Human Papillomavirus Types
in
Oral Squamous Cell Carcinogenesis

Thesis submitted for the degree of Ph.D.

University of Glasgow

by

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15th October, 1991.

Dr. E.K. Parkinson,
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Dear Ken,

Here is a list of corrections to my PhD thesis.

Page	
24	precancerous condition is **one...**
29	field theory of carcinogenesis
34	reference to SV40 integration
36	line 10 : **retinoblastoma**
52	line 3 : **casein**
68 (facing)	table of patients
77	reference for **glassmilk**
98	line 5 from bottom : **from**
99	last line 1st para : **BamHI**
103	mol. wt. markers
107	mol. wt. markers
118	line 2 : delete **"5"**
187	line 11 : **sequence**
187	line 4 from bottom : **sequence**
235(facing)	additional references **(incl. Smith & Campo)**

Best wishes,

Andrew Yeudall.

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Finally, I should like to thank Dr. D. Gillespie and Ms. S. Lowe for synthesis of oligonucleotides, and Dr. J.A. Wyke for access to facilities within such a stimulating environment.

List of Abbreviations Used.

ALV	avian leukosis virus
ATP	adenosine 3'-triphosphate
BPV	bovine papillomavirus
CRPV	cottontail rabbit papillomavirus
dATP	deoxyadenosine 3'-triphosphate
dCTP	deoxycytidine 3'-triphosphate
dGTP	deoxyguanosine 3'-triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dsDNA	double stranded DNA
DTT	dithiothreitol
dTTP	deoxythymidine 3'-triphosphate
EDTA	sodium ethylene diamine tetraacetate
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
HPV	human papillomavirus
MMTV	mouse mammary tumour virus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Rb	retinoblastoma
RNA	ribonucleic acid

RNase A	ribonuclease A
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylene diamine

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Summary.

The DNAs of human papillomavirus (HPV) types 4, 16 and 18 have been detected in biopsies of normal and malignant human oral mucosa by Southern blot hybridisation and polymerase chain reaction (PCR). By the former technique HPV-4, HPV-16 and HPV-18 DNAs were detected in three separate carcinomas, but only found in adjacent dysplastic and normal tissue by PCR. The PCR technique also allowed detection of HPV-16 and HPV-18 DNA in additional carcinomas and normal samples. The oral HPV-4 DNA has been molecularly cloned and extensive restriction analysis and nucleotide sequencing showed identity with the prototype HPV-4 DNA. The HPV-18 DNA detected by Southern blot hybridisation showed an altered restriction pattern in the E1 region of the viral genome; however direct nucleotide sequencing of PCR products from the E6 ORF showed no sequence alterations in either normal or malignant samples.

HPV-16 DNA detected in one carcinoma by Southern blot hybridisation revealed altered PstI and HpaII restriction patterns as compared with the prototype viral genome. The expected 2.6kb HpaII and 1.55kb PstI bands, which overlap, were absent, and an additional band of reduced molecular weight was visible in the HpaII digest, suggesting that the oral HPV-16 genome had undergone a deletion or rearrangement.

In a further two carcinoma samples positive for HPV-16 DNA by PCR, amplification of a late region fragment of the viral genome

produced fragments of reduced molecular weight. When these PCR fragments were used as probes, hybridisation was observed to the 1.78kb PstI and 1.81kb HpaII-BamHI bands of HPV-16 DNA, and also (as a smear) to human genomic DNA from both tumour and normal samples. This suggests that the viral DNA in these samples had undergone recombination events with repetitive cellular sequences, perhaps as a prelude to viral integration or as a means of activating cellular genes.

A keratinocyte culture (T45) derived from an oral squamous cell carcinoma was found to be non-tumorigenic *in vivo*. PCR analysis revealed that a proportion of cells in the culture contained HPV-16 early sequences. The establishment of HPV-positive and HPV-negative clones from this culture will provide an excellent system for studying the role of viral and cellular factors in oral squamous cell carcinogenesis.

1. Oral Squamous Cell Carcinoma.

1.1 Epidemiology of Oral Squamous Cell Carcinoma.

1.1.1. Incidence.

Squamous cell carcinoma is the most common form of intraoral malignant disease, accounting for 90-95% of cancers in this region and around 5% of all malignant tumours in the western world (Lucas, 1984). Oral cancer is, however, relatively uncommon compared to the incidence of cancer at other sites such as breast, bronchus and colon (Cawson, 1984). The remainder of oral malignancy comprises tumours of glandular epithelium, connective tissue lesions such as fibrosarcoma and osteosarcoma, and metastatic deposits from primary cancers at distant sites.

1.1.2. Age and Sex Incidence.

Oral cancer is mainly a disease of the elderly, with the majority of sufferers older than forty years of age (Cawson, 1984). The sex incidence of intraoral squamous cell carcinoma is approximately equal at present, with males being marginally more likely to develop the disease. However the male to female ratio has decreased from 10:1 to the current level, principally due to a decrease of disease in males,

while the incidence in females has remained static (Binnie *et al.*, 1972).

1.1.3. Site Incidence.

Squamous cell carcinoma may develop in any region of the oral cavity, but frequency of lesions is different for different sites. According to Binnie *et al.* (1972), lip is the most common site with 28.6% of oral cancers arising there, followed by tongue (24.7%), retromolar (13.4%) and floor of mouth (9.1%). Lesions of alveolar mucosa, buccal mucosa, hard palate and soft palate account for the remainder.

The high level of lip cancer (the vast proportion of which affects the lower lip) is thought to be a result of exposure to actinic radiation, as it is more common in rural populations than in city dwellers, and is frequently seen in outdoor workers in sunny climates (Miller, 1974). The lateral and ventral surfaces of the tongue together with the floor of mouth and lower alveolus form a U-shaped "sump" which extends backwards on both sides of the oral cavity, forming a region where any soluble carcinogen might pool. This may afford some explanation as to why approximately 70% of intraoral squamous cell carcinomas arise at these sites, although the area accounts for only 20% of the total mucosal surface of the oral cavity (Langdon, 1985).

1.2. Pathology of Oral Cancer.

The pathological features of intraoral squamous cell carcinoma are described well by Lucas (1984). Macroscopically, well-established lesions may appear as papillary outgrowths or, more commonly, as chronic ulcers with raised edges and a necrotic floor which bleeds easily. Earlier lesions may present as hyperkeratotic areas, or as erythematous patches.

At the histological level changes can be seen in the structure of the epithelium from normal to the malignant state. Normal oral epithelium is well-ordered, with a single layer of basal cells below the stratum spinosum, and granular and cornified layers may be above, dependent on the site. Maturation is regular, with mitotic activity being confined to the basal layer and keratinisation only occurring at the surface.

As the epithelium progresses through various stages of dysplasia towards frank carcinoma an increasing number of characteristic features (atypia) can be seen, such as increased and aberrant mitoses, and mitoses in cells other than basal cells. The cells may become pleomorphic and the regularity of maturation may be lost. Later changes may include keratin pearls throughout the epithelium and nuclear hyperchromatism. Invasion of epithelial cells through the basement membrane into the underlying connective tissue is the hallmark of malignancy. A large number of intraoral carcinomas are well-differentiated, but a whole spectrum of histological types through moderate to poorly-differentiated lesions may be observed.

Spread of these tumours is not uncommon. They are locally invasive at first, but may metastasize via local lymphatic vessels to involve regional lymph nodes. Perineural spread may occur, which clinically can cause anaesthesia or paraesthesia of the a can cause anaes involved nerve. Haematogenous spread is uncommon in head and neck cancers.

1.3. Prognosis.

Several factors are probably of importance for the prognosis of intraoral carcinomas. The site of the lesion has a marked effect on the survival of the patient, and Langdon *et al.* (1977) document a five year survival of 57% for lip lesions, compared to 38% for tongue and only 17% where the tumour affects the maxillary alveolus. These authors also observed that early stage lesions had an improved outcome over those which presented later, and that prognosis was better for smaller tumours than for large primaries. Different studies have tried to relate histological appearance of tumours to prognosis, but conflicting reports are present in the literature (Johnson, 1977; Langdon *et al.*, 1977). Some authors (Langdon *et al.*, 1977; Smith, 1973) quote a higher five year survival for females over males, but Binnie *et al.* (1972) disagree. Langdon *et al.* (1977) found that tumours in patients below sixty years of age had a better prognosis than those in the "over sixty" age group.

1.4. Aetiological Factors in Oral Cancer.

The aetiology of oral cancer is poorly understood. A number of different predisposing factors are generally believed to be of importance, although Langdon *et al.* (1977) report a lack of any obvious risk factor in 55.7% of cases in southern England.

1.4.1. Tobacco.

The majority of oral cancer patients are heavy smokers, with 96% of subjects in Wynder and Stellman's (1977) series being either smokers or ex-smokers. These authors note that the method of smoking is important, as cigar and pipe smokers are considerably more likely to develop oral cancer than non-smokers, the relative risk being four to six fold for low usage (one to five cigars or pipes daily). Smith (1979) points out that the incidence of oral cavity cancer has not increased in parallel with other smoking-associated diseases. Perhaps this reflects the relative importance of the method of smoking to development of oral cancer. As mentioned previously, the incidence of oral cancer in males has declined steadily since the 1930s while cigarette consumption has risen considerably in the same period. Lucas (1984) and Cawson (1984) suggest that the decrease in male cancer cases might be explained by a reduction in pipe smoking, whereas the disease in women, who never had this habit, does not show a similar trend. Moore (1965) has shown that, of patients who have been successfully treated for oral, pharyngeal or laryngeal cancer, approximately one third will develop new primary tumours if smoking

does not cease. There is only a small risk of developing a second tumour if the patient stops smoking after removal of the original malignancy.

A recent study carried out in northern Italy (Franceschi *et al.*, 1990) investigated the effects of tobacco and alcohol intake on development of carcinomas of the upper aerodigestive tract, including cancer of the oral cavity. These authors reported that smokers of cigars and pipes were at greater risk of intraoral malignancy than cigarette smokers, and that heavy drinkers were also at significantly greater risk, the figures relating mainly to wine consumption.

Other forms of tobacco usage may be of importance in the development of intraoral squamous cell carcinoma. Hard palate cancer, relatively common in India, has been linked to the practice of reverse smoking, during which the lighted end of the cigarette is held in the mouth (Pindborg *et al.*, 1971). Oral cancer in general is much more prevalent in Asia, accounting for around 40% of all malignancies. This level of disease is thought to be related to long term betel chewing, with cancer developing at the particular site where the betel quid is habitually held (Hirayama, 1966). Pindborg (1980) states that oral cancer is only associated with betel chewing in regions where tobacco is present in the quid. A similar relationship appears to exist in the southeastern USA where "snuff-dipping" by females is common and oral cancer mortality rates are elevated (Blot and Fraumeni, 1977), especially from carcinoma of the lower gingival and alveolar mucosa. Winn *et al.* (1981) found a four fold increase of

such cancers in snuff-dippers compared to non-users in a case control study in North Carolina.

1.4.2. Alcohol.

Several epidemiological studies from the USA provide evidence of a strong correlation between excessive alcohol consumption and development of intraoral squamous cell carcinoma (Wynder *et al.*, 1957a; Rothman and Keller, 1972; Graham *et al.*, 1977). According to Wynder *et al.* (1957a) heavy drinkers (more than six ounces of spirits or equivalent per day) have a ten to fifteen fold greater chance of developing oral cancer than occasional drinkers. The importance of the contribution made by alcohol to the aetiology of oral cancer is complicated by the fact that the majority of heavy drinkers are also heavy smokers (Rothman and Keller, 1972; Hinds *et al.*, 1980), and it is not easy to separate these two risk factors. There are conflicting reports in the literature as to whether alcohol and tobacco act synergistically or independently, which is more important, and what type of either is most likely to lead to the development of malignant oral disease (reviewed by Douglass *et al.*, 1984).

To further complicate matters, no association has been found between alcohol intake and oral cancer in the UK (Binnie *et al.*, 1972). Over the past fifty years consumption of alcohol has increased steadily while the incidence of oral cancer has decreased. Binnie (1976) points out that there may be an association between alcohol and oral cancer in the USA and France where, unlike the UK, unmatured pot-stilled

spirits (containing toxic by-products) are available. However Langdon (1985) remarks on the high number of alcohol abusers attending oral cancer clinics in the UK, in spite of the findings of Binnie and coworkers.

Experimental evidence for an effect of alcohol on oral mucosa is scant. However, in a recent study, Graham and Rennie (1987) describe a consistent biochemical change in the oral epithelium of alcoholic rats compared to control animals. These authors noted a reduction in the level of a high molecular weight glycoprotein and a concomitant increase of two smaller proteins. Histological studies (Mascres *et al.*, 1984) have detected changes in epithelial thickness after chronic alcohol consumption.

1.4.3. Liver Cirrhosis.

A few epidemiological studies have implicated cirrhosis of the liver in the development of oral cancer, notably lesions of the floor of mouth. Vincent *et al.* (1964) suggested that development of oral epithelial malignancy was accelerated in patients with liver damage induced by alcohol. Studies by Keller (1969, 1977) demonstrated an association between floor of mouth lesions and liver cirrhosis, with cancers appearing up to three years earlier in those suffering from liver damage compared to controls. It is unclear how cirrhosis might promote development of oral cancer, especially at a particular site, although one possibility might be a reduced capacity to clear toxic products from the body, leading to prolonged exposure of certain

tissues and possible promotion of malignancy. Alternatively, cirrhosis may be coincidental in oral cancer patients who consume large quantities of alcohol, which probably has some topical action on the oral mucosa in addition to systemic consequences.

1.4.4. Industrial Factors.

Reports from both the USA (Vogler *et al.*, 1962) and the UK (Binnie *et al.*, 1972) cite textile mill workers as being at greater risk of developing oral cancer than the general population. Exposure of workers to dust produced when processing raw wool and cotton results in an increased incidence of oral squamous cell neoplasms (Moss and Lee, 1974), although the nature of the responsible agent is not known.

1.5. Precancerous Conditions.

A precancerous condition is one which might render the oral mucosa more susceptible to the development of cancer. Several precancerous conditions are recognised, the more common of which are described below.

1.5.1. Syphilis.

Syphilis has traditionally been described as a risk factor for development of oral cancer (Levin *et al.*, 1942). The effect of

spirochaetes may be the production of an epithelium which is more susceptible to further modification by toxic substances in the mouth, such as tobacco (McCarthy, 1984). However it is possible that oral cancer arising in syphilitic lesions may have been due to the action of toxic chemicals previously used in the treatment of the condition (Smith, 1973). Nowadays the importance of syphilis as an aetiological factor in oral malignancy is much reduced due to the uncommon occurrence of tertiary disease.

1.5.2. Iron Deficiency Anaemia.

Paterson (1919) and Brown-Kelly (1919) first described the association of iron deficiency anaemia with carcinoma of the post-cricoid region. Ahlbom (1936) and Wynder *et al.* (1957b) confirmed the relationship of this nutritional disorder with more widespread pharyngeal and oral cancers. Brown-Kelly Paterson syndrome (Plummer-Vinson syndrome), which consists of iron deficiency anaemia, koilonychia, glossitis and dysphagia, is a common disease in middle aged Swedish females, who also have a high incidence of cancer of the upper gastrointestinal tract (Wynder *et al.*, 1957b), and who account for the increased incidence of oral cancer in Swedish women (Larsson *et al.*, 1975). Low levels of serum iron tend to result in atrophy of oral epithelium, and this is thought to be more vulnerable to the effects of irritant chemicals in much the same way as atrophic syphilitic epithelium. Prime *et al.* (1983) described an increased incidence of lingual carcinoma in iron deficient rats treated with 4-nitroquinoline-N-oxide.

1.5.3. Oral Submucous Fibrosis.

Submucous fibrosis is a chronic disease characterised by epithelial inflammation, fibroelastic change in the underlying connective tissue and epithelial atrophy, which may make the mucosa more susceptible to the topical effects of carcinogens, for example constituents of tobacco, and alcohol. A high level of epithelial dysplasia (around 13-14%) has been noted in such lesions (Pindborg, 1972). The disease affects Asian populations, mainly Indians and Pakistanis, and in a study of oral cancer patients in India 40% were found to suffer from submucous fibrosis (Pindborg *et al.*, 1967).

1.6. Oral Precancerous Lesions.

The World Health Organisation (1980) has defined the precancerous lesion as "morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart". Two such lesions are recognised in the oral cavity, leukoplakia and erythroplakia.

1.6.1. Leukoplakia.

The term "leukoplakia" is a clinical description of a white patch on the oral mucosa which does not scrape off and which can not be attributed to any other disease. There is no implication of a particular

histological appearance in this description. From the clinical picture, several types of leukoplakia exist, including homogeneous leukoplakia, verrucous leukoplakia and speckled leukoplakia (a white lesion intermingled with red patches).

Many studies have examined the incidence of malignant transformation within or associated with leukoplakias (reviewed by MacDonald, 1975). Most investigators have concluded that progression of leukoplakia to malignancy is infrequent; Cawson (1969) stated that the majority of cases (90-95%) would not develop into cancers within five years. In a Swedish study (Einhorn and Wersall, 1967) the incidence of carcinomatous change in leukoplakia was 2.4% over a ten year period, increasing to 4% over twenty years. Another large study (Banoczy and Sugar, 1972) reported that 5.9% of all leukoplakias in their Hungarian population developed into carcinomas, a similar figure to that quoted above. More interestingly, when these authors subdivided the lesions into three different types of leukoplakia, the likelihood of transformation was found to differ between variants. No cases of homogeneous leukoplakia developed into cancer; however the incidence of transformation of verrucous and speckled lesions was 4.6% and 28% respectively, suggesting that these (especially the latter) are more sinister.

1.6.2. Erythroplakia.

Erythroplakia is similar to leukoplakia in its description, although red instead of white. Again the term is a clinical one, and refers to a well-

demarcated red patch, unattributable to other causes (Mehta *et al.*, 1971). It is much rarer than leukoplakia, present in around 0.02% of an Indian study population (Mehta *et al.*, 1971) and is regarded as the most severe of precancerous lesions, often at a much later stage along the road to invasive cancer (Kramer, 1969).

1.7. Oncogene Activation in Oral Squamous Cell Carcinoma.

Only a few reports exist of the involvement of oncogenes in oral cancers. Spandidos *et al.* (1985) found elevated transcription of c-Ha-*ras*, c-Ki-*ras* and c-*myc* genes in squamous cell carcinoma biopsies compared to levels in normal tissues, but the results are complicated by the use of heterogeneous material (possibly containing normal as well as neoplastic cells) as opposed to clonal cell lines. This, however, would tend to underestimate the true level of transcription of these genes. The same authors (Field *et al.*, 1986) attempted to correlate oncogene expression with the clinical stage of tumours, but the sample number was small and no significant conclusions could be drawn.

More recently, Tadokoro *et al.* (1989) described activation of c-Ha-*ras* in two cell lines derived from oral cancers. In both lines activation occurred by point mutation, at codon 12 in one cell line and at codon 13 in the other population. Amplification of the gene coding for the epidermal growth factor receptor (c-*erb*B1) and c-*myc* was also detected in the cell line harbouring the codon 13-mutated *c*-Ha-*ras* gene. The finding of amplified EGF receptor genes and their

overexpression at the cell surface is not an uncommon finding in oral cancers, and has been noted by several authors (Cowley *et al.*, 1986; Ozanne *et al.*, 1986; Ishitoya *et al.*, 1989). Compared to other cancers however, information regarding the molecular biology of oral cancer in general and involvement of viral or cellular oncogenes in the disease is limited.

1.8. "Field of Growth" Theory.

The field of growth theory (reviewed by Walter and Israel, 1974) suggests that areas of tissue, rather than single cells, may become predisposed to tumour development. Within such a field one or more tumours may subsequently develop, perhaps from single cells which have undergone additional genetic events resulting in malignant progression. For example, multiple tumours of colon may arise from cases of polyposis coli or ulcerative colitis; fields of skin exposed to ultraviolet radiation may give rise to one or several squamous cell or basal cell carcinomas. The idea of field change may also serve to explain the source of recurrence of tumour in areas adjacent to excision sites.

The concept of an area of tissue predisposed to malignancy may reflect the nature of the aetiological agent. Exposure of tissue to chemical carcinogens or ionising radiation likely results in widespread damage to cells, making them more susceptible to further malignant progression. This may be of importance in the development of oral squamous cell carcinoma, where large areas of oral mucosa may be damaged by chemical agents and by viral infections.

2. Viral Oncogenesis.

The idea of cancer-causing viruses is not a new one. Indeed it is almost eighty years since Rous (1911) demonstrated the ability to produce highly malignant tumours in previously normal chickens by injection of a cell-free filtrate from a hen sarcoma, and that the responsible agent (Rous sarcoma virus, RSV) could be propagated by serial passage through chickens. Since then, much work has been carried out on many different tumour viruses, establishing the ways in which they contribute to the transformed phenotype of the host cell, and giving us a fascinating insight into oncogenesis in general.

2.1 RNA Tumour Viruses.

Many RNA tumour viruses (or retroviruses) have been described which infect a wide range of host animals. As the classification suggests, their genomes consist of RNA instead of DNA, in the form of two single-stranded molecules complexed with core proteins. The viral envelope comprises virally-encoded glycoproteins embedded in a lipid membrane derived from the previously infected host cell.

The retroviral particle is able to enter a susceptible host cell primarily due to the presence of specific receptors on the cell surface. On entry into the host the viral genome is transcribed into a double-stranded DNA provirus by one of the products of the viral *pol* gene, an RNA-directed DNA polymerase (reverse transcriptase). The provirus

contains identical long terminal repeats (LTRs) at both ends as a result of duplication of sequences near the 5' and 3' termini of the RNA template, and is therefore slightly longer than the original genome. Transcription of proviral DNA by host polymerase produces viral RNA which serves firstly to encode the structural proteins of the viral core (from the *gag* gene) and the envelope (*env* gene products), but also as genomic RNA of progeny virions. These then bud from the cell membrane without resulting in cell lysis.

Some distinction of oncogenicity of retroviruses may be made on the grounds of the time required for tumour production in host animals. Hence these agents can be broadly categorised as either acute or chronic, with rapid tumour induction in the former case and a longer latent period in the latter.

One mechanism by which certain retroviruses transform cells is by transducing sequences (proto-oncogenes, proto-*onc*) derived from host genetic material and inserting them into the viral genome. The viral counterpart (v-*onc*) of the cellular gene is usually altered in some way, in some cases by mutation, or alteration in length as a result of truncation or fusion to viral DNA. The v-*onc* is under control of viral regulatory elements contained within the LTRs and may be expressed at high levels, leading to cell transformation.

The transactivation of cellular genes by viral proteins is a second possible method of oncogenesis. A form of adult T-cell leukaemia, endemic in some areas of the world, is closely associated with two related retroviruses, human T-lymphotropic virus (HTLV) types I and

II (reviewed by Wong-Staal and Gallo, 1985). Leukaemogenesis caused by HTLV-I and II may be a result of trans-acting transcriptional regulation of the interleukin 2 (IL-2) receptor gene via the viral *tat* gene product (Seiki *et al.*, 1986; Inoue *et al.*, 1986); Greene *et al.*, 1986).

Thirdly, proviral DNA may act in *cis* on adjacent cellular oncogenes (c-*onc*s) to alter the cell's growth properties. Transformation by insertional mutagenesis is usually characterised by long latency, and is dependent on proviral sequences being adjacent to and in the same orientation (in the case of promotor insertion) as the cellular gene. Bursal lymphomas in chickens caused by avian leukosis virus (ALV) involve activation of the c-*myc* gene by promoter insertion (Neel and Hayward, 1981; Westaway *et al.*, 1984), the cellular gene being under control of the viral LTR, and resulting in virus-cell fusion transcripts. A few ALV-induced lymphomas are not the result of promoter insertion as the proviral DNA is integrated in the opposite orientation to c-*myc*. In such situations it is likely that the proviral DNA acts via cis enhancer activity to regulate transcription of the cellular gene (Nusse, 1986).

Oncogene activation by retroviral insertion is not limited to an increase in transcriptional activity of a particular gene. Although the gene for the epidermal growth factor (EGF) receptor, c-*erb*B1, can be activated by transduction, insertional activation resulting in a disrupted gene product has also been shown to be important. Novel chimeric RNAs are produced, which undergo splicing to create *erb*B1 mRNA coding for a protein with a truncated amino-terminal end

(Nilsen *et al.*, 1985). This reflects its activation by retroviral transduction, where a similarly truncated EGF receptor is produced (Ullrich *et al.*, 1984).

2.2 DNA Tumour Viruses.

The DNA tumour viruses comprise a heterogeneous population, from the small members of the papovavirus group with genomes of around 5 kilobase pairs (kb) in size to the large herpesviruses (up to 175kb long). Specific nucleotide sequences are again important for cell transformation, but the oncogenes carried by these virus types do not appear to be derived from host cellular DNA, unlike the RNA tumour virus group.

2.2.1. Simian Virus 40 (SV40) and Polyomavirus.

SV40 and polyomavirus, members of the papovavirus family with double-stranded DNA genomes of 5243 and 5297 base pairs (bp) respectively (Fiers *et al.*, 1978; Deninger *et al.*, 1979; Friedmann *et al.*, 1979), can interact with host cells in two ways. Firstly, a lytic cycle may occur in permissive monkey (SV40) and mouse (polyoma) cells, with production of new virus and death of infected cells. However both virus types have the ability to transform rodent cells *in vitro*, as well as some monkey and human cells (SV40 only).

Transformation of host cells by SV40 and polyomavirus depends upon the presence of a functional viral early region, which is integrated into the host genome. Integration appears to be random, with no preferred site in the cell DNA involved. The recombination event is thought to be illegitimate, with only around two to five base pairs of homology between viral and cellular sequences at the integration site (Sambrook *et al.*, 1968)

In SV40-mediated transformation expression of viral large T antigen (TAg) is necessary. This protein has been confirmed as the SV40 oncogene by analysis of mutants (Martin and Chou, 1975; Osborn and Weber, 1975) and is required for maintenance of the transformed phenotype (Steinberg *et al.*, 1978). SV40 TAg has been shown to bind the cellular proteins p53 (Lane and Crawford, 1979) and $p105^{Rb}$ (de Caprio *et al.*, 1988), both of which have tumour-suppressor functions, and the viral oncogene may act at least partly by relieving normal cellular control mechanisms.

In contrast, polyomavirus large T antigen does not transform established rodent cells and is not required for maintenance of the transformed phenotype (Novak *et al.*, 1980). This function is performed by the middle TAg (Treisman *et al.*, 1981), although large TAg is required for immortalisation of primary cells prior to transformation by middle TAg (Rassoulzadegan *et al.*, 1983). Polyomavirus middle TAg has been shown to complex with the cellular phosphoprotein $pp60^{c\text{-}src}$, the product of the c-*src* proto-oncogene, whose tyrosine kinase activity is markedly increased as a result (Courtneidge, 1985), and its transforming potential is inherent to the ability of middle TAg to bind in this way.

2.2.2. Adenoviruses.

The human adenovirus family comprises thirty one different serotypes. All are double stranded DNA viruses, with a genome length of approximately 35kb and two types of capsomers (reviewed by Pettersson and Roberts, 1986). Viral transcription can be divided into early and late phases (Fujinaga and Green, 1970) and is known to occur from both strands of DNA (Landgraf-Leurs and Green, 1973).

Adenoviruses can be divided into three groups on the basis of their relative oncogenicity, namely highly oncogenic, weakly oncogenic and non-oncogenic (Huebner, 1967). McBride and Weiner (1964) were first to report the transformation of normal hamster kidney cells *in vitro* by adenovirus type 12 (Ad12). Freeman *et al.* (1967) were able to demonstrate that even adenovirus serotypes which lack oncogenicity *in vivo* are capable of transformation of cultured cells under suitable conditions.

