity in cervical cells was demonstrated to be

similar to that observed in human lung tissue (Phillips & Nishe, 1993), which points to a possible role of these compounds in cervical carcinogenesis. When exposed to either benzo[a]-pyrene or ethyl methanesulfonate, HPV-16-immortalized human oral keratocytes were able to proliferate in higher calcium concentration, whereas normal and non-carcinogen exposed cells differentiated terminally under these conditions (Li *et al.*, 1992). Moreover, they acquired the ability to induce squamous-cell carcinomas in 20–50% of the injected animals. These carcinomas eventually regressed and it was noted that they had a wild-type c-Ha-*ras* and *p53*. When compared to their normal counterparts, these cells expressed higher levels of *E6/E7* mRNAs and transforming growth factor (TGF)-α, which may have accounted for their enhanced growth capacity both *in vitro* and *in vivo*. Further experiments have shown that exposure to *N*-nitrosamines and MNNG leads to very similar results, with higher transcription of c-*myc* and EGF receptor genes (Kim *et al.*, 1993a).

Normal and HPV-18-immortalized human foreskin keratinocytes were treated with *N*-methyl-*N*-nitrosourea either alone, or followed by 12-*O*-tetradecanoylphorbol 13-acetate, and injected into nude mice (Garrett *et al.*, 1993). Even after prolonged incubation in the animals, tumours were never generated by the chemically treated normal cells or the HPV-18-immortalized cells treated only with *N*-methyl-*N*-nitrosourea. On the other hand, poorly differentiated squamous-cell carcinomas were observed when the HPV-18 immortalized cells were exposed to both chemicals. Cell lines derived from the tumours exhibited the same copy numbers of HPV-18 DNA as found in the parental lines before chemical exposure, and no indication of alteration in the *ras* gene was detected. However, karyotypic analysis revealed several chromosomal alterations, including a deletion in 18q affecting the DCC (deleted in colon cancer) tumour suppressor gene locus, frequently deleted in colon carcinomas (see section 3.1.3). Low amounts of DCC mRNA were also found in HPV-immortalized and chemically transformed human oral keratinocytes (Kim et al., 1993b).

*(d) Radiation*

Ultraviolet (UV) radiation (see IARC, 1992) and X-radiation are known to induce mutations in cellular DNA, and exposure to UV radiation can alter immune function (Kripke & Morison, 1985). Mutations could also increase the expression of viral oncoproteins, through direct interference with viral or cellular factors that down-regulate viral transcription or replication. In general, non-melanoma skin cancers arise in body areas exposed to sunlight. This accounts for skin cancers in immunocompetent as well as immunosuppressed patients and epidermodysplasia, a very rare hereditary condition predisposing to HPV infection, and to the subsequent development of cancers within the papillomas. Since these skin cancers, particularly in immunosuppressed patients, have recently been shown to contain in part novel HPV types (Shamanin *et al.*, 1994a; Berkhout *et al.*, 1995), the localization of these cancers could suggest an interaction between sunlight and papillomavirus infections.

Experiments performed in the hairless mouse *Mus musculus* HRA/Skh suggest the participation of UV radiation in the induction of papillomas and carcinomas in association with a papillomavirus closely related to *Mastomys natalensis* papillomavirus (MnPV) (Tilbrook *et al.*, 1989).

A 16-fold increase in the risk of malignant transformation has been reported after X-ray irradiation therapy for multiple laryngeal papillomas, with a latency period of 5–40 years (summarized in Lindeberg & Elbrond, 1991). Verrucous carcinomas of the larynx present as exophytic warty tumours, some of which have been shown to contain not only HPV-16 DNA, but also low-risk HPV DNAs that are found more frequently in laryngeal papillomatosis (Byrne *et al.*, 1987; Kashima *et al.*, 1988; Brandsma & Abrahamson, 1989). A recent report shows relatively high frequencies of anaplastic transformation after irradiation of primary laryngeal carcinomas, and recommends a surgical approach to the treatment of these tumours (Hagen *et al.*, 1993).