During infection of non-permissive cells viral DNA integrates into the genome of the host cell (Doerfler, 1968). Further analysis using restriction fragments of the Ad2 genome demonstrated that only around one half of the total viral sequences were retained in transformed cells (Sharp *et al.*, 1974). The sequences necessary for transformation were located to the left hand end of the viral genome, within the HindIII 'G' fragment (Graham *et al.*, 1975). Studies by Houweling *et al.* (1980) using a smaller region of the adenovirus

genome (HpaI 'E') showed that viral transformation involved more than one step, as cells transfected with this fragment became immortal, but not fully transformed.

The transforming region of the adenovirus genome encodes the E1A and E1B group of polypeptides (Esche *et al.*, 1980). E1A is able to function as a transactivator of both viral (Berk *et al.*, 1979) and cellular (Kao and Nevins, 1983) genes, and can act together with cellular oncogenes to co-transform cells (Ruley, 1983). More recently, E1A has been shown to complex with $p105^{Rb}$, the product of the retinoblastoma gene which has tumour suppressor functions (Whyte *et al.*, 1988). Similarly, the E1B product can complex with the p53 tumour suppressor protein (Lane and Crawford, 1979; Sarnow *et al.*, 1982). These mechanisms represent important ways in which normal cellular control functions may be subverted by the presence of a transforming virus in the cell, producing an alteration in growth potential of affected host cells.

2.2.3. Hepatitis B Virus.

Hepatitis B virus (HBV) is present as a chronic infection in over two hundred and fifty million individuals world-wide, causes severe liver damage and has been linked with development of hepatocellular carcinoma (HCC) in its hosts (Beasley *et al.*, 1981). In contrast to information regarding virus structure and function, little is known of the mechanism of HBV oncogenesis. In very few cases integrated and

rearranged HBV DNA is able to activate cellular proto-oncogenes directly, such as c-*myc* (Moroy *et al.*, 1986; Hsu *et al.*, 1988).

Using a HBV transgenic mouse model, Chisari *et al.* (1989) have demonstrated that overproduction of viral large envelope protein in hepatocytes causes cell damage, followed by inflammatory and regenerative responses, which in turn lead to dysplasia, aneuploidy, adenoma, and finally HCC. The development of HCC in transgenic mice appears to mirror development of the disease in human in several aspects, including the randomness of viral integration and the presence of the same region in a functionally intact state. Malignant change is probably due to multiple secondary genetic events as a consequence of severe hepatocellular injury and hyperplasia induced by HBV.

2.2.3. Herpesviruses.

Seroepidemiological evidence points to an association of some of the human herpesviruses with specific human malignancies such as Epstein-Barr virus (EBV) in nasopharyngeal carcinoma (Raab-Traub *et al.*, 1987) and Burkitt's lymphoma (Epstein *et al.*, 1964), herpes simplex virus (HSV) type 2 in cervical carcinoma (Rawls *et al.*, 1969) and HSV-1 in oral squamous cell carcinoma (Shillitoe *et al.*, 1976).

In vivo, injection of herpesvirus into chickens produces a fatal lymphosarcoma, whereas introduction of attenuated virus does not have this effect (Okazaki *et al.*, 1970). *In vitro* studies using rodent cells and ultraviolet (UV) light-inactivated virus types have shown that herpesviruses possess transforming ability (Duff and Rapp, 1971, 1973; Albrecht and Rapp, 1973; Gelb *et al.*, 1980).

Despite the obvious oncogenic potential of HSV-1 and 2 in tissue culture systems no single viral gene has been found to be expressed universally in virally-transformed cell lines, arguing against the requirement for a virus-encoded oncogene for transformation. Indeed continued presence or expression of viral DNA does not appear to be a prerequisite for maintenance of the transformed phenotype, and the possibility of HSV-1 and HSV-2 acting via a "hit-and-run" mechanism is well-reviewed by Galloway and McDougall (1983). This theory postulates that viral DNA would enter the cell, induce certain changes resulting in transformation and then be lost again, with maintenance of the transformed state being controlled by cellular factors from this stage. Changes in viral DNA content of HSV-transformed cell lines with continued passaging have been documented by several authors (Frenkel *et al.*, 1976; Galloway *et al.*, 1980), with complete absence of viral sequences in some cases (Copple and McDougall, 1976; Hampar *et al.*, 1980).

The *in vitro* transforming activity of HSV-1 and HSV-2 has been narrowed down to specific regions of the viral genomes. In the former, the BglII "I" fragment has been shown to be oncogenic (Camacho and

Spear, 1978; Reyes *et al.*, 1979), whereas two fragments of HSV-2 (BglII "C" and "N") have transforming ability (Jarriwalla *et al.*, 1980; Galloway and McDougall, 1981). Further work by Galloway *et al.* (1984) mapped more finely the HSV-2 BglII "N" transforming activity to a 737bp stretch of DNA which could induce anchorage-independence in cultured rodent cells, although this fragment was not thought to encode a polypeptide. These authors suggest that the presence of insertion sequence (IS)-like elements within the transforming fragment may be important for transformation, and have demonstrated similar structures in a transforming fragment of cytomegalovirus (CMV) DNA.

The loss of herpesvirus DNA after the initial stages of transformation may explain the apparent lack of viral sequences in a large number of cervical carcinomas (zur Hausen *et al.*, 1974; Pagano, 1975), although recent studies have been more successful (McDougall *et al.*, 1980; Eglin *et al.*, 1981; di Luca *et al.*, 1987), and earlier work may have suffered from a lack of sensitive detection methods.

Many workers have investigated the association between HSV-1 and oral squamous cell carcinoma. Shillitoe *et al.* (1984) were able to demonstrate that oral cancer patients had an increased IgA response to HSV-1 antigens present in virally-infected HEp-2 human epidermoid carcinoma cells (Moore *et al.*, 1955) compared to a matched control population, and also a stronger IgM response, results which mirror the *in vivo* situation (Smith *et al.*, 1976; Shillitoe *et al.*, 1983). Nucleic acid hybridisation studies (Eglin *et al.*, 1983) further implicated HSV as an aetiological factor in oral cancer, with viral

RNA detected in carcinoma tissues but not in normal mucosa from the same patients, although the sample numbers were small.

HSV and other herpesvirus types clearly have some oncogenic potential, but their precise role in development of human malignancies is not yet totally apparent, and other factors must be involved in tumour progression.

2.2.5. Papillomaviruses.

Papillomaviruses have been recognised as the cause of common warts ever since Ciuffo (1907) demonstrated the presence of an infectious agent in filtered homogenates of verruca vulgaris. Since then much work has been done on this group of viruses which implicates them in the aetiology of several human diseases, including certain types of malignancy.

2.2.5.1. Physical and Biological Features.

Papillomaviruses are species-specific DNA tumour viruses belonging to the papovavirus group, along with polyomavirus, SV40, JC and BK viruses. They comprise a single circular double-stranded DNA genome complexed with histones and surrounded by a protein capsid of 72 capsomers, and lack a lipid envelope (Gissmann, 1984; Pfister, 1984).

According to Broker and Botchan (1986), the papillomaviruses constitute a distinct group separate from other *Papovaviridae* on several counts. Firstly, they have a larger particle size of 55nm diameter. Papillomavirus genomes are, on average, fifty percent larger (approximately 8kb long) than either SV40 or polyomavirus (section 2.2.1) and there is no sequence similarity. Papillomaviruses are also transcriptionally different, with all major open reading frames (ORFs) present and transcribed along one strand only, in contrast to SV40 and polyomavirus which utilise both strands of DNA. Most papillomaviruses are restricted in their tissue tropism, growing only in epithelium. Indeed the papillomavirus life cycle is closely linked to the state of differentiation of the host cell, with viral DNA replication occurring in the lower portion of the epithelium and viral capsid proteins only produced in the upper differentiating layers (for reviews see Gissmann, 1984; Pfister, 1984).

Papillomaviruses have been isolated from a diverse range of species (Pfister, 1984) including rabbits, dogs, deer, elk, cattle, donkeys, mice, chaffinches and monkeys, as well as humans. Within some of these groups several different types of papillomavirus exist, based on different types sharing less than fifty percent DNA sequence homology by reassociation kinetics under highly stringent conditions (Coggin and zur Hausen, 1979). However, later nucleotide sequence analysis of some viruses shows the differences between some related types to be much less, as in the case of HPV-6 and HPV-11 which are identical over 82% of their genomes (de Villiers, 1989). For instance, there are six different bovine papillomavirus (BPV) types known to infect cattle,

divided into two distinct subgroups (Jarrett *et al.*, 1984). Subgroup A viruses (BPV-1, 2 and 5) are fibropapillomaviruses and infect both dermal fibroblasts and keratinocytes, whereas subgroup B viruses (BPV-3, 4 and 6) are strictly epitheliotropic. Sequence data suggests that subgroup B viruses lack an E6 ORF, which may be a factor resulting in the different tissue tropisms of these two subgroups (W. Pennie and S. Campo, personal communication).

Unlike other DNA tumour viruses, no *in vitro* system exists as yet for the propagation of papillomaviruses due to the strict link between virus replication and epithelial maturation. Fortunately the advent of molecular biology and recombinant DNA techniques has allowed detailed study of these viruses through molecular cloning, sequence analysis and cell transfection experiments.

Use of this technology has enabled molecular cloning of more than sixty six human papillomavirus (HPV) types to date (reviewed by de Villiers, 1989) from a variety of pathological conditions. DNA sequence analysis has revealed a similar genome organisation between those HPV types which have been sequenced thus far, and also between animal papillomaviruses so characterised (Giri and Danos, 1986). The viral genome may be divided into three regions, coding for early and late gene products, and a non-coding region (NCR) also referred to as the long control region (LCR) or upstream regulatory region (URR). The coding regions consist of overlapping ORFs which are transcribed in all three frames, thus making the best use of the limited genomic information available.

The products of the early (E) ORFs are involved with viral replication, transcriptional regulation and cell transformation events, while the late (L) ORFs encode viral structural proteins. The NCR contains regulatory elements, including the orgin of replication, promoter sequences, binding sites for proteins involved in viral gene expression (Chong *et al.*, 1990), and perhaps for factors which confer tissue specificity on the viral type (Sibbet and Campo, 1990).

2.2.5.2. Oncogenic Activity of Papillomaviruses.

The majority of papillomavirus infections result in the production of proliferative epithelial or mucosal lesions which are characteristically benign (Gissmann, 1984), and which usually regress. However in some instances, in the presence of cofactors, lesions may progress to malignancy.

A good animal model system for papillomavirus oncogenicity is that of the cottontail rabbit papillomavirus (CRPV), the first DNA tumour virus to be isolated and characterised (Shope and Hurst, 1933; reviewed by Kreider, 1980). While the virus induces warts in its natural host, progression of these to carcinomas is approximately three times more frequent in domestic rabbits (Syverton, 1952), suggesting that perhaps some genetic factor is involved. Painting CRPV-induced warts in cottontail rabbits with chemical carcinogens also results in a higher frequency of malignant progression than with either virus or carcinogen alone (Rous and Kidd, 1938; Orth *et al.*,

1977). However no infectious viral particles were able to be isolated from primary carcinomas or metastases (Kidd and Rous, 1940).

Another example of the carcinogenic effect of papillomaviruses is afforded by the system first described by Jarrett *et al.* (1978). Bovine papillomavirus type 4 (BPV-4) induces papillomatosis of the oral cavity and upper alimentary canal of cattle. If infected animals feed on bracken fern, which contains carcinogens and immunosuppressants, progression from papilloma to carcinoma is observed. These events can be reproduced in an experimental situation where cattle infected with BPV-4 and fed on bracken are seen to develop carcinomas (Campo and Jarrett, 1986). In both cases viral DNA is present in the benign lesions but is lost as progression occurs, suggesting that the virus is not required for maintenance of the transformed phenotype (Campo *et al.*, 1985). This observation is further confirmed by work by Smith and Campo (1988), in which cultured cells transformed *in vitro* by BPV-4 DNA were seen to lose viral sequences after several passages without reversion to a non-tumorigenic phenotype.

2.2.5.3. Papillomaviruses in Human Cancers.

As mentioned above, more than sixty six types of human papillomavirus (HPV) have been isolated from mainly benign pathological lesions at various sites, including skin (Heilman *et al.*, 1980; Kremsdorf *et al.*, 1984), genital mucosa (De Villiers *et al.*, 1981; Beaudenon *et al.*, 1987a) and laryngeal mucosa (Gissmann *et al.*, 1982). However a large body of evidence has now been obtained

implicating human papillomaviruses in the aetiology of several human cancers.

The first evidence of the oncogenic potential of HPVs came from clinical cases of epidermodysplasia verruciformis (EV), a rare inherited skin disease which persists throughout the life of the patient. It is characterised by defects in cell-mediated immunity and disseminated skin warts containing specific HPV types (de Villiers, 1989) which usually appear in childhood. Around thirty percent of EV sufferers develop squamous cell carcinomas of the skin, mainly at sites exposed to UV radiation, after a variable timespan (Lutzner, 1978) which are locally aggressive and eventually fatal if left untreated.

Various workers (Orth *et al.*, 1980; Pfister *et al.*, 1983a) have demonstrated the presence of viral DNA in carcinomas from EV patients, a similar finding to CRPV in the rabbit model. In addition, Ostrow *et al.* (1982) found HPV-5 DNA in a metastatic lesion. However it is as yet unclear whether or not viral sequences are required for maintenance of the transformed state. Orth *et al.* (1980) used an *in situ* hybridisation technique to show that not all cells in a lesion were uniformly infected, implying that different amounts of viral DNA replication may occur in different cells. Ultrastructural studies did not show any evidence of productive viral infection, again paralleling the CRPV findings. Development of HPV-containing carcinomas at sites exposed to actinic radiation may be another example of carcinogenesis involving virus plus cofactors.

Other categories of immunosuppressed individuals are prone to suffer from HPV infections. Renal transplant recipients whose immune responses are necessarily depressed show an increased incidence of skin warts, and are thirty five times more likely to develop carcinomas than the population at large (Pfister, 1984). Patients with acquired immune deficiency as a result of infection with the human immunodeficiency virus (HIV) also commonly develop warts at various bodily sites (Greenspan *et al.*, 1988), and HPV-associated anogenital malignancies have been documented in male homosexuals so infected (Melbye *et al.*, 1990).

Laryngeal papillomas in children and young adults have been associated with HPV-6 (Gissmann *et al.*, 1983) and HPV-11 (Gissmann *et al.*, 1982; Mounts *et al.*, 1982). In the past, treatment of laryngeal warts (reviewed by zur Hausen, 1977) consisted of X-irradiation, but this resulted in progression to malignancy in some cases after a variable period of five to forty years, suggesting synergism between HPV types and X-rays (Pfister, 1984).

Adult onset laryngeal warts have a different potential for transformation, with progression to carcinoma occurring in over twenty percent of cases (Kleinsasser and Oliveira e Cruz, 1973); the necessary cofactor is probably heavy cigarette smoking (Pfister, 1984). Although initial investigations failed to isolate any papillomavirus sequences from the carcinomas (Gissmann *et al.*, 1982), more recent studies have been successful resulting in the detection of HPV-16 (Brandsma *et al.*, 1986) and HPV-30 (Kahn *et al.*, 1986) DNA in laryngeal carcinomas.

Epidemiological evidence has long suggested the involvement of a transmissible agent in the aetiology of human genital cancer (Rotkin, 1973), much attention in the past having been focussed on HSV-2 (section 2.2.4.) but with limited success. Warts of the genital tract were shown to contain papillomavirus particles by electron microscopy (Dunn and Ogilvie, 1968) and several authors have reported malignant conversion of these lesions (reviewed by zur Hausen, 1976). Further investigations detected viral structural antigen (Woodruff *et al.*, 1980) and viral DNA (Gissmann and zur Hausen, 1980; Gissmann *et al.*, 1982) in genital condylomata, leading zur Hausen (1977, 1982) to propose that specific papillomaviruses might be the infective agents in anogenital malignancies and that they might act synergistically with HSV to effect malignant transformation.

Much evidence has since accumulated regarding HPVs and their possible role in genital carcinogenesis. Durst *et al.* (1983) were the first authors to report the isolation of a new HPV type, HPV-16, from cervical carcinoma specimens, which was followed by the discovery of a related type in another series of tumours (HPV-18, Boshart *et al.*, 1984). Later work enabled isolation and characterisation of several additional viral types associated with genital cancers (reviewed by de Villiers, 1989), including HPV-31 (Lorincz *et al.*, 1986), HPV-33 (Beaudenon *et al.*, 1986) and HPV-35 (Lorincz *et al.*, 1987). DNAs of these papillomaviruses have been demonstrated in around ninety percent of anogenital malignancies of both sexes, although HPV-16 (50%) and HPV-18 (20%) are more prevalent (zur Hausen, 1988). Viral DNA has also been detected in cell lines developed from

cervical tumours such as HeLa (HPV-18) and Caski and SiHa (HPV-16).

Papillomavirus DNA in genital cancers is frequently, but not invariably, integrated into the host cell genome either in single copy or in multiple tandemly repeated structures (Lehn *et al.*, 1985; Matsukuru *et al.*, 1986), and this may be an early event in the process of neoplasia (Schneider-Maunoury *et al.*, 1987). As the viral genome is naturally circular, the integration event requires linearisation of HPV DNA. This split almost invariably occurs in the E2 region of the viral genome (Schwarz *et al.*, 1985), which has important consequences for regulation of viral gene expression. Integration in this way disrupts the E2 coding sequence, resulting in removal of E2-mediated repression and increasing E6 and E7 transcription, which are the major RNAs found to be expressed in cervical carcinomas (Schwarz *et al.*, 1985; Yee *et al.*, 1985; Schneider-Gadicke and Schwarz, 1986).

The continued presence and expression of HPV DNA and expression of specific viral gene products in genital cancers and cell lines derived from these indicates that papillomavirus types may play an important role in the aetiology of such tumours. More recent studies have addressed the question of the oncogenicity of genital HPV types in *in vitro* assays. Work by Matlashewski *et al.* (1987, 1988) showed that HPV-16 DNA together with an activated *ras* oncogene was sufficient for transformation of primary human fibroblasts and primary rodent epithelium in a cotransfection assay, whereas neither alone resulted in transformation, reinforcing the need for cooperative effects in HPV-induced carcinogenesis. The transforming function was localised to

the E6-E7 region of the viral genome. Cells harbouring HPV-16 DNA alone appeared to have an extended lifespan. Bedell *et al.* (1989) similarly identified the transforming genes of HPV-18 as the E6 and E7 using NIH3T3 and Rat-1 fibroblast cell lines. Further evidence has been provided for HPV-16 oncogenicity. Le and Defendi (1988) were able to fully transform NIH3T3 cells in culture using a DNA fragment containing part of the viral NCR, the E6, E7 and part of the E1, and adjacent cellular sequences. When HPV-16 and HPV-18 DNAs were transfected into primary cells (Pirisi *et al.*, 1987; Kaur and McDougall, 1988) they became immortal; human cervical epithelial cells previously immortalised by HPV-16 but lacking tumorigenicity (Woodworth *et al.*, 1988) became fully malignant when a viral Harvey *ras* oncogene was added to the system (DiPaolo *et al.*, 1989).

Using a hormone-inducible promoter construct, Crook *et al.* (1989b) demonstrated the continued requirement for expression of HPV-16 E7 protein for maintenance of the transformed phenotype of baby rat kidney (BRK) cells transformed with HPV-16 and activated *ras*. HPV-16 is also able to cooperate with v-*fos* to transform baby mouse kidney cells (Crook *et al.*, 1988), but is dependent on the continued presence of dexamethasone or progesterone in the culture medium when gene expression is driven from the viral promoter, an effect presumably mediated through the glucocorticoid responsive element in the viral NCR (Gloss *et al.*, 1987). However at later passages these cells lose the requirement for steroid hormones, with a concurrent amplification and overexpression of c-*myc* (Crook *et al.*, 1989c) which appears to confer hormone independence on HPV-16 transformed cell lines (Crook *et al.*, 1989a). Lees *et al.* (1990) extended this work by

demonstrating that cotransformation of BRK cells by HPV-16 and activated *ras* was increased when the viral E2 protein was overexpressed using an E2 construct cotransfected into the cells, which was shown to transactivate the HPV-16 early promoter.

Further analysis of the HPV-16 E7 ORF confirmed its role in HPV-mediated transformation. Edmonds and Vousden (1989) created a series of point mutations throughout the E7 which would alter the coding potential, and tested these by transfection into NIH3T3 cells. Mutation of the "zinc-finger" (Barbosa *et al.*, 1987) motif near the carboxy terminus of the E7 protein was found to considerably decrease transforming ability, while mutations in the region of homology to adenovirus E1A and SV40 large T completely abolished the E7 transformation function. Similar experiments by Watanabe *et al.* (1990) reinforced these results using point-mutated E7 constructs in a variety of different cell lines.

Whereas complete genomes or viral early regions from those HPV types frequently associated *in vivo* with genital carcinomas (HPV-16, 18 and 33) are competent in cotransformation assays of primary cells, HPV-6 and HPV-11 DNAs (mainly found in benign lesions) are not (Storey *et al.*, 1988). The reason for this may lie in the ability of the highly oncogenic HPVs to produce a spliced E7 message not possible in either HPV-6 or 11, due to lack of necessary sites within the E6 ORF (Smotkin *et al.*, 1989). Indeed, when constructs containing the HPV-6 or HPV-11 E7 ORF alone are transfected into BRK cells together with activated *ras*, these viral types give rise to transformed cells albeit at a lower efficiency than HPV-16, 18 or 33 DNAs (Storey

et al., 1990), although cells transformed by HPV-6 and HPV-11 E7 are equally as tumorigenic in immunocompetent animals as those transformed by HPV-16 plus *ras*. These authors were able to demonstrate that the cotransforming activity of HPV DNA is separate from its transactivation function, as stimulation of a chloramphenicol acetyltransferase (CAT) gene linked to the adenovirus E2 promoter was equally elevated by either HPV-11 E7 or HPV-16 E7 plasmids, suggesting that transformation by HPVs is not wholly a result of transactivation.

Two of the three regions of the HPV-16 E7 protein important for transformation (Edmonds and Vousden, 1989) show marked homology to areas of both SV40 large T antigen and adenovirus E1A (Phelps *et al.*, 1988; Dyson *et al.*, 1989), well-recognised DNA tumour virus oncoproteins (sections 2.2.1. and 2.2.2.) which have been shown to bind the retinoblastoma gene (Rb) product $p105^{Rb}$ (section 2.2.1. and 2.2.2; Whyte *et al.*, 1988; DeCaprio *et al.*, 1988). The regions of E1A and T Ag responsible for binding the Rb protein are critical for transformation and are those which share homology with papillomavirus E7, so it is not surprising that E7 is also capable of binding $p105^{Rb}$ (Dyson *et al.*, 1989). Functional similarity between these three viral oncogenes has been confirmed by Vousden and Jat (1989), who were able to rescue cells transformed with a thermolabile SV40 T Ag from growth arrest at non-permissive temperature by complementation with HPV-16 E7. Thus sequestration of $p105^{Rb}$ present in the cell is one possible mechanism by which HPVs could contribute to neoplasia, with a major role for the viral E7 gene product. Furthermore, the E7 protein of HPV-6, a type found only

rarely in malignant lesions, is able to bind only much smaller amounts of the Rb protein (3.6 to 6.5 fold less, Barbosa *et al.*, 1990). The Rb binding domain also harbours a potential casein kinase (CK) II site, and it has been shown that HPV-6 E7 is phosphorylated two to four fold less efficiently than either HPV-16 or HPV-18 E7 (Barbosa *et al.*, 1990). These authors also demonstrated that mutations in either the Rb binding domain or the CKII site of HPV-16 E7 decreased the ability of the virus to transform NIH3T3 cells.

In addition to E7, the papillomavirus E6 protein is also important for cell transformation. Hawley-Nelson *et al.* (1989) described a cooperative effect between the E6 and E7 ORFs of HPV-16 in the immortalisation of primary human keratinocytes, whereas neither alone produced this effect. Vousden and Jat (1989) showed that, although HPV-16 E7 was the major factor in rescue of cells transformed with mutant SV40 (as described above), the E6 could also perform this function. Work by Barbosa and Schlegel (1989) documented the transformation of human keratinocytes by HPV-18 E6 and E7, although they did not analyse the functions of each ORF individually. In the same way as the E7 protein has been shown to bind $p105^{Rb}$, an association between the E6 protein and p53 gene product has been reported recently (Werness *et al.*, 1990). Binding of the SV40 T Ag and adenovirus E1B oncogenes to p53 has been similarly found (section 2.2.2; Lane and Crawford, 1979; Sarnow *et al.*, 1982).

Kloster *et al.* (1988) described an animal system which mimics the pattern of development of human cervical carcinoma. A novel

papillomavirus type, the rhesus monkey papillomavirus (RHPV), was isolated from an active mating population of rhesus monkeys, around ten percent of which had invasive genital neoplasms similar to those seen in humans. These authors subsequently used cloned RHPV DNA to transform primary cells in culture with a similar efficiency to that obtained with HPV-16, and reported that RHPV has the ability to cooperate with activated *ras* in the transformation of these cells (R. Ostrow, personal communication), again similar to HPV-16.

To partly confound the above results, several authors have reported finding DNA of so-called "oncogenic" HPV types in clinically and histologically normal cervical epithelium (Cox *et al.*, 1986; MacNab *et al.*, 1986) and some doubt has been cast on the role of papillomaviruses in cervical cancer on epidemiological grounds (Munoz *et al.*, 1988). It would seem that latent infection by papillomaviruses in genital mucosa does occur, and that the majority of HPV infections do not progress to malignancy, a fact in keeping with theories of multistage carcinogenesis. However a major difference between HPV in anogenital malignancies compared with normal tissue is the amount of viral DNA present. While viral nucleic acids are readily detected in carcinomas by Southern blot hybridisation, much of the data citing HPV DNA in healthy tissue has relied on the use of a very sensitive technique, polymerase chain reaction (PCR), for detection of papillomavirus sequences, sometimes without the use of adequate controls (reviewed by zur Hausen, 1988). Therefore, as seen in the experimental systems detailed above, there may be an important dose effect of HPV on genital mucosa.

Thus much evidence has accumulated implicating specific HPV types as being of importance in the aetiology of human anogenital cancer, with recent work detailing similarities which exist between papillomaviruses and other DNA tumour viruses as regards their oncogenic mechanisms, although additional factors are clearly necessary for malignant progression of lesions induced by HPVs both *in vivo* and *in vitro*.

2.2.4.3. Human Papillomaviruses in Oral Disease.

Oral papillomas and other related benign mucosal proliferations are not an uncommon occurrence (Tyldesley, 1981). Several reports exist in the literature demonstrating papillomavirus structural antigens in such lesions (Jenson *et al.*, 1982; Syrjanen *et al.*, 1984; Jin and Toto, 1984), while Praetorius-Clausen and Willis (1971) observed virus particles in focal epithelial hyperplasia (FEH, Heck's disease), a rare familial condition which is particularly prevalent in certain populations such as North American Indians and Eskimos in Greenland, where the incidence approaches thirty five percent (Praetorius-Clausen, 1972).

DNA hybridisation studies have revealed that oral mucosa is subject to infection by a number of types of human papillomavirus including HPV-2, 4, 6, 11, 13, 16, 32 and 57 (Adler-Storthz *et al.*, 1986a; Naghashfar *et al.*, 1985; Pfister *et al.*, 1983b; Beaudenon *et al.*, 1987b; Garlick *et al.*, 1989; de Villiers *et al.*, 1989). Greenspan *et al.* (1988) found mainly HPV-7 DNA (41%) in a series of oral warts from

patients seropositive for the human immunodeficiency virus (HIV). They suggested that patients suffering from acquired immunodeficiency syndrome (AIDS) may be subject to infection by unusual HPV types, which is perhaps not surprising considering the other opportunistic infections to which this population is susceptible, and the pattern of infection in other immunosuppressed individuals (such as EV patients).

In parallel with cervical cancer, the involvement of an infectious agent has been suggested in the aetiology of oral squamous cell carcinoma, previously thought to be one of the herpes simplex viruses (Lehner *et al.*, 1973; Shillitoe *et al.*, 1976), although data has not always been consistent (section 2.2.3.) and the prevalence of HSV-1 in the general population is high.

In view of the obvious oncogenic potential of some human papillomaviruses, the close similarity between oral and genital mucosa, and evidence from other systems of malignant progression of virally-induced oral lesions (Sundberg *et al.*, 1985), the possibility of a causative role for certain HPV types in oral cancer would not seem to be remote. Initial investigations, purely at the histological level, showed features reminiscent of viral infection in pathological sections of oral verrucous carcinoma (Syrjanen *et al.*, 1983; Eisenberg *et al.*, 1985). Additionally, the former group reported positive staining of some samples using an antibody to a papillomavirus structural antigen.

Further investigations utilising hybridisation technology have enabled the viral types present in such lesions to be established. De Villiers *et al.* (1985) found largely episomal HPV sequences in three of seven tongue carcinomas by Southern blot analysis, HPV-16 being present twice while one lesion harboured HPV-2 DNA, a type not previously associated with malignancy, at a high level (around fifty copies per cell). Adler-Storthz *et al.* (1986b) also detected HPV-2 DNA in three of nine patients with verrucous carcinoma using *in situ* hybridisation on histological material. Other workers (Milde and Loning, 1986; Syrjanen *et al.*, 1988) have reported the "genital" HPV types 16 and 18 to be present in oral squamous cell carcinomas, and some parallels seem to exist with HPV in genital lesions as Syrjanen *et al.* (1988) found HPV-6 and 11 DNA in oral dysplasias but not in carcinomas. Ostrow *et al.* (1987) found HPV-16 episomes in a lymph node metastasis of a primary tongue carcinoma at a level of approximately twenty to forty copies per cell. A recent study by Chang and coworkers (1989) documented a high prevalence of HPV-16 DNA in oral carcinomas in Taiwanese patients, seventy six percent of whom were positive for viral sequences by Southern blot analysis. Only one out of seventeen controls of normal gingival mucosa contained HPV DNA; this was unable to be typed by the investigators, but was not HPV-16. The incidence of both smoking (82%) and betel quid chewing (57%) were high in cancer patients, and may reflect both a viral and chemical aetiology of oral carcinoma.

Interestingly, Maitland *et al.* (1987) reported finding a variant of HPV-16 in forty six percent of a series of oral cancer biopsies studied by Southern blot hybridisation. This viral type differed from the

prototype HPV-16 DNA in its digestion pattern using the restriction enzyme PstI, although no variation was observed on digestion with DraI. The PstI 'C' fragment, visible as a 1.55kb band in digests of prototype HPV-16, was consistently absent from the majority of biopsies examined; however on reprobing with a specific subgenomic fragment the 1.55kb band became visible, but only at very low levels (M.F. Cox, personal communication). This suggests that a small minority of viral DNA present in the samples was prototype HPV-16, the remainder having some alteration in the L2-L1 region of the genome compared to the wild-type virus.

Furthermore, these authors discovered that distribution of the variant HPV-16 was not limited to malignant epithelium alone. Seven out of eight biopsies of lichen planus, not a recognised premalignant condition of the oral cavity, contained HPV-16 sequences as did five out of twelve samples of normal oral mucosa, approximately the same percentage as virus-positive carcinomas. Other ill-defined benign lesions termed non-specific keratosis and reactive keratosis also harboured viral nucleic acids. These results were the first report of HPV DNA in clinically and histologically normal oral epithelium, in line with previous findings regarding papillomaviruses in human cervix.

From the data presented HPV-16 DNA appeared to be episomal in all but one case, a sample of normal oral epithelium; thus the situation may be somewhat different to that of HPV-16 and 18 in cervical tissues, where viral DNA is frequently integrated in carcinomas, probably from an early stage in the neoplastic process.

More recent work by Maitland and coworkers (1989) used the polymerase chain reaction to facilitate detection of HPV sequences in biopsies of oral mucosa and in cell lines derived from these, using oligonucleotide primers specific for the E6 ORF which is preferentially retained in carcinomas and which was detectable by Southern blotting. The results obtained supported those of the previous study, with around fifty percent of oral carcinomas containing HPV-16 DNA, although no data on prevalence of viral DNA in normal tissue by PCR detection were discussed, and no HPV types other than HPV-16 were screened for (N. Maitland, personal communication).

Attempts by these authors to amplify DNA from the viral late region using specific primers in the PCR were unsuccessful, either due to a lack of the DNA or to gross sequence divergence resulting in failure of the primers to anneal. They suggested that in the former case the virus would not be viable and while this may be correct, it would not necessarily preclude viral sequences from being involved in tumorigenesis as the E6 and E7 ORFs would still be present and able to be expressed, as in cervical carcinomas.

Another feature noted by this group was the apparent increase in the proportion of HPV-16 containing cells in cultures of oral carcinomas at higher passage numbers, suggesting that the presence of viral DNA might confer some growth advantage on such cells, while uninfected cells would senesce. This would be consistent with an immortalising

role for papillomaviruses in cell transformation (Hawley-Nelson *et al.*, 1989).

The precise role of HPVs in the aetiology of oral squamous cell carcinoma is not, as yet, well understood. Scully *et al.* (1988) proposed that the oral cavity might serve as a reservoir of latent human papillomaviruses which could subsequently infect anogenital epithelium following orogenital contact. However if the variant HPV-16 does indeed lack late viral sequences coding for structural proteins, as suggested by Maitland *et al.* (1989), this would not represent a feasible route for infection of genital mucosa. Although viral DNA has been demonstrated in normal epithelium, this does not exclude an involvement in oral carcinogenesis, as the effect may indeed be an early one such as an increase in cell proliferation or immortalisation, after which the viral DNA is no longer required (as for BPV-4 transformation), or the process may be dependent on viral copy number with a threshold level required to trigger further events.

2.2.6. Objectives of the Current Study.

The present work is an attempt to identify the papillomavirus types which might be present in oral squamous cell carcinomas, and therefore possibly of importance in the aetiology of such lesions. Although several workers have reported HPV DNA in oral cancers, no endeavour has been made to analyse the distribution of viral sequences in adjacent dysplastic or clinically normal tissue samples, which would seem important in the light of results obtained from screening biopsies of normal oral mucosa from patients (presumably)

without cancer (Maitland *et al.*, 1987). This would enable levels of viral DNA to be compared in epithelia of varying degrees of neoplasia, perhaps substantiating the idea of a dose effect of HPVs in transformation, and comparing samples between cancer and non-cancer sufferers.

Although HPV-16 DNA is the most widely reported type in malignant oral mucosa sequences of other HPVs have been detected, and may reflect a geographic variance. A different distribution of HPV types with area has been noted for cervical samples (Millan *et al.*, 1986; Meanwell *et al.*, 1987) and this could also be the case in oral cancers from different regions.

The possibility of new or variant viral types in oral samples must be considered. The HPV-16 variant first described by Maitland *et al.* (1987) merits further investigation to ascertain the nature of the sequence alteration and its likely effect on viral function. These authors together with Chang *et al.* (1989) found unidentifiable viral sequences showing different degrees of homology to HPV-16, and might likely represent new papillomavirus types.

To confront these possibilities, thirty nine samples of primary intraoral squamous cell carcinoma, together with adjacent normal tissue (and dysplastic and lymph node metastases where present) have been investigated for the presence of DNA of several papillomavirus types by Southern blot hybridisation and by PCR. Further analyses included cloning of a viral genome from one lesion and mapping structural abnormalities in HPV DNA present in other samples.

3. Materials and Methods.

3.1 Materials.

3.1.1. Chemicals.

All chemicals used in this work were of analytical reagent grade, and were purchased from either BDH Limited or from the Sigma Chemical Company.

Radioisotopes of α-[^{32}P]-dCTP (>111 TBqmmol^{-1}), γ-[^{32}P]-dATP (>185 TBqmmol^{-1}) and α-[^{35}S]-dATP (>22.2 TBqmmol^{-1}) were obtained from Amersham International plc, Aylesbury, Bucks.

Caesium chloride, agarose, urea and guanidinium isothiocyanate were supplied by Gibco BRL, Paisley, Scotland.

Tris(hydroxymethyl)aminoethane was obtained from Boehringer Mannheim UK (BCL), Lewes, East Sussex.

Reagents for use in polymerase chain reaction experiments were purchased from Perkin-Elmer Limited, Beaconsfield, Buckinghamshire.

Distilled phenol was supplied by Rathburn Chemicals, Walkerburn, Scotland.

3.1.2. Enzymes and Markers.

All restriction endonucleases used were obtained from Gibco BRL, as were bacteriophage T4 DNA ligase, HindIII-digested bacteriophage lambda DNA and HaeIII-digested bacteriophage ϕx174 DNA markers.

BCL were the suppliers of T4 polynucleotide kinase, calf intestinal alkaline phosphatase and random primed DNA labelling reagents.

3.1.3. Stock Solutions.

TE.	10mM Tris-HCl pH8.0
	1mM EDTA
10x TBE	0.89M Tris
	0.89M boric acid
	25mM EDTA
50x TAE	2M Tris-acetate
	0.1M EDTA (pH8.0)
20x SSC	3M NaCl
	0.3M sodium citrate
20x SSPE	3.6M NaCl
	0.2M Na_2HPO_4/NaH_2PO_4
	0.02M EDTA pH7.7

phenol/chloroform 25 vols. TE-saturated phenol
24 vols. chloroform
1 vol. isoamyl alcohol

SM 0.1M NaCl
0.05M Tris-HCl pH7.5
8mM $MgSO_4.7H_2O$

3.1.4. Growth Media.

L-broth 1% (w/v) bacto tryptone
0.5% (w/v) bacto yeast extract
170mM NaCl

L-agar 1.5% (w/v) bacto-agar in L-broth,
autoclaved and cooled to 42°C
prior to addition of antibiotics
(as required).

SOC 2% (w/v) bactotryptone
0.5% (w/v) yeast extract
10mM NaCl
2.5mM KCl
10mM $MgCl_2$
10mM $MgSO_4$
20mM glucose

NZY broth 85mM NaCl

10mM $MgCl_2$

0.5% (w/v) yeast extract

1% (w/v) NZ amine

3.1.5. Cloning Vectors and Host Strains.

Construction of genomic DNA libraries was performed using BamHI-digested arms of bacteriophage lambda EMBL3 (Frischauf *et al.*, 1983), obtained from Stratagene. Recombinants were plated on the *E. coli* host strains LE392 and P2392.

The plasmid vector pBR322 (Bolivar *et al.*, 1977) was used in molecular cloning experiments and was supplied by Gibco BRL. Recombinant clones were used to transform library-efficient *E. coli* strain DH5-α (Gibco BRL).

3.1.6. Recombinant HPV Clones.

The genomes of HPV types 1-7, 11, 13, 16 and 18 cloned in pBR322 were as originally described (Heilman *et al.*, 1980; Kremsdorf *et al.*, 1983; Kremsdorf *et al.*, 1982; de Villiers *et al.*, 1981; Oltersdorf *et al.*, 1986; Gissmann *et al.*, 1982; Pfister *et al.*, 1983b; Durst *et al.*, 1983; Boshart *et al.*, 1984).

3.1.7. Other Materials.

Nylon transfer membranes were purchased from Amersham International plc (Hybond N+) and from Biorad (Pall Biodyne A).

Eastman-Kodak supplied X-Omat S and X-Omat XAR-5 film for use in autoradiography.

3.2. Methods.

3.2.1. General Methods.

3.2.1.1. Sterilisation of Materials and Solutions.

All glass centrifuge tubes, plastic Eppendorf tubes and disposable pipette tips were sterilised prior to use, in order to prevent subsequent degradation of DNA by nucleases. This was achieved by autoclaving in a domestic pressure cooker for fifteen minutes in the presence of a suitable indicator tape (3M). Most solutions were similarly treated. Those containing heat-labile components were sterilised by filtration through Gelman Acrodisc filters of pore size 0.2mm.

3.2.1.2. Handling of Bacteria.

All bacterial cultures were manipulated under Category I containment.

3.2.1.3. Handling of Radioactive Isotopes.

All work involving the use of radionuclides was carried out according to the Local Rules as specified in the Beatson Institute Safety Manual.

3.2.1.4. Phenol/Chloroform Extraction.

Contaminating protein was removed from DNA samples by extraction with phenol/chloroform, prepared as described above. An equal volume of phenol/chloroform was added to the DNA solution and mixed by vortexing. The two phases were then separated by centrifugation in an Eppendorf centrifuge for fifteen seconds at 12000g (small volumes) or in a bench top centrifuge for five minutes at 4000g (larger volumes). The (upper) aqueous phase was removed to a fresh tube and extracted for a second time. DNA was recovered by ethanol precipitation and the pellet washed with 70% ethanol.

3.2.1.5. Ethanol Precipitation.

DNA was precipitated from solution by the following method. Two volumes of ethanol and 0.1 volume of 3M sodium acetate pH5.5 were added, mixed and stored at -20 ^{o}C for one hour. Recovery of DNA was achieved by centrifugation in an Eppendorf centrifuge (small

volumes) at 12000g for ten minutes, or by centrifugation in a benchtop centrifuge for thirty minutes at 4000g. The supernatant was decanted, and the pellet washed in 70% ethanol. After removal of the majority of the wash supernatant, the pellet was dried briefly in a vacuum dessicator and resuspended in TE buffer pH8.0.

3.2.1.6. Spectrophotometric Quantitation of Nucleic Acids.

The approximate concentrations of solutions of DNA and oligonucleotides were determined by measuring the optical density of the solution at 260nm, using the following data (Maniatis *et al.*, 1982):

An OD_{260nm} = 1.0 represents approximately the following concentrations:

$50mgml^{-1}$ for dsDNA

$20mgml^{-1}$ for oligonucleotides

3.2.1.7. Collection and Sampling of Human Tissue Specimens.

Tissue specimens were obtained from the Plastic Surgery Unit, Canniesburn Hospital, Glasgow, from patients undergoing major head and neck surgery for treatment of oral squamous cell carcinoma, under the care of Mr. D.S. Soutar and Mr. M.H.C. Webster.

(a)

sample	age	site
1	71	retromolar
2	77	commisure
3	84	palate
4	82	palate
5	66	FOM
6	73	FOM
7	72	tongue
8	46	tongue
9	82	FOM
10	66	commisure
11	74	palate
12	74	tongue
13	54	FOM
14	51	FOM
15	62	FOM
18	65	alveolus
19	79	FOM/tongue
20	55	tongue
21	65	FOM
22	53	tongue
23	77	alveolus
24	44	FOM
25	56	tongue
26	55	tongue/FOM
27	69	tongue
28	52	tongue/FOM
29	64	alveolus
30	71	buccal
33	63	FOM
34	67	tonsillar
35	45	tongue
37	47	FOM
38	68	soft palate
39	72	FOM
40	71	retromolar
41	74	buccal
42	84	alveolus
43	61	buccal/FOM
45	67	buccal

(b)

sample	age	site
N1	60	buccal
N2	69	"
N3	67	"
N4	47	"
N5	53	"
N6	69	"
N7	47	"
N8	71	"
N9	76	"
N10	54	"
N11	69	"
N12	56	"
N13	60	"
N14	60	"
N15	73	"
N16	73	"
N17	63	"
N18	47	"
N19	61	"
N20	46	"
N21	67	"
N22	57	"
N23	76	"
N24	75	"
N25	74	"

Sites of biopsies and patient ages for (a) carcinoma samples and (b) normal controls. FOM=floor of mouth.

At the time of surgery, resection specimens were collected in theatre, examined visually and obvious areas of carcinomatous, dysplastic and normal mucosa identified. A biopsy was made from the resection material to include tissues of normal appearance through to frank carcinoma. Care was taken to record the site of biopsy on the pathology form for the pathologist's ease of interpretation, and to ensure that removal of material did not hamper the histological assessment of the specimen.

Tissue samples were divided into carcinoma, dysplastic (if present) and normal, snap frozen separately in liquid nitrogen and stored at -70 ^{o}C until required. In cases where lymph nodes were removed, these were similarly biopsied and stored.

Samples of clinically and histologically normal mucosa to act as external controls were obtained from patients attending outpatient clinics for surgical treatment of various non-malignant disorders. Prior approval of the West Ethical Committee of the Greater Glasgow Health Board was obtained, as was the informed consent of each patient. A small piece (approximately three cubic millimetres) of buccal mucosa was removed by the surgeon using a sterile disposable plastic biopsy punch (Stiefel Laboratories) under local anaesthesia, and the resulting defect required no suture. The specimens were bisected, one half being snap frozen in liquid nitrogen and stored at -70 ^{o}C, while the remainder was fixed in 10% formalin and sent for histological analysis.

3.2.2. Manipulation of Nucleic Acids.

3.2.2.1. Preparation of DNA and RNA from Stored Tissue.

Nucleic acids were prepared from frozen samples of human tissue by the method of Chirgwin *et al.* (1979) with minor modifications. Samples were removed from storage at -70 ^{o}C into a container of liquid nitrogen. The tissue was then fragmented rapidly using a mortar and pestle and transferred immediately to a teflon grinding chamber containing a teflon-coated steel grinding ball 10mm in diameter, both of which had been chilled by immersion in liquid nitrogen (Krieg *et al.*, 1983). The chamber with added tissue was further cooled prior to grinding in a Mikro-Dismembrator II (B. Braun, Melsungen AG, Braun Apparate, 3508 Melsungen, FRG). This treatment reduced the tissue sample to a fine powder which could then be removed from the chamber and transferred to a 15ml sterile tube (Falcon) containing 9ml of 4M guanidinium isothiocyanate, 0.5% (w/v) sodium N-lauryl sarcosine, 25mM sodium citrate pH7.0 and 0.1M 2-mercaptoethanol.

4ml gradients of caesium chloride comprising steps of 2ml each of 1.7gcm^{-3} and 1.6gcm^{-3} CsCl in 25mM sodium acetate were prepared in 14ml polyallomer tubes (Kontron). Samples in guanidinium isothiocyanate solution were then layered carefully on top and the tubes balanced with liquid paraffin. Centrifugation was carried out in a TFT41.14 6 x 14 titanium rotor in a Centrikon ultracentrifuge (Kontron) at 20^{o}C for sixteen hours at 136,000g.

DNA was harvested from the interface between the CsCl steps using a sterile pipette, transferred to a 15ml tube and precipitated in 3 volumes of 70% ethanol at 4^{o}C for one hour. Samples were spun at 4000g for thirty minutes at 4^{o}C in a benchtop centrifuge, the supernatant decanted, the pellet washed in 70% ethanol, recentrifuged, air dried and resuspended in 1ml TE buffer pH8.0. Any contaminating protein was removed by extraction with an equal volume of phenol-chloroform as previously described, the DNA precipitated, pelleted and washed as before. After air drying, purified DNA was resuspended in a small volume of TE pH8.0, the optical density (OD) measured at 260nm and 280nm, and the 260:280 ratio calculated which allows estimation of the purity of the DNA solution, as a pure solution is expected to have a 260:280 value of 1.8 to 2.0 (Maniatis *et al.*, 1982). Any samples with a ratio outside this range were re-extracted with phenol/chloroform until satisfactory values were obtained. Samples of genomic DNA were stored at 4^{o}C prior to analysis.

The RNA pellets remaining after centrifugation through CsCl were resuspended in 1ml 7.5M guanidinium hydrochloride, 25mM sodium citrate pH7.0, 5mM DTT to ensure removal of any contaminating nuclease activity and precipitated with 0.5 volumes of absolute ethanol and 0.025 volumes of 1M acetic acid at -20^{o}C overnight. RNA was recovered by centrifugation at 12,000g in an Eppendorf centrifuge for 15 minutes at 4^{o}C. Pellets were resuspended in RNase-free distilled water and the 260:280 ratio measured, as pure RNA is known to have a value of 2.0 (Maniatis *et al.*, 1982). Samples with a value lower than this were reprecipitated with 0.1 volumes of 3M sodium acetate and 3 volumes of absolute ethanol, and RNA recovered as before. Pelleted

RNAs were resuspended in RNase-free distilled water and stored at -20 ℃ until use.

3.2.2.2. Transformation of Host Bacteria with Plasmid DNA.

E. coli strain HB101 was used for the propagation of the recombinant HPV clones detailed in section 3.1.6. using the calcium chloride method of transformation (Mandel and Higa, 1970) as described by Maniatis *et al.* (1982). 1ml of LB broth was inoculated with a single bacterial colony and grown overnight at 37°C with shaking. The following day this was added to 100ml of LB broth prewarmed at 37°C and allowed to grow until the $O.D._{550}$ was 0.5, at which point the culture was chilled on ice and then centrifuged at 4000g for ten minutes at 4°C. The bacterial pellet was resuspended in 0.5 volumes of ice-cold 100mM $CaCl_2$, recentrifuged, resuspended in 0.25 volumes of ice-cold 50mM $CaCl_2$ and allowed to stand on ice for twenty minutes. Bacterial cells were centrifuged as before, resuspended in 0.05 volumes of 50mM $CaCl_2$, aliquoted in 0.5ml volumes into chilled tubes and stored overnight at 4°C to increase the efficiency of transformation (Dagert and Ehrlich, 1979). Cells were then either used or frozen in a dry ice and ethanol bath and stored at -70 °C until required.

Transformation reactions were performed by addition of between 1 and 40ng (routinely 10ng) of HPV recombinant plasmid DNA or pBR322 control DNA in TE buffer pH8.0 to 200μl of competent HB101 cells and chilled on ice for forty minutes. Cells were heat-

shocked for two minutes in a water bath at 42°C, 1.0ml of LB broth added (containing 50μgml^{-1} ampicillin) and incubated at 37°C with shaking for one hour. Cultures were then plated on LB agar containing ampicillin and incubated overnight at 37°C.

3.2.2.3. Small Scale Plasmid DNA Preparation.

Plasmid DNA was prepared basically as described by Maniatis *et al.* (1982) with some minor modifications. Single recombinant clones grown on selective media were inoculated into 5ml sterile plastic vials containing 3ml LB broth plus ampicillin and incubated at 37°C for four hours with shaking. 1.5 ml of culture was centrifuged at 12000g in an Eppendorf centrifuge for five minutes, the supernatant decanted and the bacterial pellet resuspended in 100μl of 50mM glucose, 10mM EDTA, 25mM Tris HCl pH8.0. To this was added 200μl of a freshly-prepared ice-cold solution of 0.2N NaOH, 1% SDS, mixed by inversion and stored on ice for five minutes. Following addition of 150μl of ice-cold potassium acetate pH4.8 the tube was vortexed, stored on ice for five minutes and centrifuged as before. The supernatant was extracted twice with an equal volume of phenol/chloroform and precipitated with two volumes of ethanol. DNA was recovered by centrifugation at 12000g for fifteen minutes at 4°C, the pellet washed in 70% ethanol and air-dried. Plasmid DNA was resuspended in 50μl TE pH8.0 containing 20μgml^{-1} DNase-free RNase, a 10μl aliquot removed and digested with one unit of an appropriate restriction endonuclease at 37°C for one hour to release the insert DNA from the vector. The products were then analysed by agarose gel electrophoresis.

3.2.2.4. Large Scale Plasmid DNA Preparation.

A single bacterial colony containing the appropriate recombinant plasmid was inoculated into 10ml LB broth with ampicillin in a sterile universal container and grown overnight at 37^{o}C with shaking. The culture was then transferred to a flask containing 500ml of the same culture medium and propagated for a further 16-24 hours at 37^{o}C.

DNA was prepared by the method of Birnboim and Doly (1979), modified as described by Maniatis *et al.* (1982). Large scale cultures were centrifuged at 3000g for ten minutes, the supernatant discarded and the bacterial pellet resuspended in 10ml of 50mM glucose, 25mM Tris HCl pH8.0, 10mM EDTA, 5mgml^{-1} (w/v) lysozyme and incubated at room temperature for five minutes. Two volumes of 0.2N NaOH, 1% SDS were added, mixed gently by inversion, incubated at 0^{o}C for ten minutes, 1.5 volumes of 5M potassium acetate added, incubated for a further ten minutes and the bacterial debris pelleted by centrifugation at 10000g for twenty minutes at 4^{o}C. Plasmid DNA was precipitated for fifteen minutes at room temperature by addition of 0.6 volumes of isopropanol to the supernatant and recovered by centrifugation at 12000g for thirty minutes. The pellet was washed in 70% ethanol, dried under vacuum, resuspended in TE pH8.0 and solid caesium chloride added to a final density of 1.06gml^{-1}. 0.42ml of a 10mgml^{-1} stock of ethidium bromide was added to the solution and centrifugation was carried out at 160000g for forty hours at 20^{o}C. Supercoiled plasmid DNA was harvested from caesium chloride gradients and the ethidium bromide removed by two extractions with

water-saturated butanol. DNA was ethanol-precipitated, centrifuged, resuspended in TE pH8.0 and any contaminating protein removed by three phenol-chloroform extractions. DNA was then precipitated, the pellet washed with 70% ethanol, air-dried, resuspended in TE pH8.0 and the recovery quantified by spectrophotometry as previously described. Plasmid DNA was stored at $4^{o}C$ prior to use.

3.2.2.5. Restriction Enzyme Digestion of DNA.

DNA was digested in sterile Eppendorf tubes by addition on ice of 0.1 of the final volume of 10x digestion buffer as recommended by the supplier, $1U\mu g^{-1}$ of the appropriate enzyme and the volume made up with sterile distilled water. Samples were mixed by vortexing, spun briefly in a microfuge and incubated at $37^{o}C$ or as described in the manufacturer's data sheet. Routinely, plasmid DNAs were digested for one hour, while cellular DNAs were incubated overnight. Samples were then analysed by electrophoresis in agarose gels.

3.2.2.6. Agarose Gel Electrophoresis.

Electrophoresis of DNA in agarose was performed as described by Maniatis *et al.* (1982), using TAE or TBE buffers containing $0.5\mu gml^{-1}$ ethidium bromide. The concentration of agarose in the gel was dependent on the size of the DNA to be analysed, routinely 0.8-1% (w/v) for restriction enzyme-digested genomic DNAs. One fifth volume of 6x electrophoresis buffer consisting of 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% ficoll 400, was added to samples,

mixed by vortexing, spun briefly and loaded onto the gel. Molecular weight markers of known size were HindIII-digested bacteriophage lambda DNA and or bacteriophage øx174 RF DNA digested with HaeIII, and were co-electrophoresed with sample DNAs. Gels were run at 3-4Vcm^{-1} for 12-16 hours, examined on a UV transilluminator and photographed using Polaroid Type 57 or Type 55 film.

3.2.2.7. Polyacrylamide Gel Electrophoresis.

Electrophoresis in polyacrylamide was used primarily for analysis of polymerase chain reaction (PCR) products. Non-denaturing gels consisted of 6% acrylamide, 0.5% bis in 1x TBE buffer, polymerisation being effected by addition of 50μl of TEMED and 0.3ml of 10% ammonium persulphate per 50ml volume of solution. Polymerisation was allowed to occur at room temperature. Denaturing gels were made as described above, but with 8M urea added and dissolved by warming to 37^{o}C prior to addition of TEMED and ammonium persulphate. Sample buffer was added as previously described and electrophoresis carried out at 10 Vcm^{-1} for 1.5 hours. Gels were stained for 10 minutes in running buffer containing 0.5 μgml^{-1} ethidium bromide and examined as detailed above.

3.2.2.8. Purification of DNA by Electroelution.

DNA fragments electrophoresed in TBE-agarose were excised from the gel and transferred to individual dialysis bags prepared as

described by Maniatis *et al.* (1982). 3ml of 0.1x TBE buffer was added, the bag sealed and the DNA eluted at 200V for 1 hour. The bag was removed from the electrophoresis tank, examined by UV transillumination to ensure that all of the sample had been eluted from the gel slice, and then returned to the tank and the polarity reversed briefly to remove any DNA adherent to the side of the dialysis bag. The solution was removed to a 15ml tube, the bag washed with a further 3ml of buffer and pooled with the original sample. Solid NaCl was added to the eluate to a final concentration of 0.2M and purified by chromatography on Elutip-d columns (Anderman and Company Ltd., Kingston Upon Thames). Columns were hydrated in 2ml of high salt buffer (1.0M NaCl, 20mM Tris HCl, 1mM EDTA pH7.5), then equilibrated in 4ml of low salt buffer (0.2M NaCl, 20mM Tris HCl, 1mM EDTA pH7.5). The sample was loaded onto the column, allowing the DNA to bind, loaded on a second time and then washed with 10ml low salt buffer. DNA was eluted in 1ml of high salt buffer, precipitated with 2 volumes of ethanol at -20^{o}C, recovered by centrifugation at 12000g and the pellet washed with 70% ethanol. DNA was resuspended in TE pH8.0 and quantified spectrophotometrically.