The effect of radiation of HPV-containing human epithelial cells provides a useful model to study genetic alterations contributing to transformation. The exposure of HPV-16-immortalized human foreskin cells to X-irradiation resulted in malignant conversion after approximately 100 additional tissue culture passages (Dürst *et al.*, 1995). Human bronchial epithelial cells, immortalized with HPV-18, were exposed to ionizing radiation. The derived cell lines showed several chromosomal alterations, but they were not tumorigenic in nude mice, despite their ability to grow in soft agar (Willey *et al.*, 1993). The effect of different doses of radon-simulated α-particles was tested on HPV-18-immortalized bronchial cells (Hei *et al.*, 1994). Cells were irradiated with a single radiation dose and maintained in culture for a period of up to three months, after which they became tumorigenic. No mutation in the K-, H- or N-*ras* genes was found in four of these tumours. Since HPV DNA is occasionally found in bronchogenic carcinomas (Stremlau *et al.*, 1985), it may be important to assess the risk of environmental or occupational radon exposure on the progression of HPV lesions of the respiratory epithelium.

### *3.1.6 HPV in mice*

#### *(a) HPV recombinant retrovirus*

Sasagawa *et al.* (1992b) infected the mouse vagina with a retrovirus carrying the *E6* and *E7* oncogenes of HPV-16. Infection resulted in the onset of both low- and high-grade dysplasia: 11/39 mice developed low-grade dysplasia and 22/39 developed high-grade dysplasia. Eight of 30 mice infected with the carrier retrovirus alone developed only low-grade dysplasia. Progression to cancer occurred in 6/15 mice treated with the carcinogen MNNG and in 2/13 mice treated with TPA. Therefore, HPV-16, like animal papilloma viruses (see section 4.2.5) (Gaukroger *et al.*, 1993), can synergize with both a carcinogen and a tumour promoter.

#### *(b) HPV transgenic mice*

Studies in transgenic mice can be loosely divided into three groups: (i) those in which the HPV genome, subgenomic regions of the HPVs or other genes are expressed from the homologous HPV promoter elements; (ii) those in which the HPV genome or subgenomic regions are expressed from promoters to target expression toward organs other than the skin or to achieve diffuse expression in the transgene; (iii) those in which expression of the HPV genome or subgenomic regions is targeted toward the skin.

*(i) Transgenic animals with the HPV control region*

Choo *et al.* (1992) generated transgenic animals with the SV40 T antigen fused to the HPV-18 long control region. Low-level expression of T antigen was detected in several tissues, including the stomach and large intestine but not in the skin or in the anogenital area. In a more recent study, Comerford *et al.* (1995) expressed the *E6* and *E7* genes from the homologous HPV-18 control region and found that these transgenic mice developed genital tract disease after a prolonged latency period: 41% of the female transgenic mice developed mesenchymal cervical lesions and tumours. The transgenic males exhibited enlarged seminal vesicles and preputial glands at an age of 10 weeks. This study, in contrast to Choo *et al.* (1992), therefore suggests that the HPV-18 long control region does indeed contain elements that target the expression of a transgene to the urogenital tract. The two studies were performed in a slightly different genetic background which might, at least in part, account for the different findings.

*(ii) Transgenic animals with non-skin-specific expression of the HPV oncogenes*

Kondoh *et al.* (1991) produced transgenic mice with the HPV-16 *E6* and *E7* oncogenes under the control of the mouse mammary tumour virus (MMTV) long terminal repeat. At eight to 10 months after birth, the male mice developed testicular germ-cell tumours of the seminoma type.

Arbeit *et al.* (1993) used the HPV-16 *E6* and *E7* genes expressed from a human β-actin promoter to generate transgenic mice. A large number of mice developed neuroepithelial tumours starting at 10 weeks after birth. Expression of the HPV oncoprotein E7 was detected in the tumours but not in normal tissues of the mice. The tumour suppressor genes *pRB* and *p53*, were expressed in the tumours in their wild-type forms.

Particularly perspicacious with respect to the delineation of HPV oncogene function *in vivo* have been studies in which the expression of the HPV oncogenes was targeted to the mouse ocular lens by using the murine crystallin promoter. HPV *E6* and *E7* induced hyperproliferation and inhibition of differentiation, and adult mice displayed microphthalmia and cataracts with a very high penetrance. One particular mouse line, with a very-high level of transgene expression, developed lenticular eye tumours (Griep *et al.*, 1993). Additional studies with mice transgenic for *E6* or *E7* alone showed that both oncogenes had a distinct phenotype: *E7* transgenic mice developed cataracts and microphthalmia, and the retinoblastoma protein binding site on E7 was found to be important for this phenotype. The HPV *E6* transgenic mice had cataracts only. The normal apoptosis and denucleation programme in differentiating lens fibre cells was disrupted in *E6* transgenic animals but lens tumours developed exclusively in *E6/E7* double transgenic mice. These studies clearly demonstrated that the *E6* and *E7* genes play important roles in cellular transformation *in vivo* (Pan & Griep, 1994). Similar results in mice transgenic for *E7* expression in a *p53* nullizygous background underline the relevance of the *E6–p53* interaction in cancer development (Howes *et al.*, 1994).

One of the *E6/E7* transgenic mice lines (Griep *et al.*, 1993) also developed epidermal cancers at a very-high incidence. Development of these lesions correlated with the expression of *E6* and *E7* and often occurred at sites of wounding (Lambert *et al.*, 1993).

*(iii) Transgenic animals with skin-specific expression of the HPV oncogenes*

Skin targeted expression of the HPV oncogenes is achieved by using keratin promoters.

*E6* and *E7* expressed from the human keratin K1 promoter leads to the formation of wart-like lesions in older mice. Specific mutational activation of the *ras* oncogene was observed in the papillomas, illustrating that additional cellular events may have to occur in addition to an HPV infection for an overt lesion to occur (Greenhalgh *et al.*, 1994).

The human keratin 14 promoter was used to target HPV oncogene expression to the basal layer of the skin. Progressive epithelial neoplasia was observed in the transgenic animals and lesions appeared at multiple sites, including pinnal and truncal skin, face, snout and eyelids and anus. The phenotype progressed through discernible stages: mild hyperplasia was followed by hyperplasia and progressed to dysplasia and papillomatosis. The step-wise development of disease implicated additional cellular events to be necessary for carcinogenic progression (Arbeit *et al.*, 1994).

Expression of the early region genes of HPV-16 from the bovine keratin K10 promoter resulted in generalized epidermal hyperplasia with a marked increase of proliferating cells in the basal and superbasal layers of the skin. Expression of c-*myc* and TGF $\alpha$ was also enhanced, and negative growth regulatory functions of TGF $\beta$ were at least in part abrogated in the skin of transgenic mice (Auewarakul *et al.*, 1994).

Transgenic mice with the HPV-16 early region expressed from the bovine keratin K6 promoter developed stomach cancer consisting of multiple malignant carcinoids originating from the neuroendocrine enterochromaffin-like cells at the squamocolumnar epithelial junction. These tumours again developed after prolonged asymptomatic expression of the HPV genome indicating the necessity for additional mutations to occur (Searle *et al.*, 1994).

## 3.2 Immune mechanisms and HPV-associated neoplasia

HPVs are exclusively intraepithelial pathogens with a replication cycle that is time dependent and differentiation dependent. Productive infections are chronic and the lesions may persist for many months. The viruses are not cytolytic and no inflammation accompanies infection and replication, a phenomenon that may retard the initiation of or even prevent an effective immune response. This said, the central questions are therefore whether natural infection with HPV induces an immune response to any viral protein and if so what is the nature of this response, when and how does it occur and how does it influence HPV-associated oncogenesis. The serological data are given in section 1.2.

### *3.2.1 Immunosuppression*

Evidence from immunosuppressed individuals suggests strongly that the immune system is important in the pathogenesis of HPV-induced disease and malignant progression (Lutzner, 1985). Generalized warts have been reported in individuals with inherited immune deficiencies, specifically those in whom the T-cell arm of the response is in deficit (Lawlor *et al.*, 1974). Cutaneous and genital warts are one of the most frequent viral complications in patients immunosuppressed as a consequence of renal transplantation and these lesions are refractory to most therapeutic strategies (Benton *et al.*, 1992). Renal allograft recipients are at significantly increased risk for the development of cutaneous neoplasms but there are conflicting reports of the role of HPV in this (see section 2.5.2).

Individuals immunosuppressed as a consequence of HIV infection show similar trends with respect to anogenital neoplasia (Braun, 1994). The role of HIV infection in the pathogenesis of HPV-associated cervical neoplasia is not clear despite extensive investigation (see section 2.5.3). Unfortunately, the data from many studies are incomplete because either the HPV status of the HIV-infected individuals was not ascertained, the sample size was too small, the relevant control groups were not included, the evaluation of immune function was not attempted or clinical disease grading was not reported.