Alternatively electroelution was carried out with the aid of an LKB electroelutor (Pharmacia LKB), used in accordance with the manufacturer's instructions. Gel slices immersed in 0.1x TBE were subjected to a constant voltage of 80V for 45 minutes, the DNA being eluted into a v-shaped well containing 400μl of 7.5M ammonium acetate with 0.01 volumes of 6x electrophoresis sample buffer to aid collection. The eluate was removed by pipette, a fresh aliquot of collection buffer added to the well and elution carried out as before.

DNA was recovered by ethanol precipitation, washed and resuspended as above.

3.2.2.9. DNA Purification on a Silica Matrix.

DNA fragments separated in TAE-agarose were excised from the gel, weighed, transferred to either a 1.5ml Eppendorf tube or a 15ml Falcon tube, and cut into smaller pieces. Purification from agarose was achieved using a Geneclean kit (Bio 101, La Jolla, CA) following the manufacturer's instructions. 2.5 volumes of a 6M stock solution of NaI were added to the gel slice, mixed by vortexing and placed in a water bath at 55^{o}C for 3 minutes, removed to mix and then replaced for a further two minutes. "Glassmilk" (Vogelstein and Gillespie, 1979) was added to the solution as suggested in the supplied protocol, mixed by vortexing and incubated on ice for 30 minutes, with further mixing at 10 minute intervals. DNA bound to glassmilk was pelleted, the supernatant removed, and the pellet resuspended in 700μl of NEW wash (NaCl, Tris pH7.0-8.5, 50% ethanol) and re-centrifuged. This wash procedure was repeated twice, the pellet resuspended in TE pH8.0 and DNA eluted at 55^{o}C for 5 minutes. The glassmilk was pelleted and the supernatant containing the eluted DNA retained. Two further elution steps were performed to ensure maximum recovery of DNA. DNA was quantified by spectrophotometric analysis, after which the sample was ready for use. Typical recovery was 40-70% of the starting amount.

3.2.2.10. Southern Transfer.

DNA samples in agarose gels were immobilised on nylon membranes by the method of Southern (1975) with several alterations. Following visualisation and photography, gels were soaked for 10 minutes in 0.25M HCl, rinsed in distilled water and transferred to Hybond N+ membrane (Amersham) by capillary action using a procedure similar to that described by Maniatis *et al.* (1982), but with 0.4M NaOH as the transfer buffer. Transfer was allowed to procede for a maximum of four hours, after which the membrane was rinsed in 2x SSPE for 5 minutes and then prehybridised or wrapped in Saran-Wrap and stored at $4^{o}C$. The gel was restained with ethidium bromide and examined to ensure that all of the samples had been efficiently transferred.

3.2.2.11. Preparation of Radiolabelled DNA Probes.

Cloned HPV genomes obtained from large scale plasmid preparations as previously described were released from the cloning vector by digestion with the appropriate restriction endonuclease. Viral DNA was purified free from plasmid sequences by electrophoresis through 1% agarose. The HPV DNA was recovered either by the electroelution or glass-bead purification methods detailed above.

HPV DNA was labelled by the random primer method of Feinberg and Vogelstein (1983) using a commercially available kit (BCL). Twenty five nanogrammes of linearised viral DNA was denatured at $95^{o}C$ for 10 minutes and cooled rapidly on ice to prevent reannealing of the complementary strands. To this was added dATP, dGTP and

dTTP to a final concentration of 25μM each, 0.1 volume of a 10x mixture of random hexanucleotides, 50μCi of α-[^{32}P]-dCTP, distilled water to 19μl and 1 unit of Klenow DNA polymerase. The solution was mixed by vortexing, spun briefly and incubated at 37^{o}C for 30-60 minutes. The labelling reaction was terminated by addition of 0.1 volume of 0.2mM EDTA pH8.0.

Alternatively, probes were labelled by nick-translation (Rigby *et al.*, 1977). To 1μg of HPV DNA in an Eppendorf tube was added dATP, dGTP and dTTP to give a final concentration of 20mM each deoxyribonucleotide, 50μCi of α-[^{32}P]-dCTP, 0.1 volume of a 10x nick translation buffer solution (0.5M Tris-HCl pH7.5, 0.1M $MgSO_4$, 1mM DTT, 0.5mgml^{-1} BSA) and distilled water to 48μl. Finally 1μl of a 10ngml^{-1} solution of DNase I and 1 unit of *E. coli* DNA polymerase I were added, mixed, centrifuged and incubated at 14^{o}C for 1.5 hours. One tenth volume of 0.2M EDTA was added to stop the reaction.

Oligonucleotides to be used as probes were end-labelled with γ-[^{32}P]-dATP by the following protocol. 10ng of oligonucleotide was incubated with 50μCi of γ-[^{32}P]-dATP and 1 unit of bacteriophage T4 polynucleotide kinase in 50mM Tris-HCl pH7.6, 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA at 37^{o}C for 40 minutes. The labelled reaction product was purified as described below.

3.2.2.12. Purification of Radiolabelled Probes.

DNA fragments labelled by nick translation or random priming were purified by Elutip-d column chromatography, as described above.

The DNA was eluted into a 1.5ml Eppendorf tube in 1ml of high salt buffer. 1μl of eluate was removed and counted in a liquid scintillation counter to verify that incorporation of radiolabel had occurred and to estimate the specific activity of the probe. Random primer labelling routinely resulted in specific activities between 1-1.5 x 10^9 cpmμg^{-1} of DNA, whereas lower activities were achieved using the nick translation method (around 1 x 10^7 - 1 x 10^8 cpmμg^{-1}).

End-labelled oligonucleotides were purified by chromatography through commercially available columns (Nick^{TM} columns, Pharmacia) containing Sephadex G-50. The column was equilibrated in 10mM Tris HCl pH7.5, 1mM EDTA. The sample was added in 100μl of buffer, washed on with 400μl and eluted into an Eppendorf tube with a further 400μl.

3.2.2.13. Prehybridisation of Southern Blots.

Filters were placed in a plastic bag to which 20ml of prehybridisation buffer (5 x SSPE, 0.5% SDS, 5mgml^{-1} dried milk, 50μgml^{-1} denatured calf thymus DNA) was added, and the bag sealed using a domestic heat-sealer. Prehybridisation was carried out for a minimum of 1 hour in a shaking water bath at the same temperature as was to be used for the subsequent hybridisation reaction. The prehybridisation solution was removed prior to addition of hybridisation buffer.

3.2.2.14. Hybridisation of Southern Filters.

Hybridisation solution was essentially the same as prehybridisation buffer, but contained radiolabelled probe DNA denatured by heating at 100°C for 5 minutes and cooled on ice. For initial screening of tumour samples for HPV sequences, hybridisation was carried out under conditions of low stringency at T_m-36°C, where T_m is the melting temperature of the DNA-DNA hybrid, calculated by the formula:

$$T_m = 81.5^{\circ}C - 16.6(\log_{10}[Na^+]) + 0.41(\%G+C) - 0.63(\%\text{formamide}) - (600/l)$$

assuming an average G+C content of 40% for HPV genomes and where *l* is the length of the hybrid formed in base pairs (Sambrook *et al.*, 1989). Subsequent hybridisations were performed under conditions of high stringency, at a temperature of T_m-10°C. Hybridisation was allowed to proceed for 16-20 hours in a shaking water bath, after which the hybridisation solution was removed and stored at 4°C for further use if required.

3.2.2.15. Washing of Southern Blots.

Filters were washed in 2x SSC, 0.1% SDS for 45 minutes with three changes in a shaking water bath at the hybridisation temperature. An additional wash was performed in 0.1x SSC, 0.1% SDS for 15 minutes if a higher level of stringency was desired. Filters were partially air-dried on 3MM paper, wrapped in Saran-Wrap and exposed to Kodak X-Omat S or X-AR5 film in cassettes with intensifying screens for 10 minutes to 2 weeks at -80 °C.

3.2.2.16. Removal of Probe DNA From Southern Blots.

Following autoradiography, DNA probes were removed by immersion of filters in 0.1x SSC, 0.1% SDS at 95^{o}C and the solution allowed to cool to room temperature. To ensure adequate stripping, filters were then re-exposed to X-AR5 film overnight, wrapped in Saran-Wrap and stored until required.

3.2.3. Molecular Cloning.

3.2.3.1. Construction of Genomic DNA Libraries.

Thirty micrograms of tumour DNA was digested with the restriction enzyme BamHI, a known single-cutter enzyme for prototype HPV-4, as described above. The sample was divided 2:1 and electrophoresed in 0.8% TBE-agarose alongside a control of cloned purified linear HPV-4. The gel was stained and photographed as before, and the lanes containing the lesser amount of genomic DNA and the control were Southern blotted and hybridised at high stringency (T_m-10^{o}C) to a radiolabelled HPV-4 DNA probe. The gel slice containing the 20μg sample of tumour DNA was covered with Saran-Wrap and stored at 4^{o}C prior to excision of the area corresponding to the positive band on the Southern blot. In practice, a size-cut was selected to include DNA fragments in the 6-10kb range. DNA was purified free from agarose by electroelution into a dialysis

bag, passed through an Elutip-d column, recovered by ethanol precipitation and resuspended in TE pH8.0.

Purified size-selected DNA was ligated into the BamHI cloning site of the commercially-available bacteriophage vector EMBL3 (Stratagene). An equal molar ratio of insert to vector DNA was used, calculated as 0.2μg genomic digest to 1μg EMBL3 arms, assuming an 8kb average size for the insert and a vector size of 40kb. Insert and vector DNAs were heated to 42^{o}C and allowed to anneal for 1 hour. Ligation was carried out for 48 hours at 4^{o}C in 50mM Tris HCl pH8.0, 7mM $MgCl_2$, 1mM dithiothreitol, 1mM ATP with 2 units of T4 DNA ligase in a total volume of 5.0μl.

The ligation reaction products were packaged using Gigapack II Gold packaging extracts (Stratagene), using one extract per microlitre of ligation. Packaging was carried out for 2 hours at room temperature according to the instructions supplied. Packaged bacteriophage particles were diluted in 0.5ml SM buffer, and debris removed by addition of 20μl chloroform followed by brief centrifugation. The library was titred by adsorption and plating on host bacterial strains LE392 and P2392 (the latter being selective for recombinant bacteriophages), and plaques were counted after incubation of plates at 37^{o}C for 8 hours. Titres ranging from 5 x 10^4 to 5 x 10^5 pfuμg^{-1} of test DNA were obtained for libraries prepared in this manner.

Screening of genomic libraries was carried out by the plaque lift hybridisation procedure described by Grunstein and Hogness (1975) for identification of recombinant clones. Recombinant bacteriophages were selected by plating on P2392 host cells on 22cm^2 NZY agar

plates, incubated as described above. Plates were chilled at 4°C for several hours prior to lifting onto Pall Biodyne A nylon membrane. Filters were denatured in 1.5M NaCl, 0.5M NaOH for five minutes, neutralised in 3.0M sodium acetate pH5.5 for five minutes, air dried and baked at 80°C in a vacuum oven for 1 hour. Prehybridisation and hybridisation were carried out as previously described for Southern blots, washed and exposed to radiographic film for 30 minutes to 5 days.

Positive plaques were picked from the original plate using a sterile Pasteur pipette, transferred to an Eppendorf tube containing 0.5ml of SM buffer and allowed to stand at room temperature for 2 hours after addition of 1μl of chloroform to facilitate release of bacteriophage particles. Isolates were purified, usually through 3-4 additional rounds of screening, until free from contaminant clones.

Bacteriophage DNA was prepared according to Chisholm (1989). Ten to 100μl of eluted bacteriophages were preadsorbed on P2392 host bacteria and grown in 37ml of NZCYM for 12 to 15 hours with gentle shaking, until complete lysis had occurred. Culture were transferred to 50ml Falcon tubes, 100μl chloroform added and mixed gently. Bacterial nucleic acids were removed by addition of 0.37ml of a nuclease solution (50mg DNase I, 50mg RNase A in 10ml of 50% glycerol, 30mM sodium acetate pH6.8) at 37°C for 30 minutes. NaCl (2.1g) was added, mixed gently and the bacterial debris removed by centrifugation at 10000g for 20 minutes at 4°C. Bacteriophage particles were precipitated from the resulting supernatant with 3.7g of polyethylene glycol (PEG) on ice for 1 hour and recovered by centrifugation as above. Bacteriophages were resuspended gently in

0.5ml of SM buffer for 30 to 60 minutes and extracted with an equal volume of chloroform.

Bacteriophage DNA was prepared by incubation of the bacteriophage suspension obtained above with 20μl of 0.5M EDTA, 5μl of 20% SDS and 10μl of 5mgml^{-1} proteinase K at 65^{o}C for 30 minutes, followed by three phenol/chloroform extractions. DNA was precipitated with 170μl of 6M ammonium acetate and 0.7ml of isopropanol at room temperature and recovered by centrifugation in an Eppendorf centrifuge for 15 minutes at 4^{o}C. The pellet was washed twice in 70% ethanol, vacuum-dried and gently resuspended in TE pH8.0. An aliquot of DNA was digested with the appropriate restriction enzyme to release the insert and analysed by electrophoresis in 0.7% agarose. The gel was transferred to Hybond N+ membrane and hybridised to a radiolabelled DNA probe to confirm the presence of the desired fragment.

3.2.3.2. Subcloning of DNA Into Plasmid Vectors.

DNA cloned in bacteriophage was excised from the vector and purified free of vector sequences by gel electrophoresis and electroelution as previously described. The purified fragment was then ligated into an appropriate cloning site of the plasmid vector pBR322. The ligation reaction was carried out overnight at 15^{o}C in 50mM Tris HCl pH7.5, 7mM $MgCl_2$, 1mM DTT, 1mM ATP and 1 unit of T4 DNA ligase with a threefold molar excess of insert to vector. The contents of the reaction were diluted fivefold in TE pH7.5 and used to transform library efficiency DH5-α competent cells (Gibco BRL)

using the supplier's modification of the procedure of Hanahan (1983). Transformed cells were grown in 1ml SOC at 37°C for 1 hour, plated on LB agar plates containing ampicillin and incubated overnight at 37°C.

Colonies were picked, grown in 5ml of LB broth with ampicillin for 4 hours to overnight, and plasmid DNA prepared as described above. The insert DNA was released from the plasmid vector by restriction endonuclease digestion and analysed by gel electrophoresis followed by Southern blotting and hybridisation, together with appropriate controls. Large scale production of the desired plasmid was then carried out.

3.2.4. Sequencing of Double-stranded DNA Templates.

Nucleotide sequencing of double-stranded DNA was carried out by the dideoxy chain termination procedure of Sanger *et al.* (1977) using a commercially available kit (Sequenase version 2.0, United States Biochemical Corporation, Cleveland, Ohio, USA).

Sequencing templates were prepared as follows. Plasmid DNA (4μg) in a total volume of 18μl was denatured for 5 minutes at room temperature by addition of 2μl of 2M NaOH. DNA was precipitated with 0.4 volumes of 5M ammonium acetate pH7.5 and 5 volumes of absolute ethanol on a dry ice-ethanol bath for 5 minutes, and recovered by centrifugation at 12000g for 15 minutes at 4°C. The pellet was washed with 70% ethanol, the supernatant removed and the

denatured template dried in a vacuum dessicator. DNA was either used immediately in a sequencing reaction or stored dry at -70°C.

Template DNA was resuspended in 7μl of distilled water, 2μl of 5x sequencing buffer (200mM Tris HCl pH7.5, 100mM $MgCl_2$, 250mM NaCl) and 1μl (0.5pmol) oligonucleotide sequencing primer added, and the primer and template annealed at 37°C for 20 minutes. To the template-primer mix was added, on ice, 1μl 0.1M DTT, 2μl of a 1:20 dilution of 5x labelling mix (7.5mM each of dGTP, dCTP and dTTP), 0.5μl of α-[^{35}S]-dATP (1000Cimmol^{-1}) and 2μl of a 1:8 dilution of Sequenase v.2.0 enzyme in TE pH8.0. The sample was mixed by pipetting and incubated at room temperature for 3 minutes before termination by addition of equal volumes (3.5μl) of labelling reaction to each of four tubes containing 2.5μl of A, G, C or T termination mixes (80μM of each dNTP, 50mM NaCl and 8μM of one dideoxynucleotide) preheated to 37°C. Termination was carried out for 2 minutes at 37°C and stopped by addition of 4μl of stop solution which comprised 95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol.

Sequencing reaction products were denatured at 80°C for 2 minutes and loaded immediately onto a 6% polyacrylamide, 8M urea denaturing gel which had been pre-run for 30 minutes. Samples were electrophoresed at 1400V for 1 to 6 hours, depending on the distance to be read from the primer sequence. Following electrophoresis, sequencing gels were fixed in 10% acetic acid, 12% methanol for 20 minutes and dried under vacuum onto 3MM paper at 80°C for 1 to 2 hours. Autoradiography was carried out at room temperature from overnight to several days.

3.2.5. Polymerase Chain Reaction.

3.2.5.1. Precautions.

Due to the high sensitivity of the PCR technique, several general precautions were taken to avoid contamination of reagents and samples. As far as was practically possible, the suggestions of Kwok and Higuchi (1989) were followed. Disposable gloves were worn when dealing with samples or reagents. In addition, a disposable mask and cap were worn. PCR products were analysed on a separate bench from that used to set up reactions. Positive displacement pipettes, dedicated for PCR work, were used which avoided the production of aerosols, and sample and reagent tubes were briefly centrifuged prior to careful opening to ensure no splashing occurred. Mixes of reagents were made, sufficient for the number of reactions being undertaken, to ensure consistency between samples and controls. Reaction mixes were aliquoted into tubes, with target DNA being added last (except for the enzyme) to reduce the possibility of accidental transfer between samples, and a new capillary and piston assembly was used for each pipetting action.

Positive and negative controls were included in each set of reactions performed. The use of cloned DNA as a positive control was avoided, where possible, to minimise the risk of contamination, with HPV-containing genomic DNA samples being preferred. Where these were not available 1 to 10pg of HPV plasmid was used, but set up in isolation from the remaining samples. A known negative control such as commercially available calf thymus DNA or salmon sperm DNA was included, as was a control without template DNA in order

to exclude possible contamination of PCR reagents with potential target sequences.

3.2.5.2. Reaction Conditions.

Polymerase chain reaction experiments were performed using GeneAmp kits (Cetus Corporation, Emeryville, CA, USA) or a similar set of reagents. Reactions were carried out in final volumes of 50μl or 100μl in 10mM Tris HCl pH8.3, 50mM KCl, 1.5mM $MgCl_2$, 2.5 units of Taq polymerase (Cetus or BRL), with 0.5μg of each oligonucleotide primer and 1μg of genomic DNA. Sample buffer minus DNA and enzyme was aliquoted into 0.5ml Eppendorf tubes and overlayed with 0.5 volumes of light paraffin oil to prevent evaporation during the reaction procedure. Target DNA was then added, mixed by vortexing, centrifuged briefly and denatured in a water bath for 10 minutes at 95^{o}C. Samples were re-centrifuged, placed on ice and Taq polymerase added. Tubes were again mixed, spun briefly and placed in a thermal cycler (Cetus Corporation) pre-programmed to perform a specified number of cycles (usually 30 to 40) of denaturation, annealing of primers and template, and extension. Denaturation was routinely carried out at 94^{o}C for 1 minute. The annealing step also lasted for 1 minute, but the temperature was dependent on the primers being used and was determined empirically for each primer pair to allow the minimum of non-specific binding, and was usually in the range of 50-60^{o}C. Extension of newly primed DNA strands occurred at 72^{o}C, the duration of this step being dependent on the length of the DNA being synthesised. For short (50-400bp) PCR products an extension time of 1 minute was used. Extension for

products of intermediate size (up to 2.5kb) was 2.5 minutes, whereas longer syntheses required 5 minute extension times. All PCR protocols were concluded with annealing (1 minute) without denaturation and a final extension step of 10 minutes' duration to complete all strands being synthesised. Completed reactions were stored at $4^{o}C$ prior to analysis.

3.2.5.3. Analysis of PCR Products.

Routinely one tenth of the total reaction product was analysed by electrophoresis in 6% polyacrylamide under non-denaturing conditions, as described above. Occasionally denaturing gels were used which contained, additionally, 8M urea. Electrophoresis was carried out at 4-5Vcm^{-1} for 1 to 2 hours, depending on the anticipated size of the PCR product. In cases where the expected fragment size was 1.0kb or greater, 1% to 2% agarose gels were used to improve resolution. Products were visualised on a UV transilluminator following staining with ethidium bromide (0.5μgml^{-1} in TBE), and photographed.

Polyacrylamide gels were blotted onto Hybond N+ membrane as previously described, although transfer times were halved (2 hours) in view of the small DNA fragment size. Filters were treated as before and hybridised to γ-[^{32}P] endlabelled oligonucleotide probes internal to the amplified fragment overnight in 6x SSC, 0.1% SDS at T_m-10 ^{o} C, washed for 30 minutes at room temperature in 2x SSC, 0.1% SDS with two changes followed by 5 minutes at $50^{o}C$. Autoradiography was

carried out for 20 minutes to 2 days as previously described for ^{32}P-labelled probes.

3.2.5.4. Sequencing of Polymerase Chain Reaction Products.

PCR products were sequenced using the Sequenase method described above, with a few modifications to the protocol. DNA fragments were purified from polyacrylamide gel slices by elution into 100μl to 200μl of 0.5M NH_4Cl, 10mM EDTA overnight at 37^{o}C. Supernatant was removed to another tube and the eluted DNA precipitated with 0.1 volume of 7.5M ammonium acetate and 2 volumes of absolute ethanol at -20^{o}C for 1 hour. DNA was pelleted at 12000g for 10 minutes in a microfuge, washed once with 70% ethanol and dried under vacuum.

Purified DNA was resuspended in 7μl of distilled water, sequencing buffer and primer added as before, and 0.1 volume of DMSO included to counteract reannealing of complementary strands of template DNA following denaturation (Winship, 1989). DNA was denatured by heating at 100^{o}C for 5 minutes and cooled rapidly on a dry-ice-ethanol bath for 5 minutes. To the thawed primer-template mix sequencing reagents were added as before, with additional DMSO (0.1 volume), and the labelling reaction performed on the bench for 3 minutes. Termination was carried out as described above, again in the presence of 10% DMSO. Denaturation, electrophoresis and autoradiography were as previously described.

3.2.6. Culture of Keratinocytes from Oral Squamous Cell Carcinomas.

3.2.6.1. Preparation of Feeder Cells.

Swiss 3T3 cells (Todaro and Green, 1963) were grown in DMEM supplemented with 10% (v/v) bovine serum in an atmosphere of 10% CO_2/90% air at 37^{o}C. Cells were subcultured by exposure to 0.1% trypsin for 5 minutes and harvested in growth medium, after which they were replated at 1 x 10^4 cells per 9cm dish. The remainder was exposed to 6000 rads of γ-irradiation from a ^{60}Co source. Irradiated feeder cells were plated at a density of 5 x 10^5 cells per 9cm dish and incubated in DMEM with 2% FBS and 0.4μgml^{-1} hydrocortisone for 24 hours prior to seeding of keratinocytes.

3.2.6.2. Preparation of Cells from Carcinoma Biopsies.

Biopsy specimens of approximately 5mm^3, obtained at the time of resection, were transferred to sterile universals containing 10ml of DMEM supplemented with 2% serum, penicillin (50Uml^{-1}), streptomycin (50μgml^{-1}), kanamycin (50μgml^{-1}) and amphotericin B (2.5μgml^{-1}). Samples were stored at 4^{o}C until ready for use, up to seven days later. No adverse effect of storage was noted on the ability of samples to establish a primary culture.

Tissues thus obtained were washed twice in fresh culture medium supplemented as above, transferred to a culture dish and dissected into fragments of approximately $1mm^3$. These were transferred to a sterile universal container, washed twice in culture medium and then plated onto a feeder layer of one-third confluent lethally-irradiated Swiss 3T3 murine fibroblasts, known to suppress the proliferation of dermal fibroblasts (Rheinwald and Beckett, 1981).

Alternatively, the dissected tissue was incubated in culture medium supplemented with 2% FBS, antibiotics as described above, and $200Uml^{-1}$ collagenase type VII (Sigma) at 37^oC for one to five days, as described by Freshney (1987). The supernatant containing collagenase and fibroblasts was aspirated, and the epithelial cell clusters washed by resuspension and settling. Keratinocytes were then plated onto irradiated feeder cells as described above, in DMEM supplemented with 2% FBS and $0.4\mu gml^{-1}$ hydrocortisone (Rheinwald and Green, 1975; Rheinwald and Beckett, 1981) in order to inhibit growth of normal keratinocytes.

3.2.6.3. Subculture of Keratinocytes.

Cultures grown on feeder layers were incubated briefly (30 seconds) in a solution of 0.02% EDTA at 37^oC followed by vigorous pipetting of PBS against the dish to remove the feeder cells. Keratinocytes were harvested by incubation with equal volumes of 0.1% trypsin and 0.02% EDTA at 37^oC for 10 minutes. Cell counting was performed using a haemocytometer and replated (5×10^4 cells per 9cm dish) onto a fresh batch of feeder cells.

To ensure that a stock of cells was available, cells were cryopreserved at each passage by addition of 10% (v/v) DMSO to 1 x 10^6 cells in a sterile plastic ampoule on ice. In order to ensure good viability, cells were frozen slowly, wrapped in cotton wool, first at -20 ^oC for 30 minutes and then at -70^oC overnight. The following day ampoules were placed in liquid nitrogen for permanent storage.

3.2.6.4. Immunocytochemistry.

Cultured keratinocytes were stained with an anti-keratin antibody, to prove that they were truly of epithelial origin. Cells were grown on feeder layers on 13mm round glass coverslips for several days, under the conditions described above, and fixed in 50:50 acetone and methanol (Lane, 1982) for 10 minutes at room temperature. SCC25 oral carcinoma cells (Rheinwald and Beckett, 1981) and Swiss 3T3 murine fibroblasts were used as positive and negative controls, respectively. Coverslips were incubated with normal goat serum from a Vectastain anti-mouse IgG kit (Vector Laboratories), washed three times in PBS and then incubated overnight at 4^oC with a mouse antikeratin monoclonal antibody (LP34, a kind gift from Dr. E.B. Lane). The antibody solution was removed by washing three times in PBS for 10 minutes and the second antibody, an anti-mouse IgG antibody conjugated to alkaline phosphatase (Vector) was added for one hour at room temperature. After removal of the second antibody with PBS as before, the alkaline phosphatase substrate was added and the coverslips were incubated in the dark for 25 minutes at room temperature. Final washing was carried out in PBS and the coverslips

mounted using an aqueous mounting medium. The cells were examined using a light microscope.

Alternatively, cells were treated as described above, but a FITC-conjugated second antibody was substituted for the alkaline phosphatase-linked IgG second antibody, and allowed to bind in the dark at 37^{o}C. In this case, fluorescence microscopy was used to visualise the cells.

3.2.6.5. Preparation of Nucleic Acids from Keratinocyte Cultures.

Cells were trypsinised as described above, transferred to a universal container, pelleted, resuspended and washed twice in PBS. The cell pellet was resuspended in 9ml of guanidinium isothiocyanate solution (section 3.2.2.2.) and centrifuged through caesium chloride gradients as for tissue biopsy material.

3.2.6.6. *In Vivo* Tumorigenicity Studies.