Overall, however, the evidence from transplant recipients, inherited immunodeficiencies and HIV-infected individuals suggests that it is the absolute deficit in $CD4^+$ cells that is important in HPV infection and associated neoplastic progression. This implies a central role for $CD4^+$ cell-mediated mechanisms in the control of HPV infection. The role of the humoral response in HPV infection remains open to debate. Disorders of humoral immunity do not result in an increased susceptibility to HPV-induced lesions (Lutzner, 1985), which suggests that antibody has little to do with the maintenance of HPV infections. There is persuasive evidence from animal studies in rabbits and cattle that antibodies directed against the major capsid protein L1 are protective (Campo, 1994).

### *3.2.2 Histological studies*

The involution and regression of benign cutaneous and genital warts is accompanied by a noticeable histological reaction characteristic of a delayed type hypersensitivity response with a pronounced influx of mononuclear cells dominated by $CD4^+$ cells and macrophages (Stanley *et al.*, 1994). Immunohistological studies have shown that non-regressing anogenital warts are characterized by a relative lack of immune activity: mononuclear cells are present predominantly in the stroma and the few intraepithelial lymphocytes are mainly $CD8^+$ cells. Spontaneously regressing lesions are characterized by a mononuclear cell infiltrate dominated by $CD4^+$, $CD45RO^+$ T cells and macrophages (Stanley *et al.*, 1995). The wart keratinocytes express HLA-DR and ICAM-1 and endothelial cells in the stromal capillaries immediately underneath the infected epithelium express the adhesion molecules E selectin and VCAM and the cytokine RANTES. Interestingly, no statistical difference in the numbers of Langerhans' cells in either active warts or regressing warts was observed in this study (Stanley *et al.*, 1995), although the morphology of Langerhans' cells in active warts was characterized by a loss of dendritic arborizations, a phenomenon reported also in HPV-associated cervical lesions (Barton *et al.*, 1988; Hughes *et al.*, 1988; Morelli *et al.*, 1994). A similar immune infiltrate and expression of MHC class II antigen in keratinoytes were also observed in regressing CRPV warts (Okabayashi *et al.*, 1991).

The situation in cervical lesions differs from that of genital warts and is related to the grade of disease and neoplastic status. Low-grade cervical lesions (CIN I) are to a large extent immunologically quiescent. There is general agreement that there is a reduction in the number of Langerhans' cells (Morris *et al.*, 1983; Tay *et al.*, 1987a; Hawthorn *et al.*, 1988) and changes in Langerhans' cell morphology (Hughes *et al.*, 1988) have been described. High-grade intraepithelial cervical lesions are also characterized by a reduction in Langerhans' cells (Tay *et al.*, 1987a; Hawthorn *et al.*, 1988; Viac *et al.*, 1990). Several groups have investigated T-cell numbers in lesions with varying results. Tay *et al.* (1987b) described a significant reduction in

intraepithelial T-cell numbers in all grades of CIN with a preferential decline in the $CD4^+$ subset. Morris *et al.* (1983) showed a reduction in intraepithelial T cells in low-grade lesions but an increase in the $CD8^+$ subset in CIN III. Similarly, Viac *et al.* (1990) reported increased numbers of both stromal and epithelial lymphocytes in CIN II–III with a dominance of $CD8^+$ cells in the epithelium but equivalent numbers of $CD4^+$ and $CD8^+$ cells in the stroma.

Surveillance and defence against viral infection and tumours are mediated via both MHC-restricted and non-restricted effector mechanisms. Nearly all of the latter are mediated via large granular lymphocytes and include natural killer cells. Large granular lymphocytes with the national killer (NK) phenotype $CD56^+$,$CD16^+$,$CD3^-$,$CD2^{variable}$,$CD57^{variable}$ are rarely found within the normal cervical squamous epithelium or CIN (Syrjanen *et al.*, 1986; Viac *et al.*, 1990; McKenzie *et al.*, 1991) although they are present in the stroma and the endocervix. A separate and small subset of large granular lymphocytes, $CD56^+$,$CD3^+$,$CD2^+$,$CD16^-$, is found within the ectocervical epithelium, and this subset dominates the intraepithelial population in high-grade CIN (McKenzie *et al.*, 1991).