Trypsinised oral keratinocytes were washed twice in PBS and resuspended at concentrations of 4 x 10^6 or 4 x 10^7 cellsml^{-1}. Cell suspensions were injected subcutaneously into athymic mice at levels of 1 x 10^6 or 1 x 10^7 cells per animal. On the first occasion the lower number of cells was used. The experiment was repeated using a tenfold greater cell number in the event of no tumour formation. Positive and negative controls of SCC25 and Swiss 3T3 cells were

used. Tumorigenicity experiments were performed in quadruplicate. Tumour development was allowed to occur for twelve weeks, or until the lesion was 1cm in diameter, at which time the animals were sacrificed.

3.2.7. Computer Analysis of DNA Sequence Data.

Nucleotide sequences of PCR products or cloned DNAs were analysed on an Opus PCV Turbo microcomputer running the VAX/VMS operating system and the University of Wisconsin Genetics Computer Group (UWGCG) sequence analysis software package (Devereux *et al.*, 1984). Sequences were entered using SEQED. Homology studies were performed by means of the programmes BESTFIT and GAP. Screening of sequences against those already present in the EMBL and Genbank databases was carried out using WORDSEARCH. Nucleotide sequences were translated into peptide sequences using TRANSLATE and compared using BESTFIT.

4. Results.

4.1. Southern Blot Analysis of Human DNA Samples.

Genomic DNA was prepared from tissue samples collected from thirty nine patients undergoing major head and neck surgery for the treatment of oral squamous cell carcinoma. Samples included both malignant and normal oral mucosa; adjacent clinically dysplastic tissue and resected lymph nodes were also obtained, where present. Additionally, DNA was prepared from samples of normal buccal mucosa from twenty five consenting volunteers who were cancer-free.

Southern blots of 10μg aliquots of each DNA sample digested with PstI or HpaII were probed sequentially with DNA probes of cloned HPV types 1-7,11,13,16 and 18, together with appropriate HPV controls. Initial hybridisation was carried out under conditions of low stringency to facilitate detection of non-homologous HPV types. Subsequently blots were hybridised and washed at high stringency to positively identify any HPV types present in cellular DNA samples.

Using this strategy, three out of thirty nine samples from oral cancer patients were shown to harbour HPV sequences. One carcinoma specimen (C1) was positive for HPV-18 DNA, another (C2) for HPV-16 DNA and the third (C4) contained HPV-4 sequences, as shown in Figure 4.1. No viral sequences were detected in samples of DNA prepared from dysplastic or normal epithelia adjacent to these three cancers, or in the twenty five external control samples of normal buccal mucosa by this method.

Sample C1 contained HPV-18 DNA, as judged by hybridisation to a homologous DNA probe at high stringency. The PstI digestion pattern (Figure 4.1, lane C1a) revealed an additional restriction site compared to prototype HPV-18 DNA, with the expected 6.33kb fragment being cleaved into two smaller fragments. An extra HpaII site was also present (lane C1b). Polymorphism of restriction sites of HPV-18 has been reported previously in a cervical carcinoma biopsy from the west of Scotland (Millan *et al.*, 1986), perhaps reflecting a geographical variation of this viral type.

In sample C2 (Figure 4.1, lanes C2a and b) HPV-16 sequences were detected. The PstI restriction pattern (lane C2a) is atypical, as the 1.55kb PstI "C" fragment of prototype HPV-16 DNA, which maps to the L2-L1 region of the viral genome, is absent. The HpaII digestion pattern of the HPV-16 DNA in C2 was also variant: the expected 2.6kb fragment was absent, but an additional band of approximately 0.9kb was visible. The 2.6kb HpaII and 1.55kb PstI fragments overlap (Figure 4.2) and, as viral DNA was episomal rather than integrated, these data point to an alteration in the late region of the HPV-16 genome. This has been investigated further (section 4.5).

HPV-4 DNA was found in cancer sample C4 (Figure 4.1, lanes C4a and b). No variation from the wild-type HPV-4 restriction patterns was noted for either enzyme. The presence of HPV-4 DNA in this sample was the first association of this viral type with a malignant lesion, although it has previously been reported in one lesion of oral verruca vulgaris by *in situ* hybridisation (Adler-Storthz *et al.*, 1986a).

In all three cases viral DNA was present as episomes with no evidence of integration (Figure 4.3), although integration of a single copy into the cellular DNA can not be ruled out. Multiple copies of each HPV type were present in the cancer DNAs, as judged by the relative intensities of the viral sequences compared to the 10ng of cloned DNA controls run in parallel. Assuming that one viral genome per cell is represented by 26pg of viral DNA in 10μg of cellular DNA, the controls in Figure 4.1 represent 384 genomes of HPV DNA per cell. Therefore approximately 50 copies of HPV-18 were detected in C1, 200 copies of HPV-16 in C2 and 50 copies of HPV-4 in C4. The controls were viral genomes linearised at the EcoRI site (HPV-18) or the BamHI site (HPV-4 and HPV-16) and digested with PstI or HpaII, thus in each case additional bands are visible than would be expected on digestion of the normally circular viral DNA; the 1.55kb PstI band of HPV-16 is cleaved to produce bands of 0.91kb and 0.64kb, the 6.33kb HpaII band of HPV-18 is reduced to two fragments of 4.0kb and 3.33kb, and the 0.35kb HpaII band of HPV-4 is cut on linearisation withBamHIinto bands of 0.2kb and 0.15kb.

Exposure of the Southern blot in Figure 4.1 for up to two weeks did not result in the appearance of the wild type HPV-16 bands which were absent. In addition, reprobing the blot with the 0.91kb and 0.64kb PstI-BamHI subgenomic fragments of HPV-16 failed to detect any more viral sequences. No viral DNA was detected in dysplastic or normal DNA samples after a longer exposure, and no HPV sequences were found in any of the other thirty six sets of DNAs from oral cancer patients or in any of the twenty five normal control samples with any of the HPV probes used, even under conditions of low stringency. These data suggest that these samples do not harbour

HPV sequences, or are present below the limit of detection of this technique (one HPV genome per cell in 10μg of genomic DNA), or that other HPV types which do not cross-hybridise to any of the probes used, even at low stringency, were present. The use of a more sensitive detection method, involving amplification of low abundance viral sequences in the polymerase chain reaction (section 4.4), has enabled these questions to be addressed.

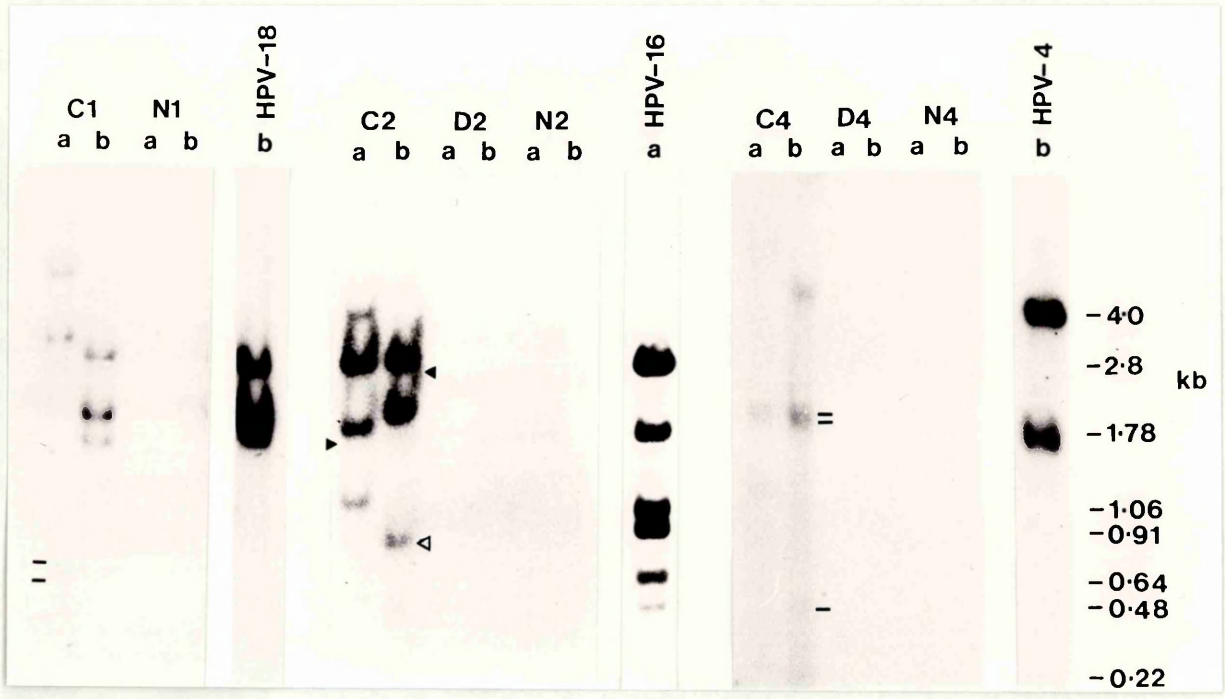


Figure 4.1.

Southern blot of cellular DNA (10μg) from carcinoma (C), dysplastic (D) and normal (N) tissue from oral cancer biopsies 1, 2 and 4 digested with (a) PstI or (b) HpaII. The blot was sequentially hybridised to ^{32}P-labelled DNAs of HPVs 1-7, 11, 13, 16 and 18 as described in the text. In C1, the position of the two small PstI fragments of HPV-18 are indicated by lines to the left of lane (a). In C2, the closed arrowheads point to the missing PstI and HpaII fragments of HPV-16, whilst the open arrowhead indicates the additional HpaII band. In C4, the HpaII doublet and the smallest band of HPV-4 are indicated by lines to the right of lane (b). Molecular weights indicated are the sizes of the PstI-BamHI fragments of HPV-16 and the large HpaII band of HPV-4 (4.0kb).

Figure 4.2.
Map of prototype HPV-16 DNA showing the location of PstI and HpaII restriction sites and the relationship between the 1.55kb PstI and 2.6kb HpaII fragments, neither of which were visible in C2 by Southern blot hybridisation. The alteration appears to be upstream of the 0.22kb PstI fragment and 3' to the 1.9kb HpaII fragment, as both were detected by Southern analysis.

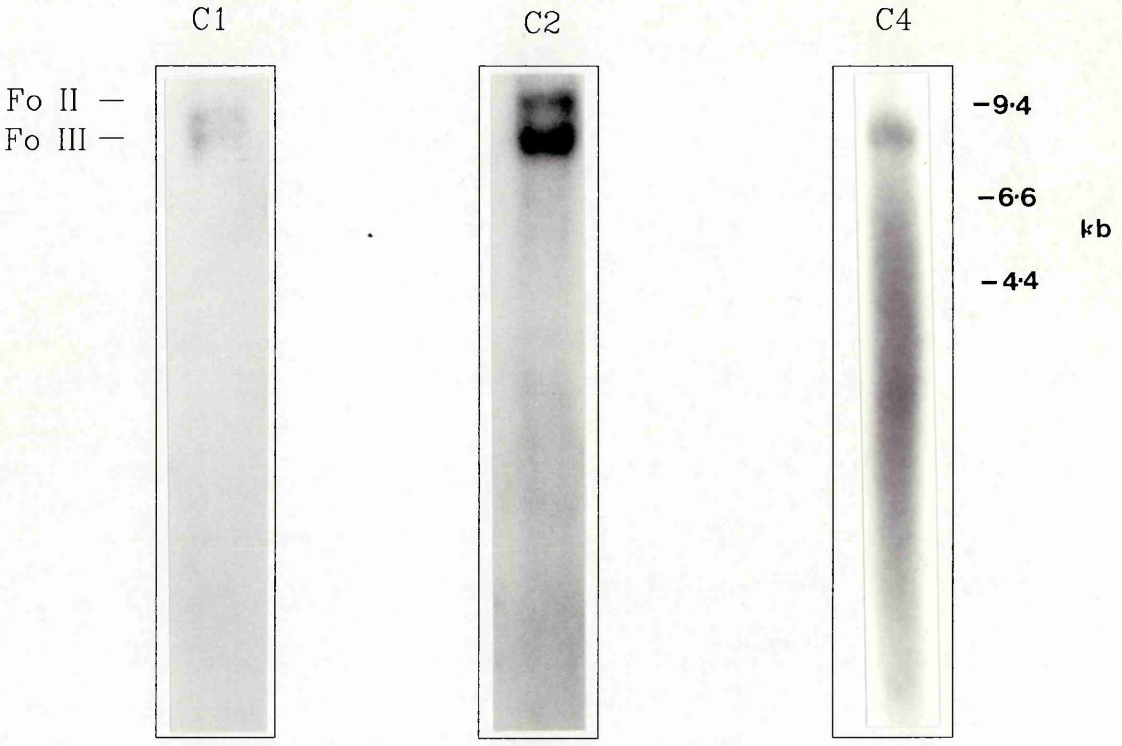


Figure 4.3.

Southern blot of 10ug aliquots of undigested C1, C2 and C4 genomic DNA, hybridised to HPV-18, HPV-16 and HPV-4 respectively. Linear (form III) and relaxed circular (form II) DNA can be seen. No evidence of integration was observed in any of the samples. HPV-4 DNA in C4 was badly degraded, and only linear DNA was visible.

4.2. Molecular Cloning of HPV-4 DNA From Cancer C4.

The cancer DNA sample C4 contained HPV-4 sequences, the first demonstration of this viral type in a malignant lesion. As such, it was decided to molecularly clone the viral DNA to enable a more detailed analysis to be carried out.

4.2.1. Isolation of HPV-4 DNA.

Cellular DNA from C4 was digested with BamHI, a known single-cutter enzyme for HPV-4, and DNA fragments in the size range 6-10kb ligated into the BamHI site of the bacteriophage lambda replacement vector EMBL3 (Figure 4.4; Frischauf *et al.*, 1983). Screening of 5×10^4 recombinant plaques resulted in detection of two positive clones. These were further purified, bacteriophage DNA prepared and digested with BamHI or SalI to release the insert DNA.

Figure 4.5(a) shows the restriction pattern of two clones, A and B, which were positive for HPV-4 sequences on plaque lift analysis, together with a control of linearised HPV-4 DNA. Clone A failed to digest with an excess of BamHI, even when incubated for up to 24 hours, and only partial release of the right vector arm was achieved with SalI. Clone A did not hybridise to HPV-4 probe on the Southern blot shown in Figure 4.5(b), which suggests that the plaque isolated was not one containing viral sequences, or that the HPV-4 DNA was lost during the growth of the bacteriophage in culture. Digestion of

clone B with BamHI or SalI produced left and right vector arms plus three insert bands, implying that the bacteriophage stock was not homogeneous. The three BamHI insert bands, a,b and c, of approximately 9.0kb, 8.0kb and 6.0kb respectively, hybridised to HPV-4 DNA at high stringency (Figure 4.5(b)). Band b corresponds to full-length HPV-4 DNA.

Full-length HPV-4 DNA purified free from bacteriophage sequences was subcloned into the plasmid vector pBR322. Colonies were screened for the presence of recombinant plasmid by small-scale plasmid DNA preparation, restriction enzyme digestion, Southern blotting and hybridisation to a HPV-4 probe. Six identical clones were positive for HPV-4 DNA (Figure 4.6(a)). The 9.0kb and 6.0kb bands were similarly subcloned, as can be seen in Figure 4.6(b), but blotting and hybridisation of these samples to a HPV-4 probe detected only the 6.0kb band (Figure 4.6(c)). It is likely that the 9.0kb band which was subcloned was the right vector arm.

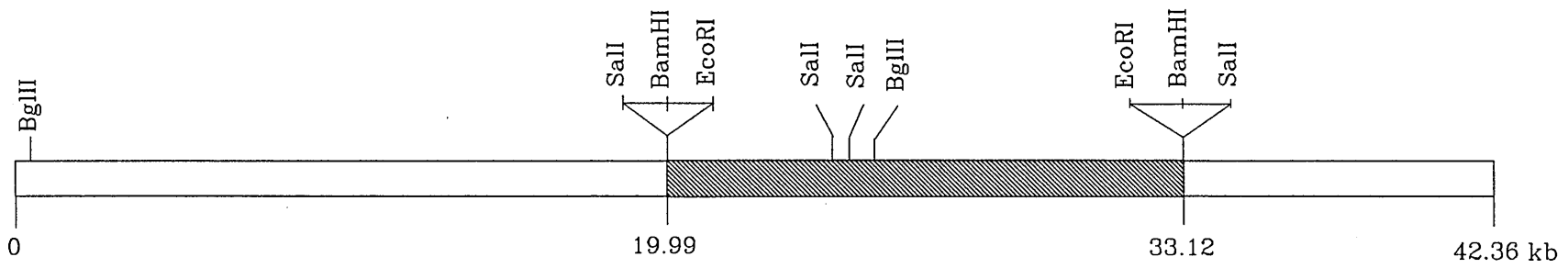


Figure 4.4.

Map of the bacteriophage vector EMBL3 (Frischauf et al., 1983) used to molecularly clone HPV-4 DNA from tumour C4. Left and right EMBL3 arms predigested with BamHI minus the 13.13kb stuffer region (hatched) were obtained from Stratagene.

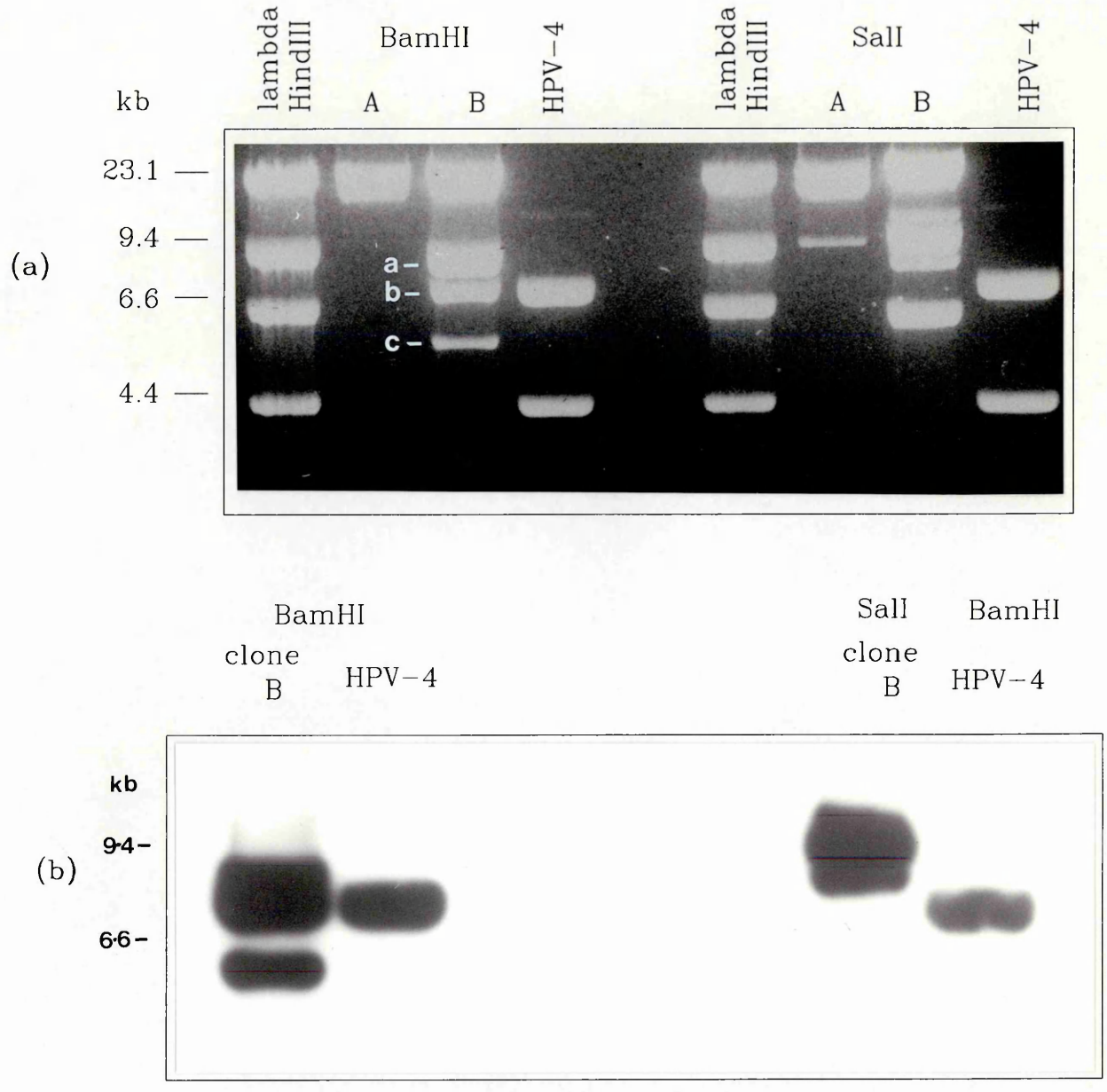


Figure 4.5.

(a) Restriction enzyme analysis of two clones, A and B, which hybridised to a HPV-4 probe on plaque lift analysis. Both cloned DNAs were digested with BamHI (left lanes) and SalI (right lanes) which cuts outwith the cloning site. A control HPV-4 plasmid was digested with BamHI and co-electrophoresed. Clone A did not digest with BamHI and only partially with SalI. Clone B gave three insert bands (arrowed a,b and c) on digestion with BamHI, of approximately 9.0kb, 8.0kb and 6.0kb, in addition to left and right vector arms, suggesting that the 'clone' was not a pure isolate. (b). Southern blot of the samples in (a) hybridised to HPV-4. No hybridisation was detected to clone A. All three BamHI insert bands hybridised to the HPV-4 probe, and were subcloned. Band b is full length HPV-4.

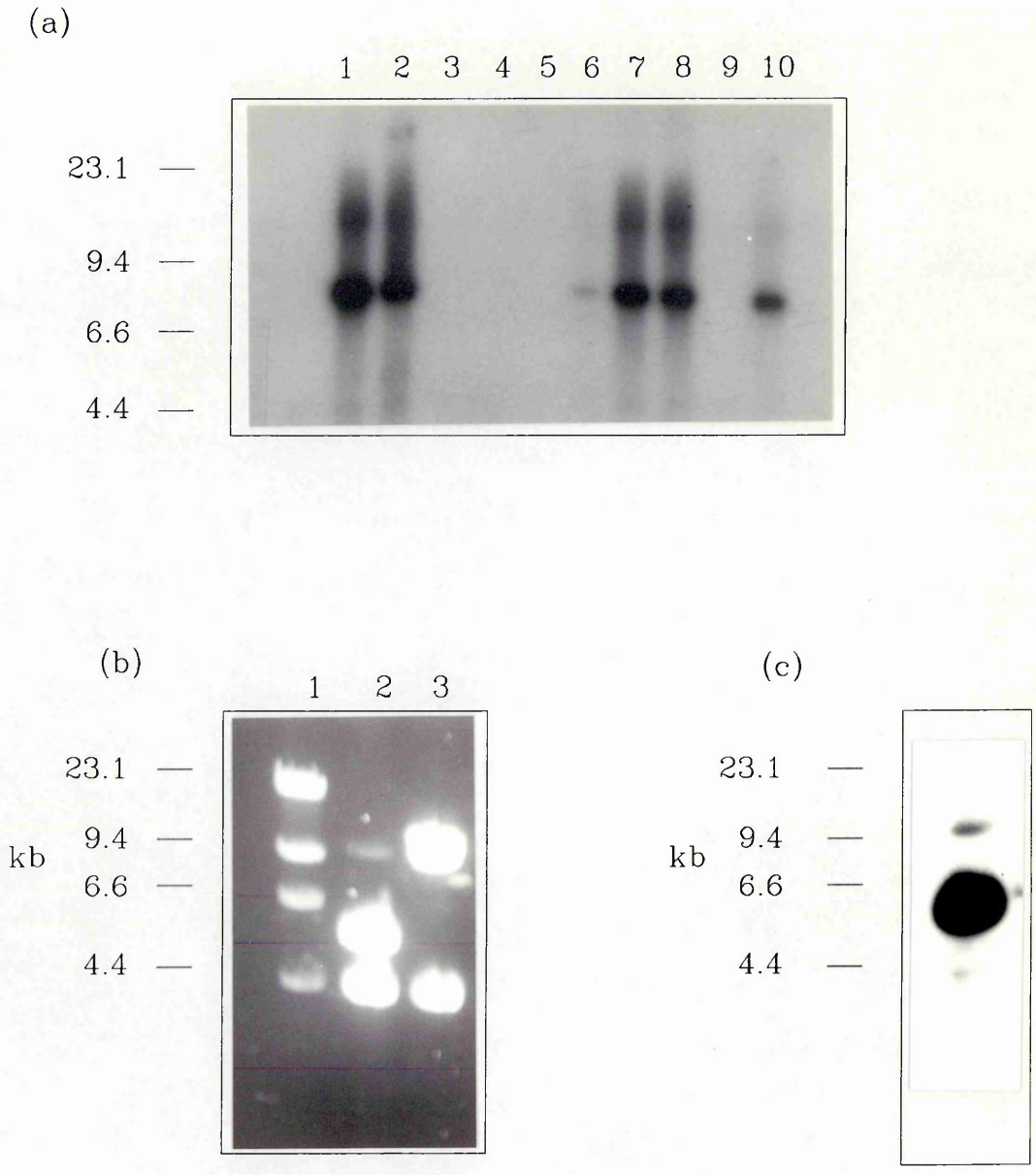


Figure 4.6.

(a) Southern blot of BamHI-digested miniprep DNA from 10 clones, hybridised to HPV-4 DNA at high stringency. Lanes 1,2,6,7,8 and 10 show HPV-4 sequences to be present. (b). Ethidium bromide-stained agarose gel of subcloned DNA of the HPV-4 positive 9.0kb and 6.0kb bands seen in Figure 4.5, digested with BamHI. Inserts of the expected sizes appear to have been cloned. (c). Southern blot of the gel in (b) hybridised to the HPV-4 probe. Only the 6.0kb band hybridised.

4.2.2. Comparative Restriction Analysis of HPV-4 DNA.

Cloned prototype HPV-4 DNA and the oral HPV-4 clone, linearised at the BamHI site and purified free from the cloning vector, were digested with twelve restriction endonucleases in an attempt to detect any polymorphisms which might be present between the two isolates.

Figure 4.7 is a Southern blot of restriction endonuclease-digested prototype and oral isolates of HPV-4 DNA hybridised to a HPV-4 DNA probe at high stringency. No differences in restriction pattern are visible in any of the lanes. The additional bands in the Sau3AI digest of prototype HPV-4 DNA are products of partial digestion.

The absence of polymorphism for these few enzymes does not imply that the two isolates of HPV-4 are identical. Obviously sequence differences may be present which do not alter these specific restriction sites, and would therefore not be apparent on such a limited analysis. Although further restriction mapping might possibly reveal restriction enzyme polymorphisms, a definitive answer would be provided by obtaining the complete nucleotide sequence of both HPV-4 isolates.

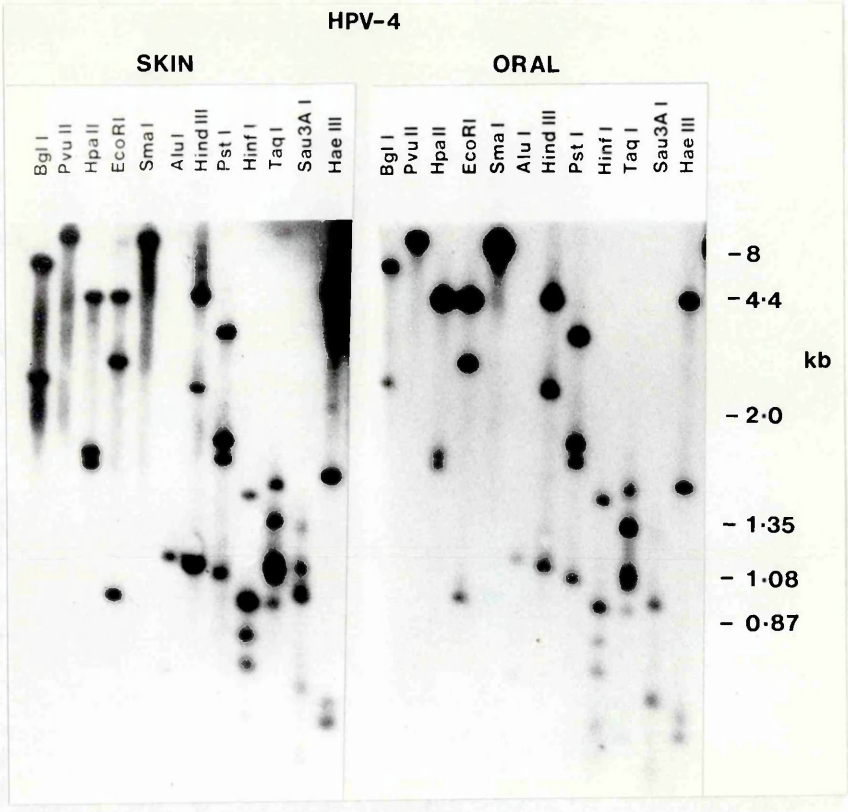


Figure 4.7.