Increased numbers of T cells are seen locally in squamous-cell carcinoma of the cervix (Ferguson *et al.*, 1985; Hilders *et al.*, 1993; Ghosh *et al.*, 1994) with a dominance of the $CD8^+$ subset. These $CD8^+$ tumour-infiltrating cells can cause effective and specific in-vitro killing of autologous tumour targets (Okada *et al.*, 1989). However, bulk cultures of tumour-infiltrating cells generated from cervical squamous-cell carcinomas showed non-MHC-restricted cytotoxicity in the majority of cases (Ghosh *et al.*, 1994). T-cell clones isolated from two cases had the phenotype $CD3^+$,$CD4^+$,$CD56^+/CD56^-$ and showed low cytotoxicity to autologous tumour cells (Ghosh & Moore, 1992).

### *3.2.3 Cell-mediated immunity*

#### *(a) Helper T-cell responses*

Although the histological evidence indicates that HPV-associated lesions elicit an immune response, the target antigens in this response are unknown. However, there is evidence from both experimental animal models and humans that viral proteins can be immune targets. Murine keratinocytes expressing HPV-16 *E6* or *E7* can be grafted onto the flanks of syngeneic immunocompetent mice to reform a differentiated epithelium. Subsequent challenge of the recipients by intradermal inoculation in the ear with a recombinant vaccinia virus expressing HPV-16 *E6* or *E7* results in a delayed-type hypersensitivity response (McLean *et al.*, 1993; Chambers *et al.*, 1994a). This response is $CD4^+$ cell-dependent (McLean *et al.*, 1993). Intradermal challenge with HPV-16 E7 protein also elicits a delayed-type hypersensitivity response provided that a nonspecific inflammatory stimulus, such as the phorbol ester TPA, is applied to the ear in concert with the protein challenge (Chambers *et al.*, 1994b), which illustrates the crucial role of inflammation, at least in this model system. The ability to prime the immune system and elicit a delayed-type hypersensitivity response is critical and is dependent upon antigen dose in this model. Thus, there is a threshold graft inoculum of HPV-16 expressing keratinocytes, below which, although an epithelium reforms, a delayed-type hypersensitivity response cannot be elicited despite repeated antigen challenge (Chambers *et al.*, 1994b). A delayed-type hypersensitivity response has also been detected in regressor rabbits against the structural proteins of

CRPV (Hopfl *et al.*, 1993) and in CIN patients against the L1 protein of HPV-16 (Hopfl *et al.*, 1991).

Using synthetic peptides and fusion proteins in lymphoproliferation assays, murine helper T-cell epitopes in HPV-16 E7 have been determined in several studies (Davies *et al.*, 1990; Comerford *et al.*, 1991; Tindle *et al.*, 1991; Shepherd *et al.*, 1992). A public $T_h$ epitope (DRAHYNI) which provides cognate help for B cells in all strains tested has been located at aminoacids 48–54 in HPV-16 E7 (Tindle *et al.*, 1991). However, it does not appear to hold this property in humans (Tindle & Frazer, 1994). As this latter observation shows, peptides recognized by the murine T-cell response repertoire may not have the same identity for humans.

The analysis of T-cell responses for HPV proteins has been hampered by the heterogeneity of the circulating T-cell population. In one of the first studies to be reported, Strang *et al.* (1990) showed proliferative responses to peptides from the E6 and L1 proteins of HPV-16 when peripheral blood mononuclear cells from healthy donors were tested. Peptide-specific clones and T-cell lines were then used to define the HLA restriction of these responses. Using a similar approach, three $T_h$ epitopes in HPV-16 E7 were identified that were recognized in association with at least two different HLA haplotypes (Altmann *et al.*, 1992). In a study using overlapping peptides spanning the entire HPV-16 E7 protein, lymphoproliferative responses to a carboxyl-terminal peptide 72–97 were significantly related to ongoing infection with HPV-16 and related types (Kadish *et al.*, 1994). Specific T-cell responses to HPV-16 L1 have been detected in patients with cervical dysplasias (Shepherd *et al.*, 1994). In this context, it is of interest that T-cell responses to structural proteins of CRPV have been shown to increase in the papilloma–carcinoma conversion with a dramatic increase in the response to L2 (Selvakumar *et al.*, 1994).