Southern blot of prototype and oral isolates of HPV-4 DNA digested with twelve restriction enzymes and hybridised to a HPV-4 probe. No alteration was visible in any of the restriction patterns. The Sau3AI digest of prototype HPV-4 shows partial digestion.

4.2.3. Restriction Analysis of the 6kb Fragment.

The 6kb band which was seen to hybridise to HPV-4 in Figure 4.6(c) was purified free from vector sequences and digested with PvuII, HpaII and EcoRI, as detailed in Figure 4.8.

PvuII did not cut within the cloned sequence (as is the case for full length HPV-4 DNA), as judged from its co-migration with unrestricted DNA. In the HpaII digest, 4.4kb and 1.6kb prototype bands were visible, but the 1.5kb band, which maps to the right of the BamHI-linearised HPV-4 genome, was absent. Digestion with EcoRI produced the 0.9kb prototype band plus a band of approximately 5.1kb, not seen on digestion of prototype HPV-4 DNA with this restriction enzyme; two prototype EcoRI bands of 4.4kb and 2.9kb were also missing. These map to the middle (4.4kb) and right (2.9kb) of the BamHI-linearised prototype HPV-4 genome; it is likely that the variant 5.1kb EcoRI band described above reflects the prototype 4.4kb band plus part of the 2.9kb band, as the 4.4kb HpaII fragment and the 4.4kb EcoRI fragment overlap (Figure 4.8(b)). This suggests that a region of viral DNA to the right of the BamHI-linearised genome is missing in the 6kb clone. The most likely explanation is that the 6kb band hybridising to the HPV-4 probe at high stringency represents the leftmost region of full-length HPV-4 DNA, and as such is an artefact of the cloning procedure. Alternatively it could represent a second, deleted viral genome present in the cancer DNA sample; however no evidence of such is visible by Southern blot analysis (Figure 4.1).

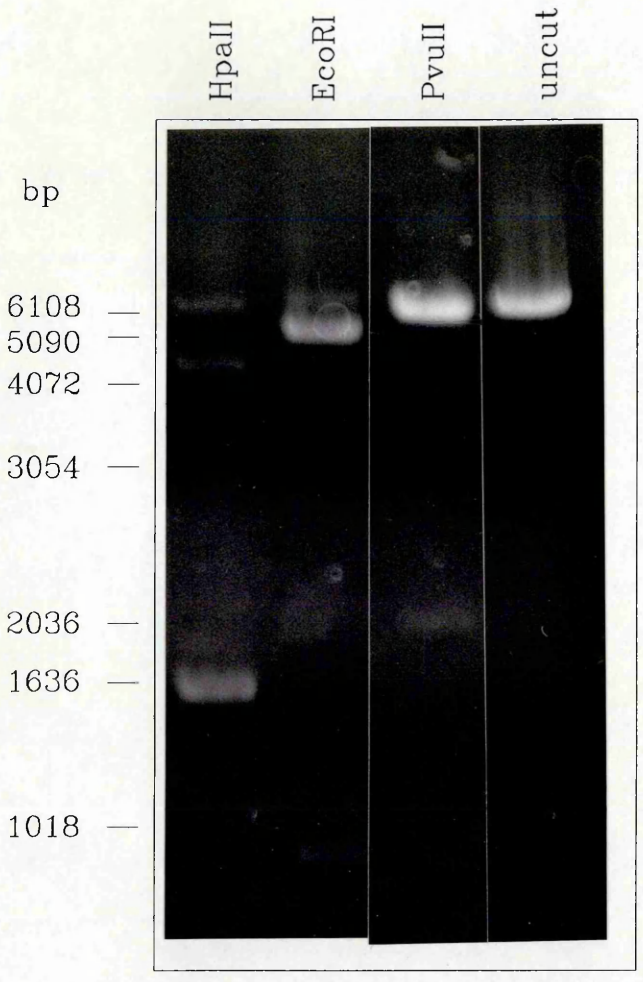


Figure 4.8(a).

Restriction digestion of the 6kb band (Figure 4.6(c)) with the enzymes indicated. Molecular weight markers were a 1kb ladder.

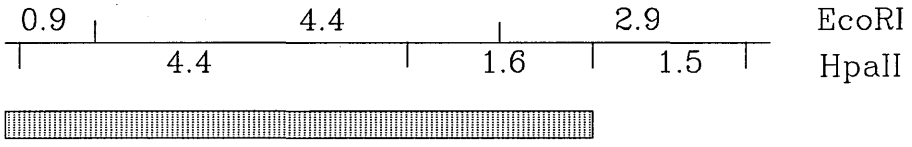


Figure 4.8(b).

Restriction map of prototype HPV-4 DNA showing the EcoRI and HpaII sites within the viral genome, linearised at the BamHI site. Shaded regions represent the areas present in the 6kb clone. Sizes of restriction fragments are in kb.

4.3. Nucleotide Sequencing of HPV-4 Isolates.

4.3.1. The Partial Nucleotide Sequence of HPV-4.

A region of the nucleotide sequence of prototype HPV-4 DNA was determined, primarily to facilitate the design of specific oligonucleotide primers for use in polymerase chain reaction screening of genomic DNA samples. In addition, comparison of this with a similar region of sequence from the oral isolate of HPV-4 described above might reveal base changes not apparent on a limited restriction endonuclease analysis.

Three hundred and eighty bases of the prototype HPV-4 sequence immediately adjacent to the vector-viral junction were determined, using the seventeen base oligomer 5'-GCCGGCCACGATGCGTC-3' as sequencing primer, which corresponds to nucleotides 406-390 of the pBR322 genome (Sutcliffe, 1979). The sequence obtained read from 5' to the HincII site at map coordinates 0.018 through the HindIII site at 0.036 (Heilman *et al.*, 1980).

The prototype HPV-4 sequence determined is listed in Figure 4.9(a), and the restriction sites indicated. Homology comparison analysis was performed in both orientations with the HPV sequences present in the EMBL and Genbank databases, using the GAP programme in the UWGCG sequence analysis software package. No convincing regions of homology were revealed at the nucleotide level with any of the HPV sequences available. This reflects and confirms the isolated position of HPV-4 in the phylogenetic tree of papillomaviruses as

previously reported (Pfister, 1984). The full-length clone of oral HPV-4 was similarly sequenced. The data obtained demonstrated identity between these two isolates over the region sequenced. This suggests that the oral and prototype isolates of HPV-4 are the same, or are at least identical in this region.

Using the TRANSLATE programme, it was possible to identify a putative protein coding region within the available HPV-4 sequence, reading in frame one up to the TAA stop codon at nucleotides 232-234 as shown in figure 4.9(a). No ATG start codon could be found for this sequence; therefore the protein is likely to represent the product of the 3' end of a larger coding region starting 5' to the available sequence.

Using BESTFIT to compare this region with protein sequences from other sequenced papillomaviruses, homology was noted with the E2 proteins of HPV-6b, HPV-16 and HPV-18, as shown in Figure 4.9(b). The putative HPV-4 protein is homologous to the 3' end of the coding region in each case, consistent with the sequence obtained above. As can be seen from the alignment several conserved amino acid motifs are present, probably reflecting their functional importance.

4.3.2. Sequence Analysis of the 6kb Clone.

A region of the 6kb clone (Figure 4.6(b and c)) was sequenced using the same oligonucleotide primer as above. Over the 150 bases sequenced, the sequence was identical to that obtained for HPV-4 DNA (not shown). These data support the conclusion that the 6kb fragment is an artefact of cloning, although this will only be confirmed by comparison of the entire nucleotide sequences of the full-length clone and the 6kb band, or by heteroduplex analysis.

```
                                                                    48
   TTG TTA AAG GGC ACA GCA AAT TCT TTG AAA TGT TGG AGA TAT AGA AAA
1    L   L   K   G   T   A   N   S   L   K   C   W   R   Y   R   K
2      C   -   R   A   Q   Q   I   L   -   N   V   G   D   I   E
3        V   K   G   H   S   K   F   F   E   M   L   E   I   -   K

    HincII                                                          96
   GTT AAC TCA AAT TGC TGC AAC TTC TTA TTC ATG AGT ACT GTT TGG AAC
     V   N   S   N   C   C   N   F   L   F   M   S   T   V   W   N
   K   L   T   Q   I   A   A   T   S   Y   S   -   V   L   F   G
    S   -   L   K   L   L   Q   L   L   I   H   E   Y   C   L   E

                                                                   144
    TGG GTT GGA GAT TGC TCA CAT AAT CAT CGT CGC ATG CTT ATT GCA TTT
      W   V   G   D   C   S   H   N   H   R   R   M   L   I   A   F
    T   G   L   E   I   A   H   I   I   I   V   A   C   L   L   H
     L   G   W   R   L   L   T   -   S   S   S   H   A   Y   C   I

                                                                   192
    GAT AGC ACT GAC CAA AGA GAC GCT TTT GTA AAA CAC AAC CTT TTT CCT
      D   S   T   D   Q   R   D   A   F   V   K   H   N   L   F   P
    L   I   A   L   T   K   E   T   L   L   -   N   T   T   F   F
     -   -   H   -   P   K   R   R   F   C   K   T   Q   P   F   S

                                                                   241
    AAA CTG TGT ACA TAT ACC TAC GGC TCA TTG AAT AGT TTA TAA AAT CGA
      K   L   C   T   Y   T   Y   G   S   L   N   S   L   -   N   R
    L   N   C   V   H   I   P   T   A   H   -   I   V   Y   K   I
     -   T   V   Y   I   Y   L   R   L   I   E   -   F   I   K   S

    HindIII                                                        288
    AAG CTT GAG TAG AAG GAA AGA GAT TCA GTT CCA AAC AGT ACT CAT GAA
      K   L   E   -   K   E   R   D   S   V   P   N   S   T   H   E
    E   S   L   S   R   R   K   E   I   Q   F   Q   T   V   L   M
     K   A   -   V   E   G   K   R   F   S   S   K   Q   Y   S   -

                                    HincII                         336
    TAA GAA GTT GCA GCA ATT TGA GTT AAC TTT TCT ATA TCT CCA ACA TTT
      -   E   V   A   A   I   -   V   N   F   S   I   S   P   T   F
    N   K   K   L   Q   Q   F   E   L   T   F   L   Y   L   Q   H
     I   R   S   C   S   N   L   S   -   L   F   Y   I   S   N   I

                                                                   380
    CAA AGA ATT TGT CTG TGC CCT TTA ACA ATA TCA TAG GCG GAT CC
      Q   R   I   C   L   C   P   L   T   I   S   -   A   D
    F   K   E   F   V   C   A   L   -   Q   Y   H   R   R   I
     S   K   N   L   S   V   P   F   N   N   I   I   G   G   S
```

Figure 4.9. Partial nucleotide sequence of prototype HPV-4 DNA reading from 5' of the HincII site at map coordinate 0.018 through the HincII site at 0.05 map units. Restriction sites are indicated, and the sequence translated in all three reading frames. In frame one, a potential coding region can be identified reading through to the TAA stop codon at nucleotides 233-235. The oral isolate of HPV-4 was identical to the prototype DNA over the region sequenced.

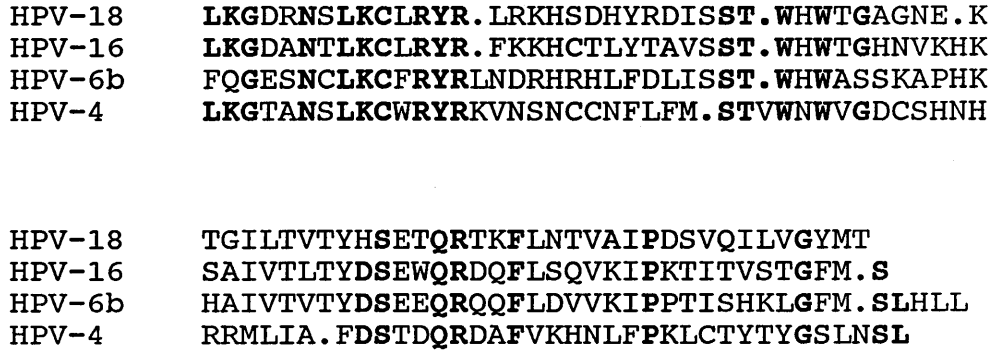


Figure 4.9(b). Alignment of a putative HPV-4 peptide with regions of the E2 proteins of human papillomavirus types 6b, 16 and 18. Highly conserved amino acids between the HPV-4 sequence and the other papillomavirus types are highlighted. Dots in the sequences indicate spaces which have been inserted in order to align the proteins with maximum homology.

4.4. PCR Screening of Genomic DNA Samples For HPV DNA.

Polymerase chain reaction amplification of specific fragments of HPV DNA was used to increase the amount of material available prior to Southern blotting and hybridisation, thus increasing the overall sensitivity of the screening procedure to considerably less than one viral genome per cell. According to Maitland *et al.* (1989) this should enable detection of one copy of a specific sequence in a background of up to 1×10^4 uninfected cells.

Areas of the E6 ORFs of HPV-16 and HPV-18, selectively retained in cervical carcinomas and detectable in oral samples C2 and C1 (section 4.1), were chosen for amplification, as was a region of the HPV-4 sequence determined previously (section 4.3). Oligonucleotide primer pairs used in the PCR are listed in Table 4.1, together with the expected sizes of the amplified fragments for each set of primers. Listed in Table 4.2 are the sequences of two 40-base oligomers (16-Int and 18-Int), internal to the HPV-16 and HPV-18 amplification products, which were used as hybridisation probes to ensure that the sequence amplified was the correct one.

Oligonucleotide Primers for Polymerase Chain Reaction

			Location		Expected fragment size
5'	TTAATTAGGTGTATTAACTG	3'	401–420	16E6	166bp
	TGCATGATTACAGCTGGGTT		566–547		
	GATACACCTACATTGCATGA		571–590	16E7	270bp
	GATGGGGCACACAATTCCTA		840–821		
	GATTTAAGTACTATTGATCCTGC		5220–5242	16La	1816bp
	GCGTCCTAAAGGAAACTGATCTAGG		7035–7011	16Lb	
	ATCTGTGCACGGAACTGAAC		151–170	18E6	100bp
	AATGCAAATTCAAATACCTC		250–231		
	AAAGGGCACAGCAAATTCTT		4–23	H4	137bp
	ATGCAATAAGCATGCGACTA		140–121		
	GACGGAATATAAGCTGGTGG		1668–1687	HA12	63bp
	TGGATGGTCAGCGCACTCTT		1730–1711	HB12	

Table 4.1

Oligonucleotide primers used in the polymerase chain reaction.

Internal Oligonucleotide Probes		
GACAAAAAGCAAAGATTCCATAATATAAGGGGTCGGTGGA	461–500	INT–16
CTGCAAGACATAGAAATAACCTGTGTATATTGCAAGACAG	177–215	INT–18

Table 4.2. Oligonucleotides used as probes for HPV–16 and HPV–18 E6 region DNAs.

4.4.1. PCR Screening For HPV-4 Sequences.

PCR screening of case and control DNA samples did not increase the percentage positive for HPV-4 DNA (3%; one out of thirty nine cases), with only sample 4 harbouring HPV-4 sequences. However, by increasing the sensitivity of the detection technique, it was possible to demonstrate that viral sequences were not confined to the DNA from the cancer biopsy alone.

Figure 4.10 is a Southern blot of PCR products from carcinoma (C), dysplastic (D) and normal (N) DNAs from sample 4, amplified using the HPV-4 primers and hybridised to a ^{32}P-labelled probe comprising the complete genome of HPV-4. Viral sequences are present in lanes D4 and N4 as well as in C4, which was positive by conventional Southern blot (Figure 4.1). Lane C4 contains one tenth of the amount of PCR product of either lanes D4 or N4. Thus it appears that viral sequences are present in non-malignant epithelia, albeit at lower copy number.

(a)

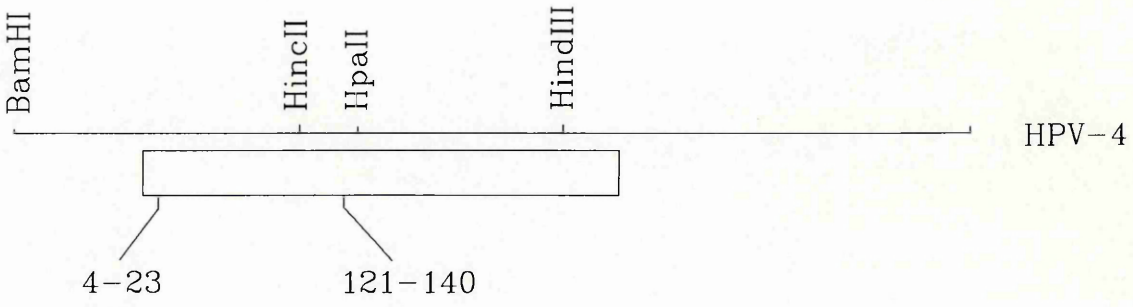
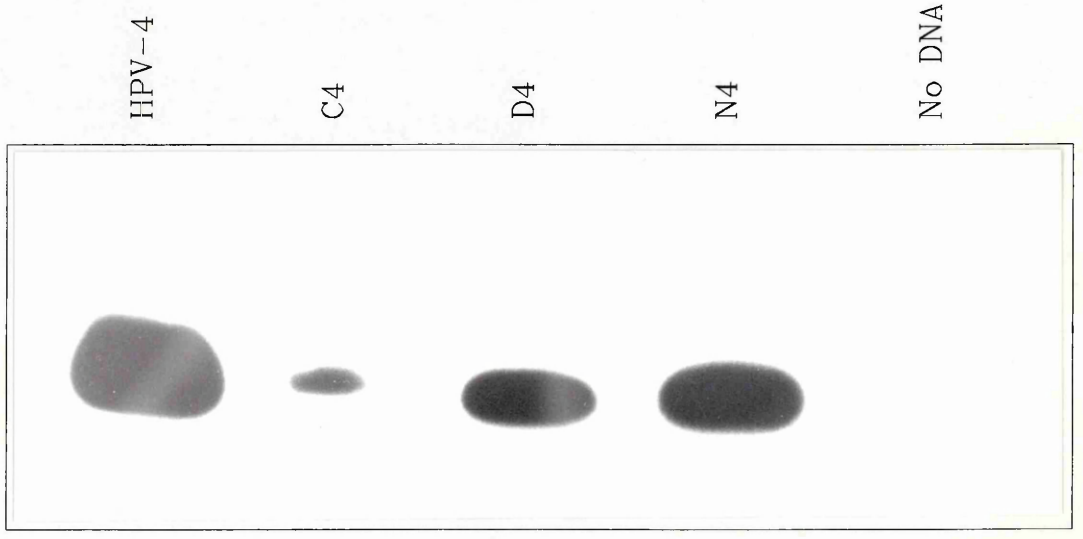


Figure 4.10

(a) HPV-4 PCR primers derived from the nucleotide sequence in Figure 4.9, expected to amplify a 136bp fragment. (b) Southern blot of PCR products from sample 4; the probe was total HPV-4 DNA. C=carcinoma, D=dysplastic, N=normal. Lane C4 has tenfold less sample than D4 or N4.

(b)



4.4.2. Screening For HPV-16 Sequences By PCR.

By Southern blotting of PCR products obtained using the HPV-16 E6 primers detailed above and subsequent hybridisation to an internal oligonucleotide probe, ten out of the thirty nine case DNA samples (25.6%) were shown to contain HPV-16 E6 sequences. A representative blot is shown in Figure 4.11. None of the twenty five normal control samples from cancer-free individuals were found to harbour HPV-16 DNA sequences.

Where HPV-16 DNA was present in carcinoma DNA samples, adjacent normal epithelia were also found to contain viral sequences. Figure 4.12 shows a Southern blot of PCR products from dysplastic (D) and normal (N) DNAs from tissues adjacent to carcinoma C2, which was positive for a variant of HPV-16 by conventional Southern blot analysis (Figure 4.1). HPV-16 related sequences are clearly present in these tissues, although apparently at lower copy number than in the carcinoma.

The nucleotide sequence of the HPV-16 fragment amplified from sample D2 was determined in both orientations, using both PCR primers as sequencing primers. No base changes were found when compared to cloned HPV-16 DNA in either the forward orientation (Figure 4.13) or the reverse (data not shown). Although the entire 166 nucleotides were not sequenced, data were obtained to within five bases of the primer.

In the light of results obtained by other workers (Maitland *et al.*, 1987;1989) and the altered restriction digestion patterns apparent in sample C2 by Southern blot analysis, sequencing of a region of the HPV-16 E7 ORF amplified from samples of cellular DNA by PCR was carried out using the 16E7 primers (Table 4.1), in order to establish whether or not the E6 and E7 ORFs were retained unaltered. In each case, no base changes were found when compared to prototype HPV-16 DNA. Figure 4.14 shows the nucleotide sequence of a region of the E7 PCR product from the HPV-16 control and sample N30 (DNA from normal epithelium adjacent to a carcinoma). Although the entire fragments were sequenced, only those bases from nucleotides 736 to 833 are shown. Sequencing primer was the forward PCR primer. This suggests that the E7 ORF and the region of the E6 ORF sequenced are conserved between oral and prototype isolates of HPV-16, probably reflecting their functional importance.

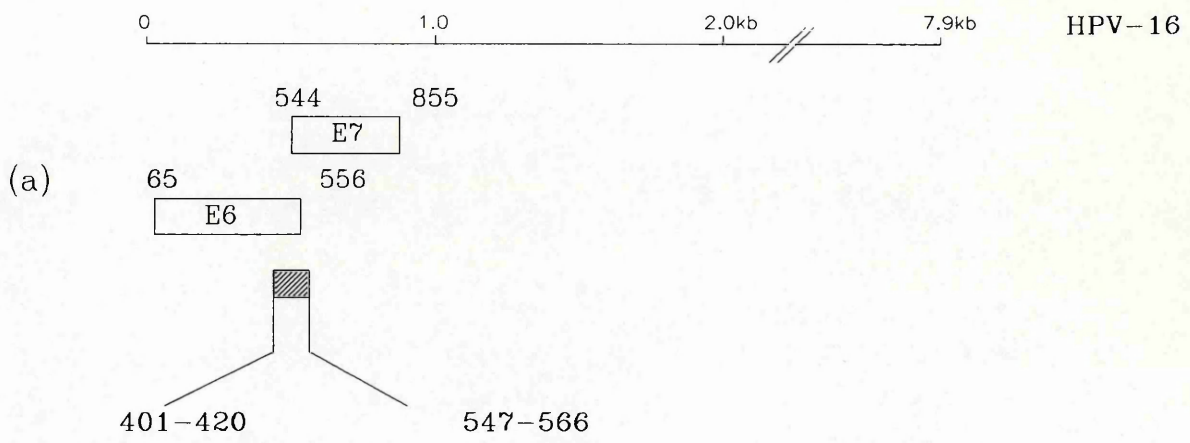
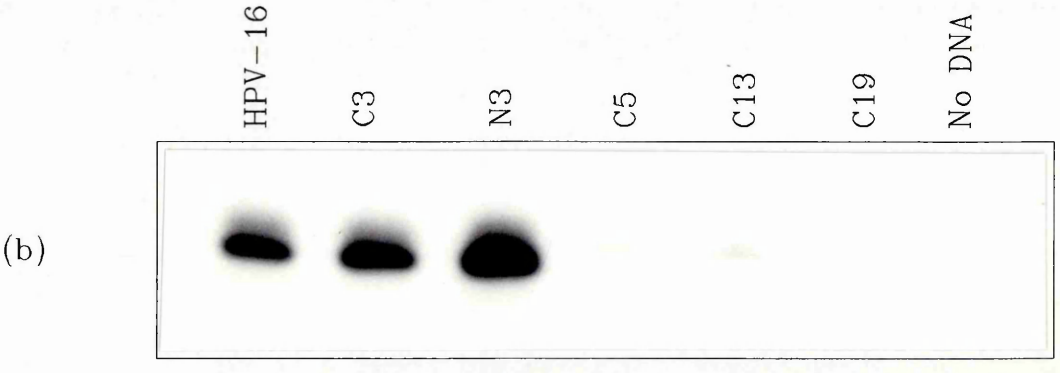


Figure 4.11.

(a) HPV-16 PCR primers (16E6), expected to amplify a 166bp fragment from the HPV-16 E6-E7 region. (b) Southern blot of PCR products amplified from samples 3,5,13 and 19, using the primers in (a). C= carcinoma, N=normal, HPV-16=control template of 1pg of HPV-16 plasmid, No DNA=control sample minus DNA template.



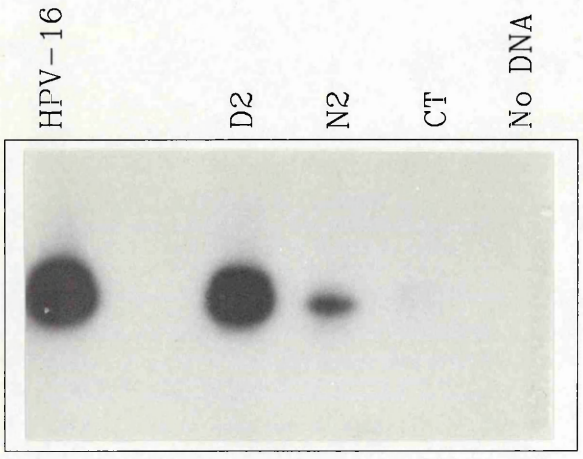


Figure 4.12.

Southern blot of PCR products from samples D2 and N2 using the 16E6 primers. D=dysplastic, N=normal, Controls were HPV-16 plasmid, calf thymus DNA (CT) and a reaction containing no DNA. Hybridisation probe was the internal oligonucleotide 16-Int.

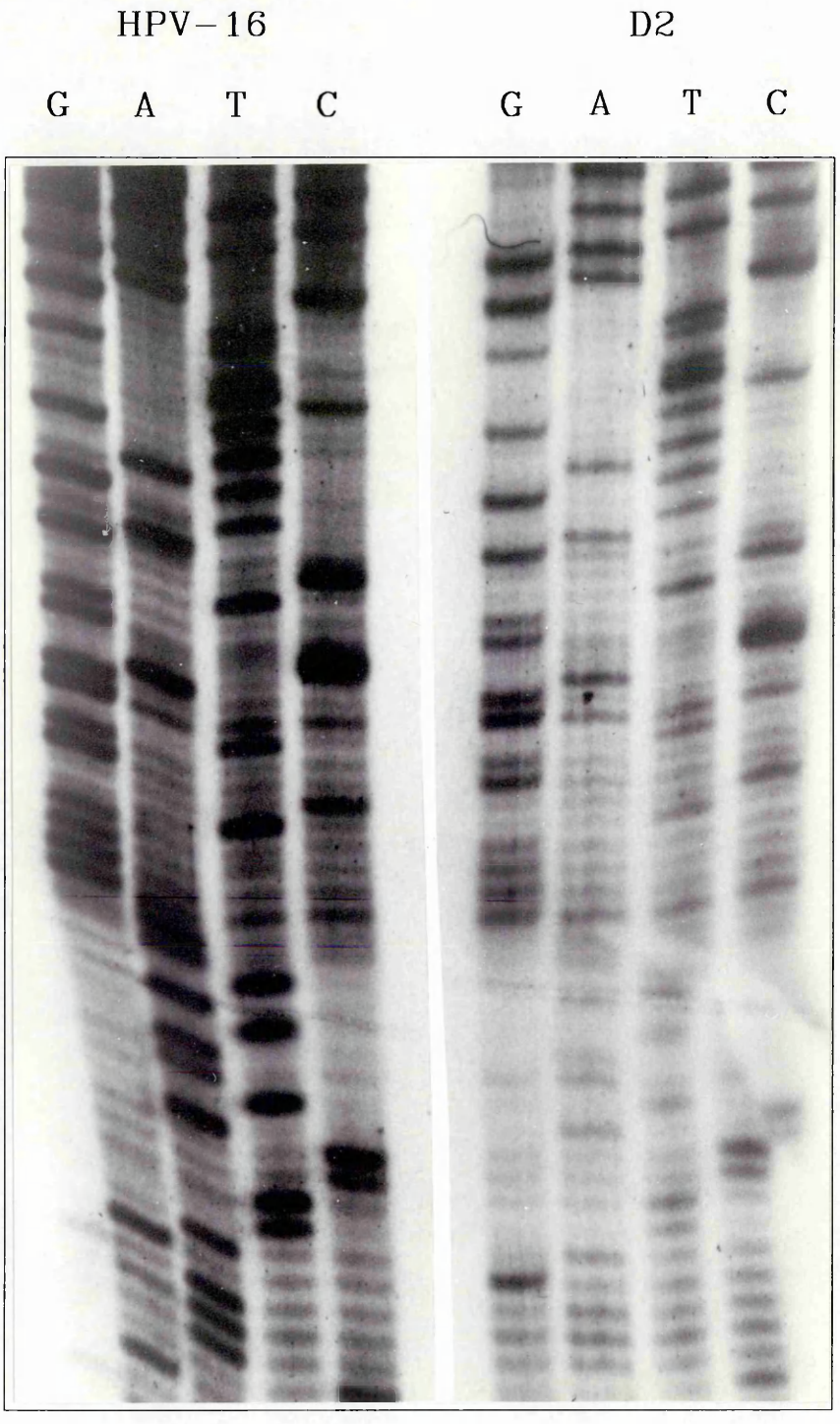


Figure 4.13.

Direct nucleotide sequence of PCR products obtained from HPV-16 plasmid and sample D2 (Figure 4.12). No nucleotide differences were observed.

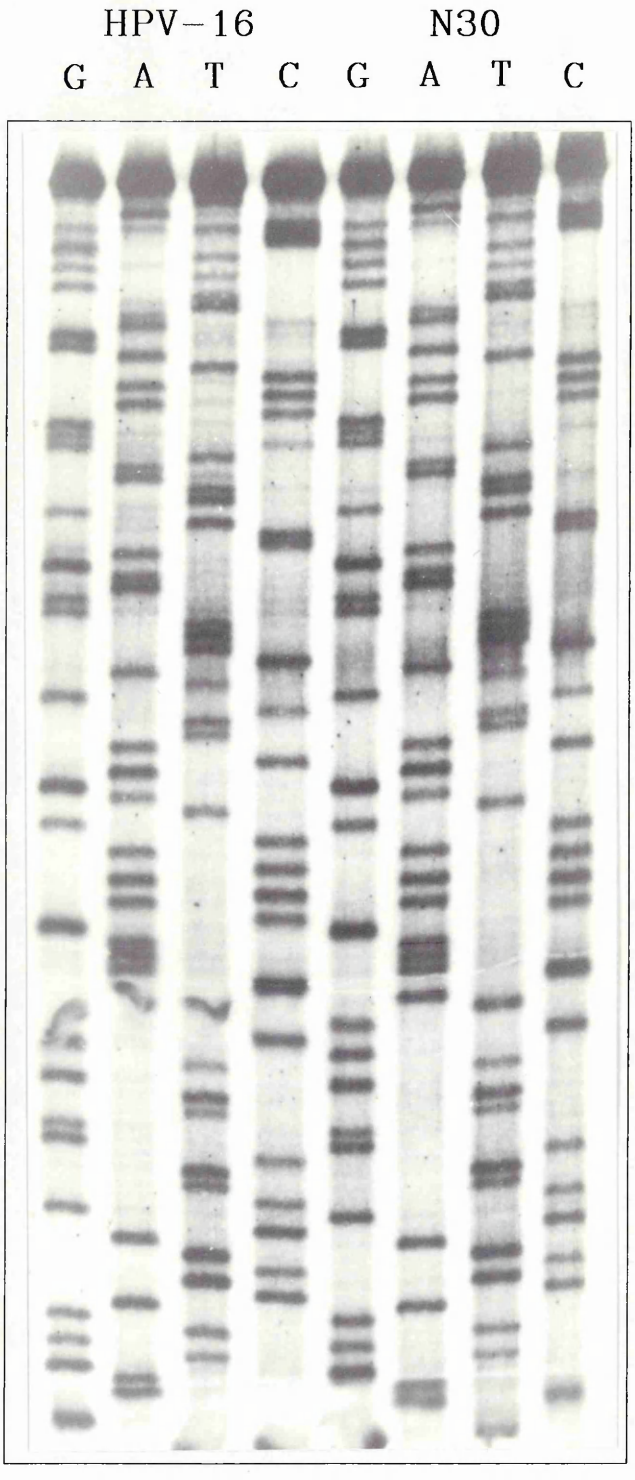


Figure 4.14.

Direct nucleotide sequence of HPV-16 E7 region PCR products amplified from HPV-16 plasmid and sample N30, using the 16E7 primer pair (Table 4.1). The entire fragment was sequenced, although only a section is shown. No changes in base composition were detected.

4.4.3. PCR-Mediated Detection of HPV-18 DNA.

Eight out of thirty nine cases (20.5%) were found to harbour HPV-18 sequences using the HPV-18 E6 region PCR primers described above (Table 4.1). As was the case for detection of HPV-4 and HPV-16 DNAs, where viral DNA was present in the carcinoma sample it was also present in the corresponding normal sample, except in two cases. Figure 4.15 is a representative Southern blot of samples containing HPV-18 E6 DNA hybridised to an internal oligonuleotide probe. The negative control of DNA from T45 cells, an oral carcinoma cell culture (Parkinson and Yeudall, in press) containing HPV-16 DNA by PCR, and the control reaction of reagents minus template DNA show that the amplification was specific for HPV-18 sequences.

In addition to HPV-18 positive cancer case samples, two out of twenty five (8%) control samples of DNA from normal buccal mucosa of healthy volunteers also contained DNA of this viral type, the only HPV type found in cancer-free subjects in this study.

In order to discover if, as in the case of HPV-16 positive samples, the amplified HPV-18 E6 region was identical to the wild-type sequence, direct nucleotide sequencing was carried out on PCR products from carcinoma sample C1 and from the two HPV-18 positive normal samples N12 and N18. As Figure 4.16 shows, no nucleotide differences were observed between these samples and the PCR product amplified from cloned prototype HPV-18 DNA.

(a)

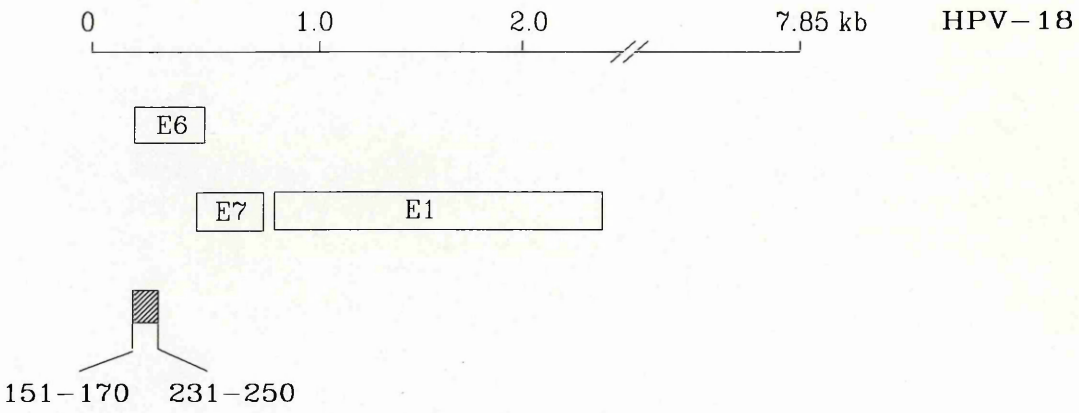
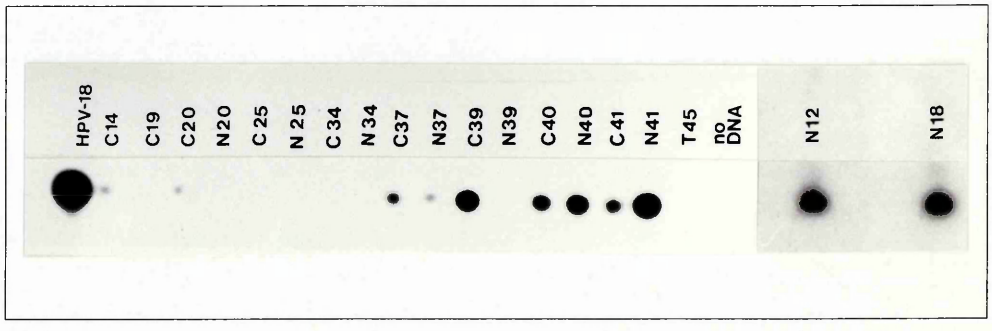


Figure 4.15.

(a). HPV-18 oligonucleotide primers (18E6) used to amplify a 100bp fragment of the viral E6 ORF by PCR. (b). Southern blot of HPV-18 specific PCR products from case samples and two external controls (N12 and N18). Controls were HPV-18 plasmid (1pg), DNA from a HPV-16 containing cell line (T45) and a reaction minus DNA. The internal oligonucleotide 18-Int was used as hybridisation probe.

(b)



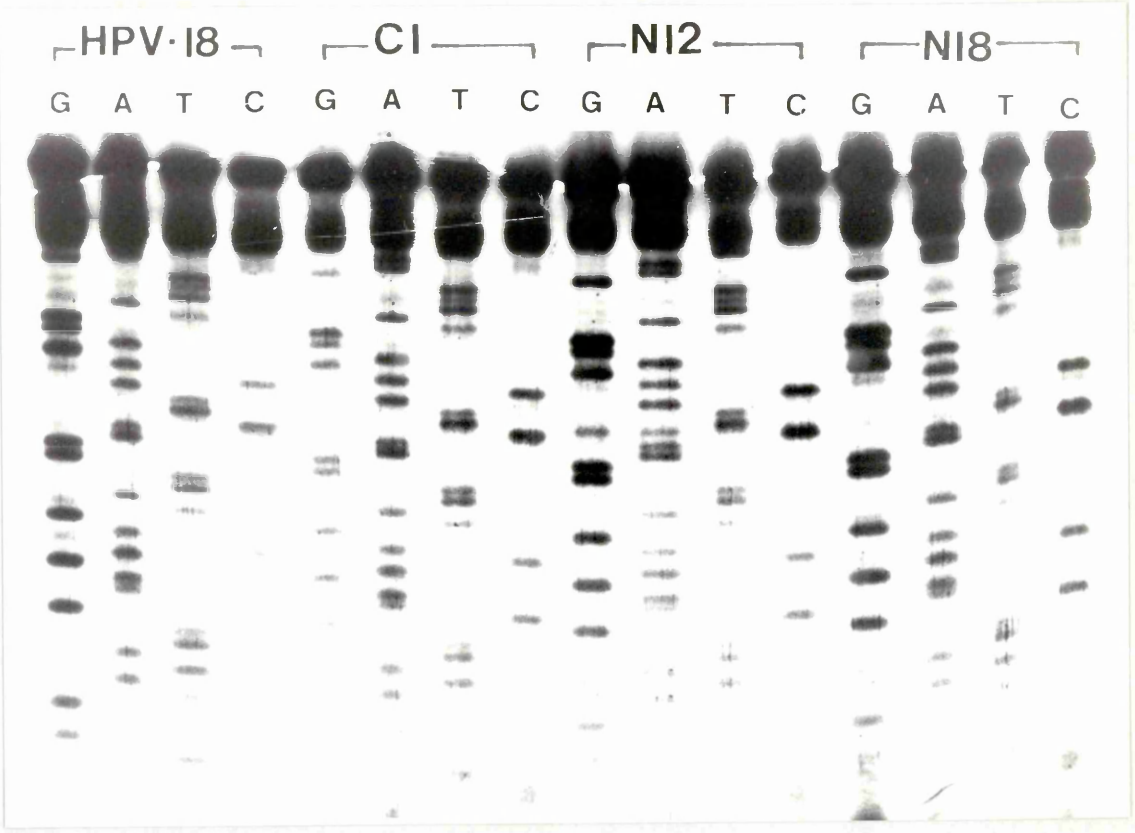


Figure 4.16.

Direct nucleotide sequence of HPV-18 E6 PCR products from HPV-18 plasmid, carcinoma C1, and external control samples N12 and N18. The upstream PCR primer was used as sequencing primer. No alterations in nucleotide sequences were seen over the region sequenced.

4.4.4. Use of Anticontamination Primers.

To control for possible contamination of samples by cloned HPV DNAs present in the laboratory, two sets of anticontamination primers were used, one pair for HPV-18 and the other for HPV-16, and were as detailed in Table 4.3. These were designed to amplify a fragment of DNA spanning the cloning site, and in the case of plasmid contamination the size of the amplified product would be greater by the length of the cloning vector, 4365bp for DNAs cloned in pBR322, as outlined in Figure 4.17.

As the length of any potential contaminating plasmid fragment amplified by these primers was considerably greater than the HPV target sequence, and thus less likely to be copied in total by the polymerase, the sensitivity of the anticontamination primers was tested using cloned HPV-16 and HPV-18 DNA templates over a range of concentrations. The extension step of each cycle was carried out at 72^{o}C for five minutes to maximise completion of each copy of the long (4545bp for pHPV-18) sequence. Figure 4.18 shows the amplification products for both pairs of primers using template DNA concentrations of 100ng, 10ng, 1ng, 100pg, 10pg and 1pg in a 100μl reaction volume. In both cases the amplification product was visible in all but the 1pg sample after hybridisation and a short (10 minutes) exposure to XAR-5 film. No amplification was detected in the samples containing 1pg of template DNA, even after several days' exposure. This suggests that the sensitivity of the anticontamination primers to amplify a large fragment of DNA is much reduced, compared to the ability to amplify a short sequence of a few hundred nucleotides.

Although the sensitivity of these primers was reduced when amplifying longer sequences, their use did not present any problems in the case of samples positive for HPV-18 E6 DNA. All such samples were shown to be true positives as the fragment amplified was of the expected size for wild type HPV-18 DNA (170bp). No evidence of plasmid contamination was found. A representative autoradiograph of amplified HPV-18 DNA hybridised to a ^{32}P-labelled probe comprising the complete genome of HPV-18 is shown in Figure 4.19.

Annealing of the anticontamination primers to sequences in genomic DNA samples shown to contain HPV-16 E6 and E7 DNA proved, however, to be impossible, although no problem was encountered in annealing to wild-type HPV-16 sequence. In view of the Southern blot results for sample C2 (section 4.1) and those of Maitland *et al.* (1989) for oral mucosal biopsy DNAs, this may be due to sequence rearrangement or deletion in this region of the viral genome.

No evidence of plasmid contamination was found in any of the samples positive for the HPV-16 early region DNA. However this was inferred by the lack of amplification of the viral-vector PCR product.

Anticontamination Primers

	Location		Expected fragment size
TGCTAGTGCTTATGCAGCAA	6030–6049		
		16AC	151bp
ATTTACTGCAACATTGGGTA	6180–6162		
AAATCATATTTTGGAATGAG	2381–2400		
		18AC	170bp
GCATTTCTCATATAGGTATC	2550–2531		

Table 4.3.

Oligonucleotide primers flanking the cloning sites of HPV–16 (16AC) and HPV–18 (18AC), used to control for contamination of PCR samples by HPV plasmids present in the laboratory. In the event of plasmid contamination, the size of the amplified fragment would be greater by 4365bp, the length of the plasmid vector.

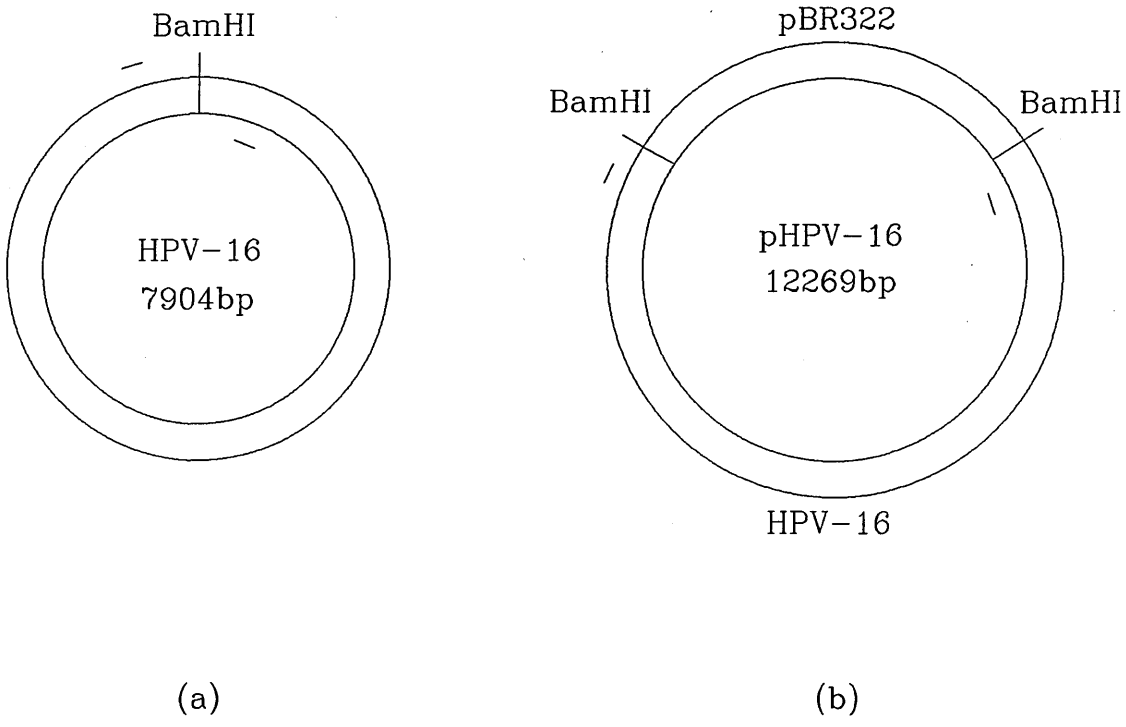


Figure 4.17.

Anticontamination primers (Table 4.3), situated on either side of the cloning site were used to amplify across this region of HPV−16 (diagram a) and HPV−18. In the case of contamination of samples by HPV plasmids (diagram b) the amplified fragment would be longer by the size of the pBR322 vector (4365bp).

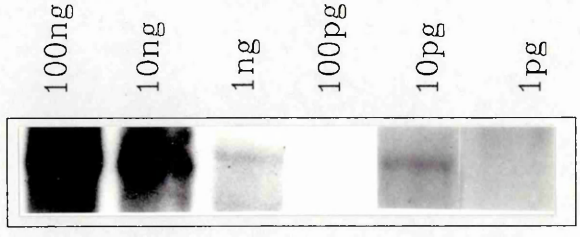


Figure 4.18.

Southern blot of PCR amplification products using the anticontamination primers for HPV-16 as listed in Table 4.3. Template DNA consisted of of dilutions of HPV-16 plasmid from 100ng to 1pg of DNA as indicated. The blot shown above was exposed for 10 minutes. A signal was detected for each concentration of template DNA except that which contained 1pg of starting material. A longer exposure (2 days) did not reveal a band in the 1pg sample.

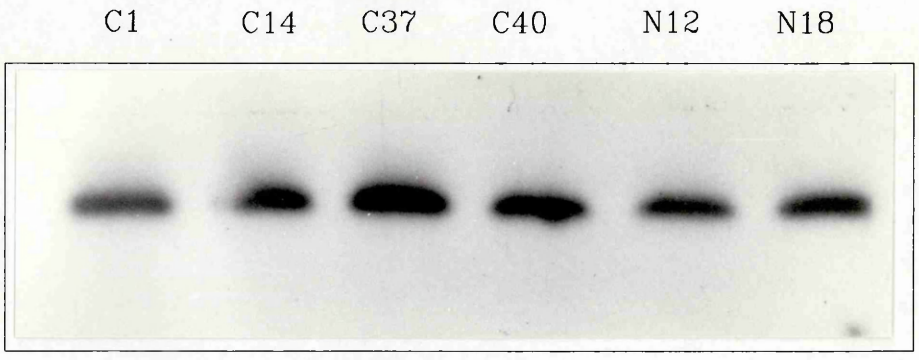


Figure 4.19.

Southern blot of PCR products amplified from case and control samples using the HPV-18 anticontamination primers. All bands were of the expected size for episomal viral DNA minus plasmid. No evidence of plasmid contamination was found.

4.5. Late Region Alterations in HPV-16 From Oral Samples.

The data obtained from the Southern blot hybridisation pattern of HPV-16 DNA in sample C2 implies that the L2-L1 region of the viral genome has undergone a deletion or recombination event, as the prototype PstI 'C' fragment of 1.55kb and the 2.6kb fragment in the HpaII-restricted sample are absent; an additional band of approximately 0.9-1.0kb is present in the HpaII lane (Figure 4.20).

Possibly the best way in which to address this question would have been to molecularly clone the entire viral genome from the tumour sample and then obtain the nucleotide sequence. Due to the small biopsy size, however, no sample DNA remained from which to isolate the variant HPV-16 DNA. It was decided, therefore, to use a PCR-based strategy in order to amplify a fragment of DNA spanning the area of interest, using a pair of oligonucleotide primers further upstream and downstream of the alteration, presumably in regions of unchanged sequence. The strategy is outlined briefly and the primers (16La/b, Table 4.1) detailed in Figure 4.21. These primers also serve as anticontamination primers, as they are situated on either side of the BamHI cloning site of prototype HPV-16.

4.5.1. PCR Amplification of HPV-16 Late Region Sequences.

Initially two samples, C3 and C5, positive for HPV-16 sequences using the E6-specific primers were used to attempt the amplification

procedure described above. Figure 4.22 shows the result of electrophoresis of the PCR products of C3, C5 and a control of recircularised prototype HPV-16 DNA in a 2% agarose gel, stained with ethidium bromide. In the HPV-16 control lane the expected amplification product of 1.81kb is visible. This fragment is absent in both C3 and C5 lanes, but a band of approximately 0.9-1.0kb is present. No evidence of plasmid contamination of these samples was apparent. No amplification of a similar product occurred in the negative control sample, which lacked template DNA in the reaction mix. This fragment of DNA was unable to be amplified from seven other samples of genomic DNA previously shown to contain HPV-16 sequences by PCR using the E6 ORF primers (section 4.4.2). Additionally, PCR performed on paraffin-embedded tissue sections from cancer C2 (which contained HPV-16 DNA detectable by Southern blot hybridisation) failed to amplify any HPV-16 late region sequence, although part of exon 1 of the human *c*-Ha-*ras* gene could be easily amplified using primers HA12 and HB12 (Table 4.1; Figure 4.23).

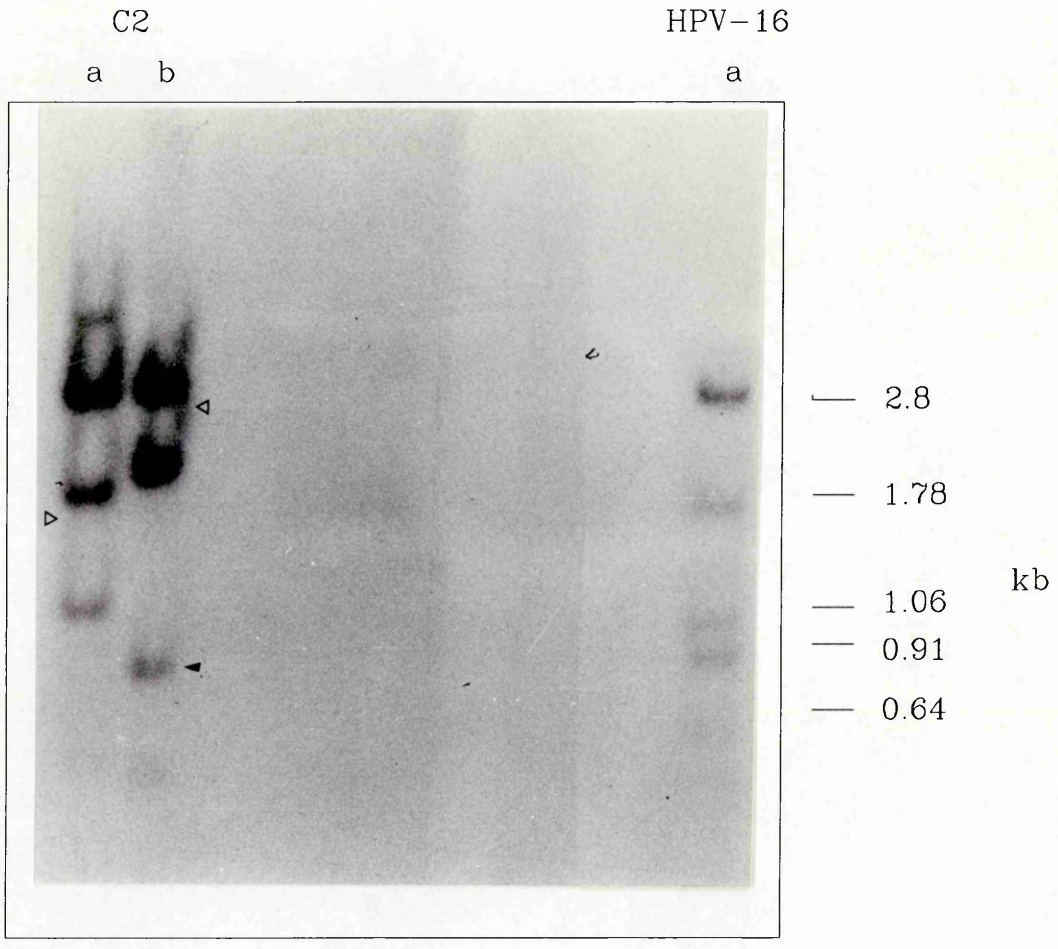


Figure 4.20.

Southern blot of total cellular DNA from sample C2, digested with (a) PstI and (b) HpaII hybridised to a probe comprising the complete genome of prototype HPV-16. The control was 10ng of BamHI linear HPV-16 DNA cut with PstI. The 1.55kb PstI and 2.6kb HpaII wild type bands are absent from the sample (open arrowheads), and an additional band of approximately 0.9-1.0kb is present in lane b (closed arrowhead).