*(b) Cytotoxic T-cell responses*

Cell-mediated cytotoxicity is a phenomenon mediated via a range of cells which include $CD4^+$ and $CD8^+$ cells, LAK cells and NK cells. Classically, cytotoxic T cells are class I restricted $CD8^+$ cells and the role of these cells in HPV infection is under intense investigation. Since transformed cells from HPV-associated cancers consistently express the E6 and E7 viral proteins, these would represent targets for cytotoxic T cells *a priori*. Various investigators have attempted to determine the immunogenicity of these proteins in rodents in experiments in which cells transfected with HPV genes are used as tumour challenge. Using this approach it has been shown that HPV-16 E6 and E7 can act as tumour rejection antigens (Chen *et al.*, 1991; Meneguzzi *et al.*, 1991; Chen *et al.*, 1992a). In an extension of these studies, it was shown that HPV-16 E7-transfected melanoma cells grew progressively in the immunocompetent host, but that transfection of these cells with B7 (the counter receptor for CD28) induced rejection of both the B7 transfectant and the parental E7 expressing line (Chen *et al.*, 1992b); $CD8^+$ antitumour cytotoxic T cells could be isolated from protected mice (Chen *et al.*, 1992b). Evidence for the induction of HPV-16 E7-specific cytotoxic T cells has come from the studies of Feltkamp *et al.* (1993), in which vaccination with a synthetic peptide of the HPV-16 E7 sequence, amino acids 49–57, protected against challenge with HPV-16-transformed tumour cells and induced cytotoxic T cells which lysed tumour cells *in vitro*.

However, in other studies the E6 and E7 proteins have been weakly immunogenic (Gao *et al.*, 1994; Sadovnikova *et al.*, 1994). Thus, mice immunized with HPV-16 E6- or E7-

transfected cells did not generate detectable cytotoxic T-cell responses, which were only seen in mice immunized with E6 or E7 recombinant vaccinia virus. These studies also illustrate the limitations of predictive peptide motifs. Thus, a motif-positive peptide of HPV-16 E6 that showed strong MHC class I binding in the RMA-S assay was not recognized by anti-E6 cytotoxic T cells generated by an E6 recombinant vaccinia virus. Instead, these T cells recognized a motif-negative peptide that showed weak class I binding (Gao *et al.*, 1994). Similar observations were made for HPV-16 E7 (Sadovnikova *et al.*, 1994).

Nevertheless, motif predictions have been useful in humans. Tarpey *et al.* (1994) used the HLA A2.1 motif to locate putative cytotoxic T-cell epitopes in HPV-11 E7. Of the three nonapeptides tested, one primed cytotoxic T cells to recognize and lyse cells infected by HPV-11 E7 recombinant vaccinia virus. Potential HLA-A T-cell epitopes in HPV-16 E6 and E7 have been identified for five HLA-A alleles using a set of overlapping nonapeptides and T2 binding assays (Kast *et al.*, 1994). However, the authenticity of these peptides as cytotoxic T-cell targets remains to be proven.

An alternative strategy for defining cytotoxic T-cell targets in humans is to use mice transgenic for a human HLA allele, immunized with recombinant HPV proteins to generate cytotoxic T-cell precursors *in vivo*. This approach has been taken by Beverley *et al.* (1994) using HLA-A2 transgenics and HPV-16 E6. Their preliminary data suggest that an E6 epitope can be presented by HLA-A2. However, as these workers point out, there is a paradox between the murine and human systems. The murine experiments show that E6 and E7 contain cytotoxic T-cell epitopes and conventional immunization procedures generate T cells against them, but, to date, there are no reports of human cytotoxic T cells that recognize HPV antigens in association with HLA-A2 or any other HLA allele. It is worth re-emphasizing that HPV is an exclusively intraepithelial pathogen; viral gene expression is confined to keratinocytes, which are incapable of delivering accessory signals to T cells — a situation that could induce tolerance rather than prime an active immune response (Bal *et al.*, 1990; Chambers *et al.*, 1994b).

### *3.2.4 Major histocompatibility complex (MHC) expression*

#### *(a) MHC class I*

Loss or downregulation of class I MHC expression is a well recognized mechanism whereby viruses escape immune detection, and the expression of MHC molecules in relation to HPV-associated neoplasms has been closely investigated (Stern & Duggan Keen, 1994). Immunohistological studies on cryostat sections using the monoclonal antibody W6/32 provided little evidence for class I modulation in benign HPV lesions or CIN (Viac *et al.*, 1990; Glew *et al.*, 1993a). Similar results were obtained by Torres *et al.* (1993) using the monoclonal antibody HC10 in the analysis of paraffin sections from a large series of premalignant cervical lesions. However, in an extensive study analysing HLA expression in paraffin-embedded material using a polyclonal antibody RaHC and the monoclonal antibody HC10, disturbed HLA class I heavy chain expression was found in all grades of CIN and cancer of the cervix (Cromme *et al.*, 1993a). The monoclonal antibody W6/32 recognizes monomorphic determinants of the heterodimeric HLA class I molecule; the monoclonal antibody HC10 recognizes HLA, B and C locus products preferentially; RaHC is specific for HLA A, B and C heavy chains. The

differences in staining patterns observed in these studies could reflect allele-specific downregulation, which would be detectable by loss of staining with HC10 and RaHC, or alternatively the presence of incomplete or modified heavy chains, which would stain positively with W6/32 but not with HC10/RaHC. They may also reflect the inadequacies of assessing HLA expression by immunohistochemistry on small biopsies where fixation and processing artifacts could distort the analysis.

There is no dispute that class I expression is downregulated in cervical squamous-cell carcinoma (Connor & Stern, 1990; Cromme *et al.*, 1993a; Glew *et al.*, 1993a; Torres *et al.*, 1993; Hilders *et al.*, 1994). These changes occur in both HPV-positive and HPV-negative lesions and have been shown, in a proportion of tumours, to be controlled post-transcriptionally (Cromme *et al.*, 1993b). In HPV-16/18-positive tumours, this post-transcriptional loss of HLA class I expression is related to the loss of peptide transport due to downregulation of expression of TAP-1 protein (Cromme *et al.*, 1994). This is an important observation, but it is unlikely that all class I downregulation in cervical cancer is due to this single mechanism. Connor & Stern (1990) observed that loss of β2 microglobulin was often accompanied by the absence of HLA heavy chains and this suggests that the regulation of several MHC-associated gene products may be altered in invasive cancers. Whatever the mechanism of downregulation, these changes may be of central importance functionally, since the absence of class I or allele-specific downregulation would be expected to interfere with T-cell recognition of target antigens (whether of host or viral origin) and disable cytotoxic T-cell effector mechanisms. In support of this, there is evidence from preliminary studies that patients with early cervical cancers with downregulated class I have a poorer clinical outcome (Connor *et al.*, 1993).

*(b) MHC class II*

Normal ectocervical epithelium does not express class II antigens, although HLA-DR expression has been reported in the transformation zone (Roncalli *et al.*, 1988). The expression of class II by HPV-infected keratinocytes in low-grade cervical lesions is variable with some studies finding no expression (Hughes *et al.*, 1988; Warhol & Gee, 1989) and other patchy focal expression (Fais *et al.*, 1991; Coleman & Stanley, 1994). Class II expression is seen in high-grade CIN, although the extent of expression varies from patchy HLA-DR positivity (Ferguson *et al.*, 1985) to diffuse extensive staining (Glew *et al.*, 1992; Cromme *et al.*, 1993a; Coleman & Stanley, 1994). Expression of class II molecules occurs in at least 80% of cervical cancers (Glew *et al.*, 1992; Cromme *et al.*, 1993a; Glew *et al.*, 1993a). Glew *et al.* (1992) postulated that such expression in high-grade CIN and cervical cancer is a reflection of the transformation of squamous metaplastic cells, which they have shown to be class II-positive. An alternative explanation is that this expression is in part induced rather than constitutive. This explanation is supported by the observation of increased numbers of T cells in the subepithelial stroma of HLA-DR-positive as compared to HLA-DR-negative CIN (Coleman & Stanley, 1994) and an increase in tumour-infiltrating cells in DR-positive regions of cervical squamous-cell carcinomas (Hilders *et al.*, 1994). In addition, no constitutive expression of HLA-DR could be shown on fully transformed HPV-16-expressing keratinocytes *in vitro*, although it could be induced by γ-interferon (Coleman & Stanley, 1994). A large proportion of high-grade CIN express ICAM-1, as well as HLA-DR, although co-expression of these molecules is not inevitable and an ICAM-1

positive lesion can be HLA-DR negative (Stanley *et al.*, 1994b). Evidence from in-vitro studies with HPV-16-expressing keratinocyte cell lines indicates that this expression in high-grade lesions is constitutive rather than induced (Coleman *et al.*, 1993b), suggesting that ICAM-1 expression *in vivo* in CIN III is unlikely to be a virally induced phenomenon but rather a consequence of neoplastic transformation *per se*.

### *3.2.5 HLA polymorphisms: association with cervical cancer risk*

The recognition of foreign as opposed to self antigen depends upon the recognition by the T-cell receptor of subtle changes in the MHC/peptide complex as presented on the cell surface. Fundamentally many immune responses are controlled by genes of the MHC complex and, crucially, there is evidence that host resistance or susceptibility to pathogens is dependent in part on the dynamic interaction between the host MHC and permissivity for the presentation of pathogen peptides (Parham, 1994). For any one protein, different alleles of the MHC will present different peptides to the immune system. Thus, the loss or upregulation of different alleles could influence the natural history of HPV infection and the risk of neoplastic progression. If this were the case, then it could be reflected in different HLA frequencies in patients with cervical carcinoma when compared to the appropriate normal population.

Associations between HLA haplotype and cervical carcinoma have been reported in recent studies. Using serological typing, Wank and Thomssen (1991) showed association with HLA-DQw3 and increased risk for cervical cancer but decreased risk in association with DR6 in a German patient group. A similar association but with a smaller relative risk was found in a Norwegian study (Helland *et al.*, 1992). Serological typing does not discriminate alleles as precisely as molecular typing, and typing by PCR and single-strand oligonucleotide probes in the German group revealed that DQB1*0301 and *0303 were the risk alleles for cervical cancer (Wank *et al.*, 1993). However, in a study in the United Kingdom of 57 patients with cervical squamous-cell carcinoma and 857 controls using molecular and serological typing, no such association was found (Glew *et al.*, 1993b).

Gregoire *et al.* (1994) showed an association between DQB1*0303 and cervical cancer risk in African American women but could not show an association between HPV type in the tumour and HLA. This is in contrast to the recent studies of Odunsi *et al.* (1995) who found a strong association between HLA DQB1*0301 and CIN and HPV. Apple *et al.* (1994) investigated the role of the HLA class II loci and HPV type in cervical cancer. This case–control study included biopsies from 98 Hispanic patients with cervical cancer, and cervical scrapes from 220 Hispanic control women with normal Pap smears. All patients were from the same geographic area in the South West of the USA. In this group, certain HLA class II haplotypes, including DB1*150 – DQB1*0602, were significantly associated with HPV-16-containing cancer, whereas DR13 haplotypes were negatively associated. If the type specificity of these associations are confirmed, this may be of fundamental importance since it implies that specific HLA class II haplotypes influence the response to HPV-encoded epitopes and the risk of neoplastic progression. It is of interest that class II haplotypes have been implicated when MHC class I is downregulated in the majority of cervical squamous-cell carcinomas. The high frequency of HLA-B locus downregulation seen in both premalignant and malignant lesions (Cromme *et al.*, 1993a) raises the suspicion that these locus products may be important for the presentation of

target host or HPV peptides. Evidence in support of this has recently been presented (Ellis *et al.*, 1995). In this study, potential cytotoxic T-cell epitopes in HPV-16 E6 for HLA-B7 were identified by T2 binding assays. Sequence analysis of the E6 region from HPV-16 isolates derived from HLA-B7 cervical cancer patients identified a consistent mutation in the N-terminal corresponding to two of the B7 binding epitopes. The mutation, a single base change from guanosine to adenosine results in a change in the amino acid sequence from arginine to glycine in the binding peptide at position 3. This amino acid substitution does not affect binding, but does alter the residues exposed to the T-cell receptor and would be likely to alter the affinity of the TCR/MHC peptide binding. It seems likely that the mutant is a true HPV-16 viral variant with a wide geographical distribution, implying that the HLA-B7 allele is important in the immunological control of HPV-16 infection in a manner analogous to other HLA alleles involved in antiviral surveillance (Gavioli *et al.*, 1993). A role for HLA haplotype and susceptibility to HPV-associated cancer is strongly supported by data from the rabbit, with the observation that regression or progression of papillomas induced by CRPV is linked respectively to the MHC DR and DQ phenotypes of the animals (Han *et al.*, 1992).