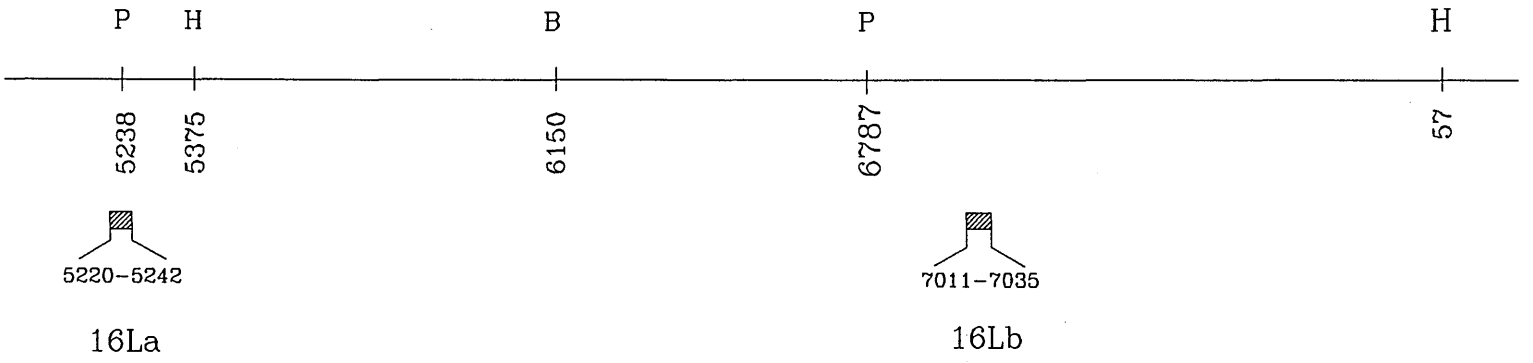


Figure 4.21. Location of oligonucleotide primers for amplification across the deleted/rearranged region of oral HPV-16 by polymerase chain reaction. PstI (P), HpaII (H) and BamHI (B) sites are shown. The expected fragment would be 1815bp long from a prototype HPV-16 template.

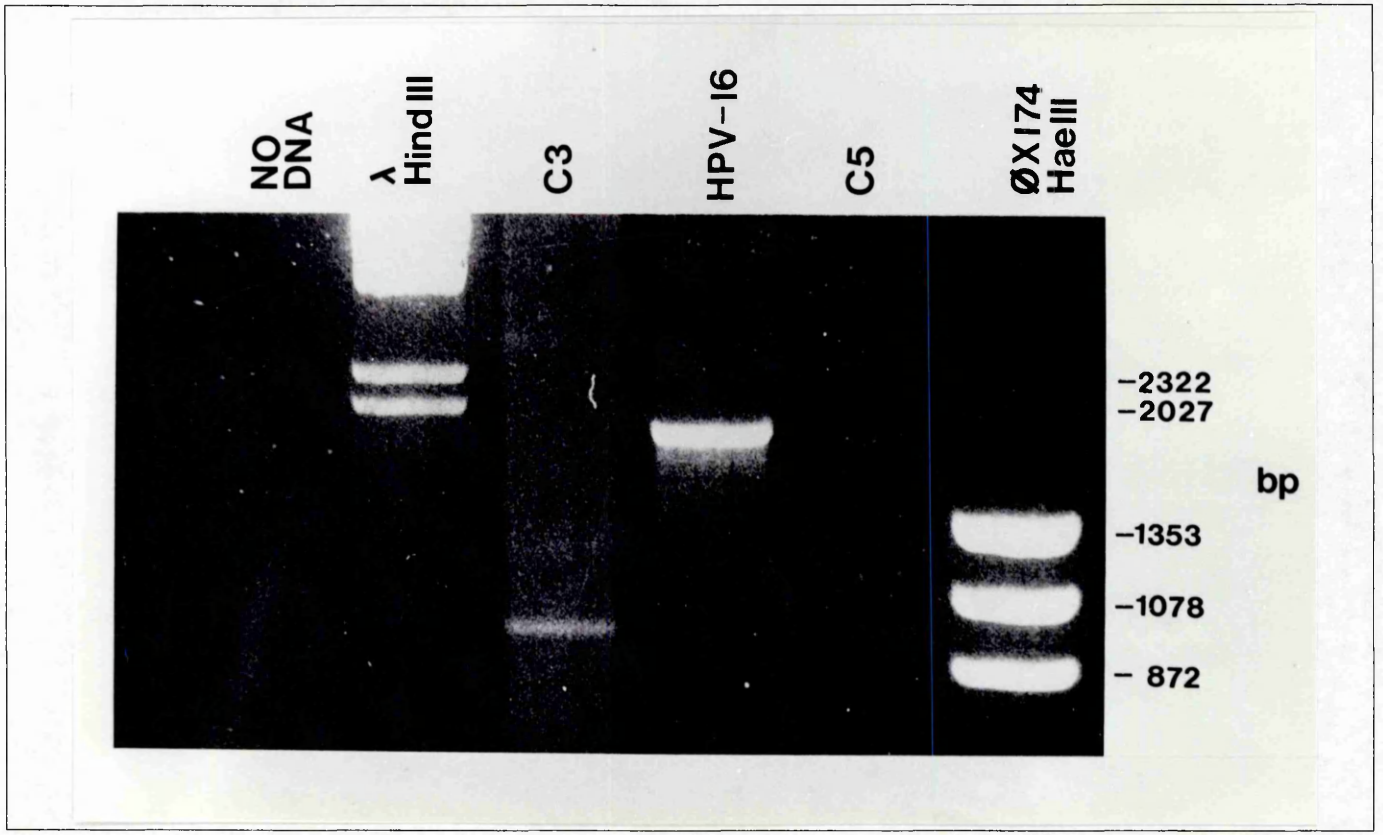


Figure 4.22

Ethidium bromide stained agarose gel of PCR products amplified using the 16La/16Lb primer pair (Table 4.1). Molecular weight markers are bacteriophage lambda DNA cut with HindIII and HaeIII-digested øx174 DNA. HPV-16=control of recircularised HPV-16 DNA; no DNA=control reaction minus target DNA. The expected 1.81kb band is amplified from wild-type HPV-16 DNA, but not from samples C3 or C5 which were positive for HPV-16 DNA using the E6 region primers. However a single band of lower molecular weight is visible in both of these lanes, of approximately 950-1000bp (arrowed).

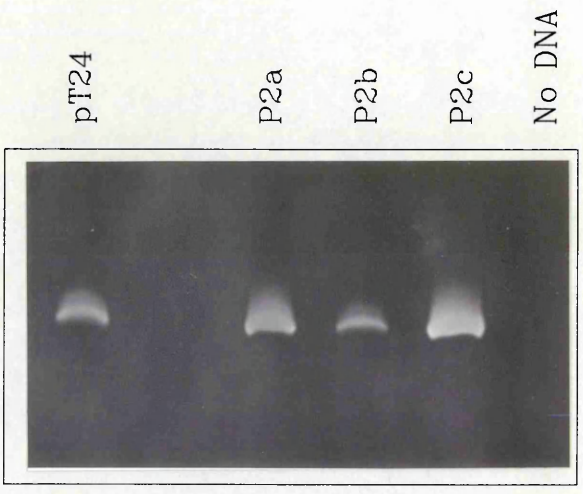


Figure 4.23.

Ethidium bromide stained gel showing the amplification of a 75bp fragment of DNA from three different tissue sections from a paraffin-embedded specimen from carcinoma C2 (lanes P2a-c). 10pg of the pT24 c-Ha-*ras* plasmid (Santos et al., 1982) was used as a positive control for the reaction. A negative control of reaction mix minus template DNA was also included, as before.

4.5.2. DNA Sequence Analysis of HPV-16 Late Region PCR Products.

PCR products amplified from tumour DNAs C3 and C5 using the 16La/16Lb primer pair were gel-purified, isolated and partially sequenced using oligonucleotide 16La as the sequencing primer. The nucleotide sequences thus obtained are listed in Figure 4.24

Homology comparison with the expected 1.81kb sequence from prototype HPV-16 was performed for both C3 and C5 sequences using the UWGCG software. Both sequences showed only very poor homology with HPV-16 DNA, in the regions shown in Figure 4.25, and very poor homology with each other. However only a short region of each DNA was successfully sequenced, and it is possible that a greater degree of sequence similarity might be observed if the entire nucleotide sequences of these PCR products were available. The poor homology is suggestive of recombination of viral sequences in this region.

(a) C3

TGTGTCCTTG TTAAGGATTG TACGTTTAAT CTTGATTCCG AGCCTTGTGC

ATGGAGTCCC ACTGTTTCTG GTGGTCAAGT GCGTAGCTAA AGATGATACT

GCAACGTGGT AGCTAAAGAT GATACTGCAA CCTGATTAGC GCGAAAGAGC

AAAGGGCGCG ACACAGGCAG A

(b) C5

ATTTGCTATT TTCATGGTGG TGGGGTTCAT ACTGTTTGCG TTTACGTCAT

AATATAGAAT GAATCAATAG TCCAATAACT CGTACTTTTA TAGATCTCTA

GTTGCTATAC AAGCATTAGG CTAAATATTA TAGTGATTGT CTAGCTATGG

ATTATCTCTA CTTAATAGAG ATTTATAGGT CACGATACGT TCTAAGTCTA

TTATCAGTAG CGTGTCGTAT ACGAGAGATC TCGGATTATA AGAAGTTATA

TAGCTCATCA CTTCCGATAC GATTGAGTTT GAGATATCTA GCTCTTGCTC

ATATATAAGC TAACATACGA TCTAGATCTC AATTC

Figure 4.24.

Nucleotide sequences obtained by direct sequencing of the 1.0kb PCR products from (a) C3 and (b) C5 (Figure 4.22). Sequencing primer was the 16La oligonucleotide. Sequence comparison revealed only poor homology with the prototype HPV-16 late region in both cases.

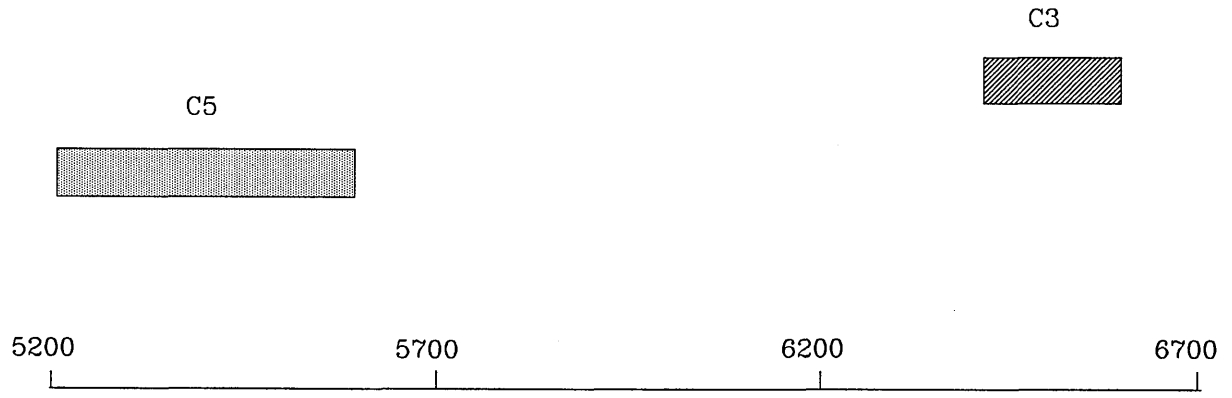


Figure 4.25.

Schematic respresentation of the areas of prototype HPV-16 DNA which show limited homology by nucleotide sequence comparison with the 1.0kb PCR products amplified from oral carcinoma samples C3 and C5 using the 16La/b PCR primers. Homology was low (40%); comparison was carried out using the GAP programme in the UWGCG software package.

4.5.3. Hybridisation of C3 and C5 DNAs to Genomic Sequences.

Genomic DNAs from tumour and normal biopsy samples were digested with PstI and analysed by Southern blotting and hybridisation to ^{32}P-labelled C3 and C5 PCR products initially under conditions of low stringency (T_m-40^{o}C), washed and autoradiographed for 2 days. Blots were subsequently washed at high stringency (Tm-10 ^{o}C) and exposed to radiographic film as before. The results of hybridisation of a C3 probe to 10μg aliquots of tumour DNAs and to normal DNAs (10μg) can be seen in Figures 4.26 (a) and (b) respectively. No discreet bands were observed in any of the samples. The probe hybridised to the smear of genomic DNA in both cases. The difference in signal intensity between the two autoradiograms is probably due to decay of the probe as both blots were hybridised independently, the filter containing normal samples being hybridised after that containing the tumour DNAs. However the possibility of amplification of specific sequences in the tumour DNA samples can not be ruled out. Hybridisation of a C5 probe to the same filters produced a similar result (not shown). This suggests that the DNA fragments amplified from samples C3 and C5 by polymerase chain reaction were either repetitive human genomic elements, or were viral products recombined with repetitive cellular sequences.

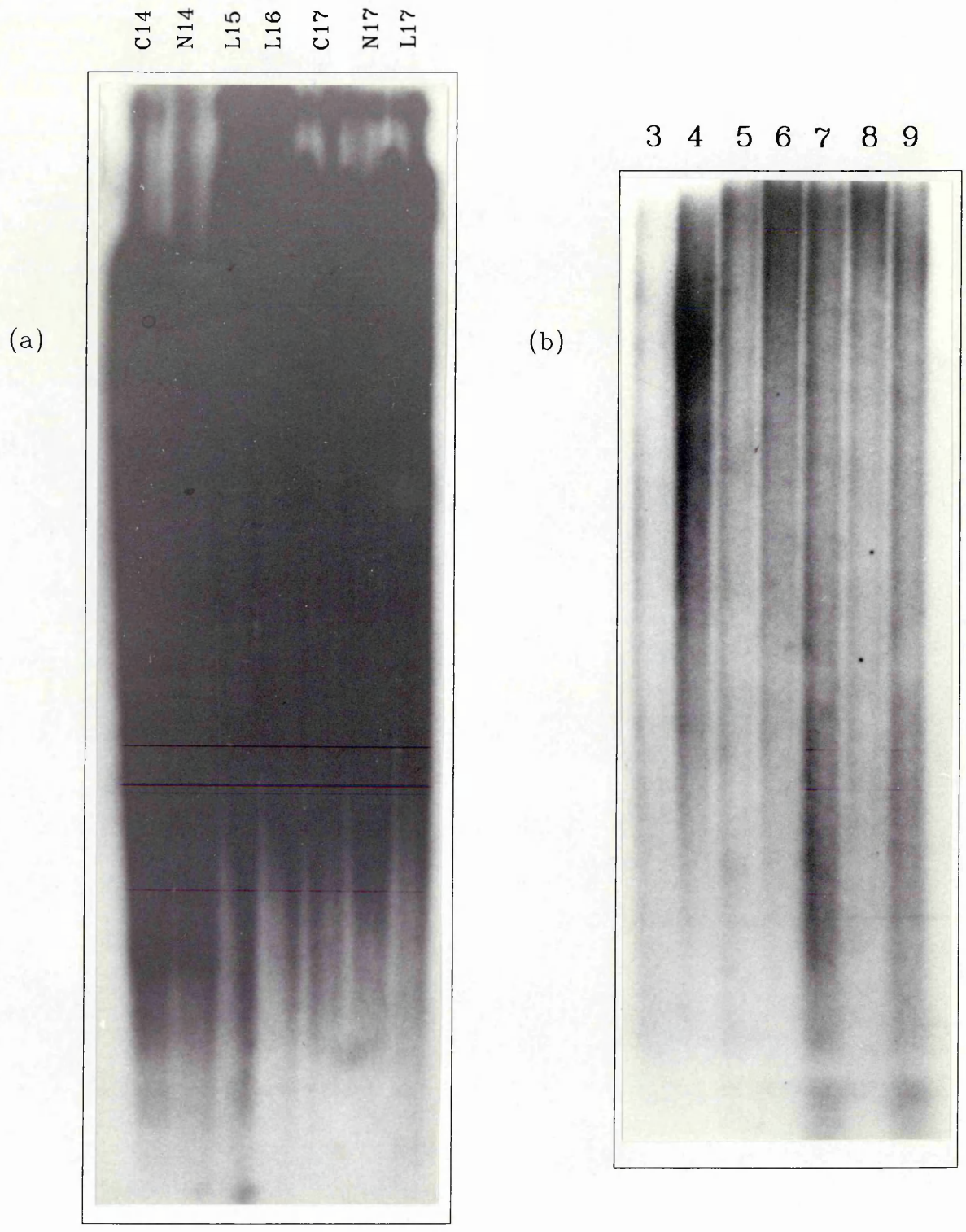


Figure 4.26.

Southern blot of PstI-digested total cellular DNAs from (a) carcinoma and (b) normal samples to the C3 1.0kb PCR fragment probe at high stringency. The same result was obtained probing with the C5 1.0kb PCR product.

4.5.4. Hybridisation of C3 and C5 PCR Products to Prototype HPV-16 DNA.

To further analyse the 1.0kb DNA fragments amplified from tumours C3 and C5 in the PCR, each was isolated, labelled and hybridised to Southern blots of BamHI linear prototype HPV-16 DNA digested with PstI and HpaII under conditions of low stringency. The hybridisation pattern for the C3 fragment is shown in Figure 4.27(a). Both PCR products hybridised to the 1.78kb PstI fragment, but not to the 0.91kb or 0.64kb BamHI-PstI fragments of prototype HPV-16. Hybridisation also occurred in both cases to the 1.81kb BamHI-HpaII fragment of HPV-16, but not to the 0.8kb BamHI-HpaII fragment, which is colinear with the 1.55kb PstI fragment between map coordinates 5375 to 6787 in the prototype genome (Figure 4.27(b)). In view of the nucleotide sequence data obtained for these DNA fragments, which showed little homology with HPV-16 DNA in this region, the lack of hybridisation to the 1.55kb PstI and 0.8kb BamHI-HpaII fragments is not surprising. These data suggest that viral sequences are conserved in the late region of the HPV-16 genomes present in tumours C3 and C5, downstream from position 6787.

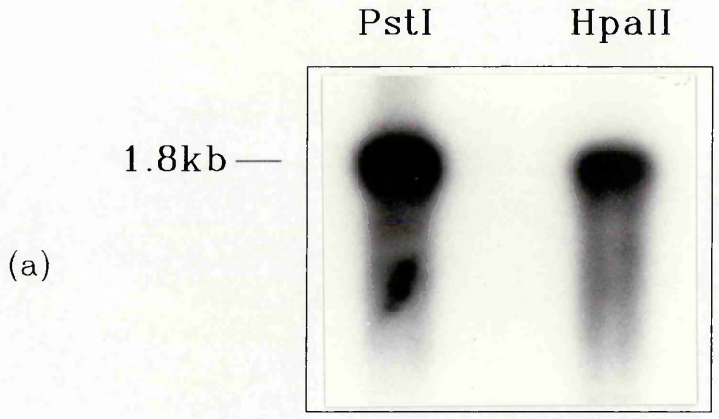
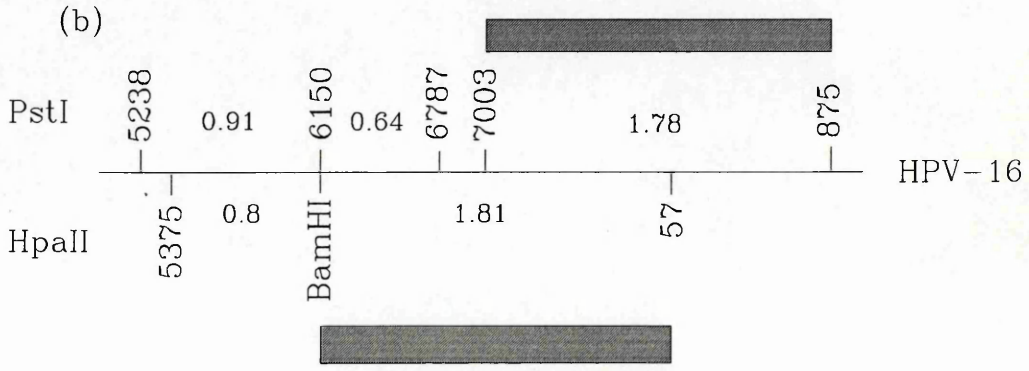


Figure 4.27.

(a) Southern blot of BamHI linear HPV-16 DNA digested with PstI and HpaII and hybridised to labelled C3 PCR product at moderate stringency. No hybridisation was observed to the 0.91kb or 0.64kb BamHI-PstI bands, or to the 0.8kb BamHI-HpaII band. Hybridisation occurred to the 1.78kb PstI and 1.81kb BamHI-HpaII fragments of HPV-16, which are situated 3' to the rearrangement. The relationship of these restriction fragments is shown in (b). PstI and HpaII sites are shown. Fragment sizes are listed in kilobases. The shaded boxes represent the hybridising regions seen in (a) above.



4.6. Culture of Keratinocytes from Oral Tumour Biopsies.

4.6.1. Establishment of Cells in Culture.

One primary culture of cells was established from an oral squamous cell carcinoma biopsy (C45). The cells, designated T45, were dependent on 3T3 feeder cells for growth, and were cultured in medium supplemented with 2% bovine serum, a level at which normal keratinocytes would not be expected to grow. A colony of T45 cells is shown in Figure 4.28, which exhibit morphology typical of keratinocytes in culture.

To verify that the cultured cells were indeed human keratinocytes, they were grown on coverslips on 3T3 feeders for 2 to 3 days, fixed and stained with a mouse monoclonal antibody (LP34) to human keratin, followed by an enzyme-linked secondary antibody. Figure 4.29 shows that the T45 cells stain positively with the LP34 antibody. 3T3 fibroblasts present adjacent to the keratinocytes remained unstained.

4.6.2. Tumorigenicity of T45 Cells.

T45 cells were injected subcutaneously into athymic mice, with appropriate positive and negative control cell lines, firstly at a lower cell number (1×10^6 cells per animal) and secondly using a tenfold

greater number of cells. Tumours were allowed to develop for twelve weeks, or until they reached 1cm in diameter.

Injection of T45 cells, even at ten million cells per mouse, never produced tumours. SCC25 malignant oral keratinocytes always resulted in tumour development after two weeks, even at 1×10^6 cells per animal, as did A431 epidermoid carcinoma cells. Swiss 3T3 fibroblasts were consistently negative for tumour formation. This suggests that, while the T45 cells were derived from a malignant lesion and grow at low serum concentrations, they are not overtly malignant, and may require additional events to be capable of tumour production *in vivo*.

4.6.3. HPV Status of T45 Cells.

Genomic DNA prepared from T45 cells was analysed by PCR using the HPV-16 E6 region primers, together with appropriate controls. A Southern blot of the PCR products was hybridised to a ^{32}P-labelled internal oligonucleotide probe, and Figure 4.30(a) shows that HPV-16 DNA can be amplified from the cell line DNA as well as from the original tumour biopsy DNA (C45). No viral sequence was amplified from negative controls of A431 DNA, calf thymus DNA, or from a sample lacking any DNA.

The PCR products shown in Figure 4.30(a) were isolated and sequenced using the forward PCR primer as sequencing primer. The nucleotide sequences obtained from the HPV-16 control and from T45 cell DNA are shown in Figure 4.30(b). No alterations in

nucleotide sequence were observed in this region of the viral genome, suggesting that the E6 ORF is conserved.

Use of the HPV-16 late region PCR primers consistently failed to amplify any sequences from the T45 DNA sample, or from the original C45 carcinoma DNA. This may reflect rearrangement of the viral genome in this region, perhaps to a greater extent than seen in samples C3 and C5. Alternatively, the viral DNA could have become integrated into the host genome near this site, thus preventing amplification of the expected fragment of DNA by PCR.

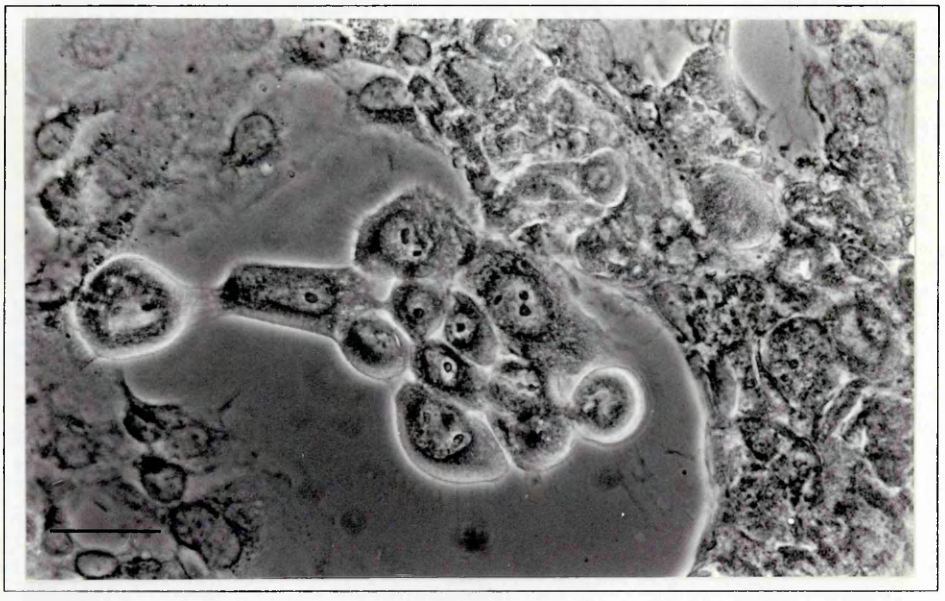


Figure 4.28.

Phase contrast micrograph showing a colony of T45 cells, grown on a feeder layer of lethally-irradiated Swiss 3T3 fibroblasts. Bar =200um.

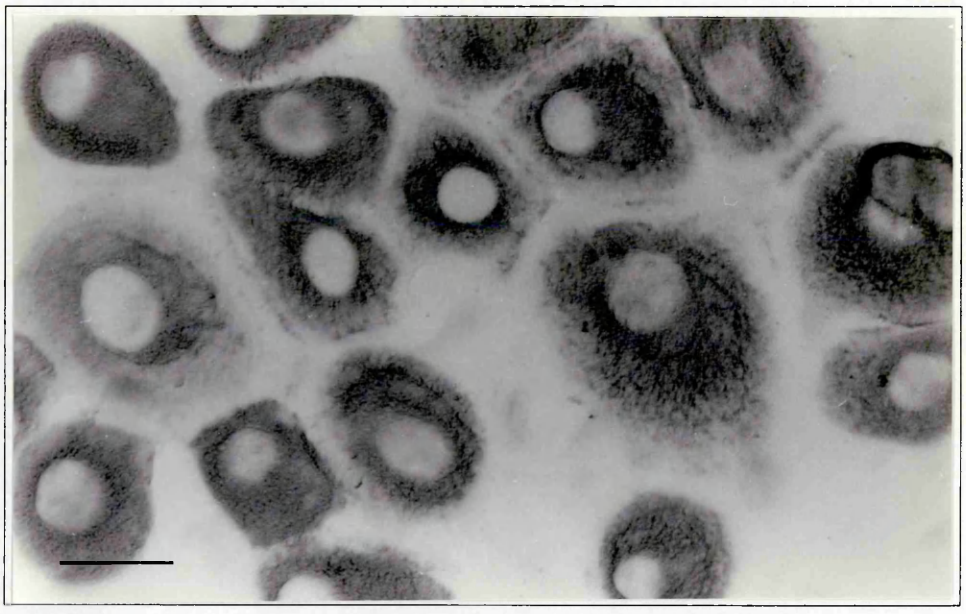


Figure 4.29.

T45 cells stained with the LP34 antikeratin monoclonal antibody and an alkaline phosphatase-conjugated secondary antibody, confirming that they are a keratinocyte population. Bar =100um.

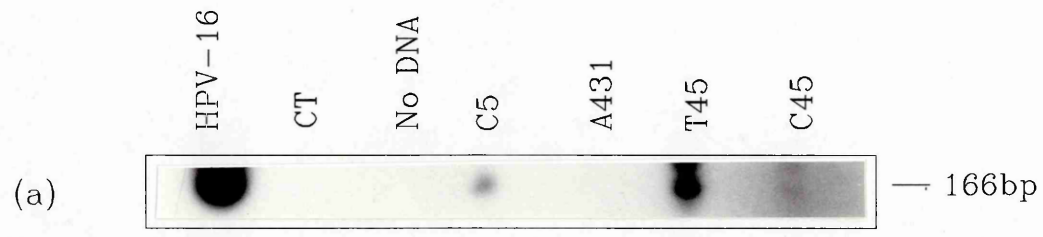


Figure 4.30.

(a). Southern blot of PCR products amplified from a HPV-16 plasmid control, T45 cell DNA and carcinoma samples C5 and C45, using the 16E6 PCR primers. CT=calf thymus DNA; no DNA=control reaction minus target DNA; A431= DNA from A431 epidermoid carcinoma cells.

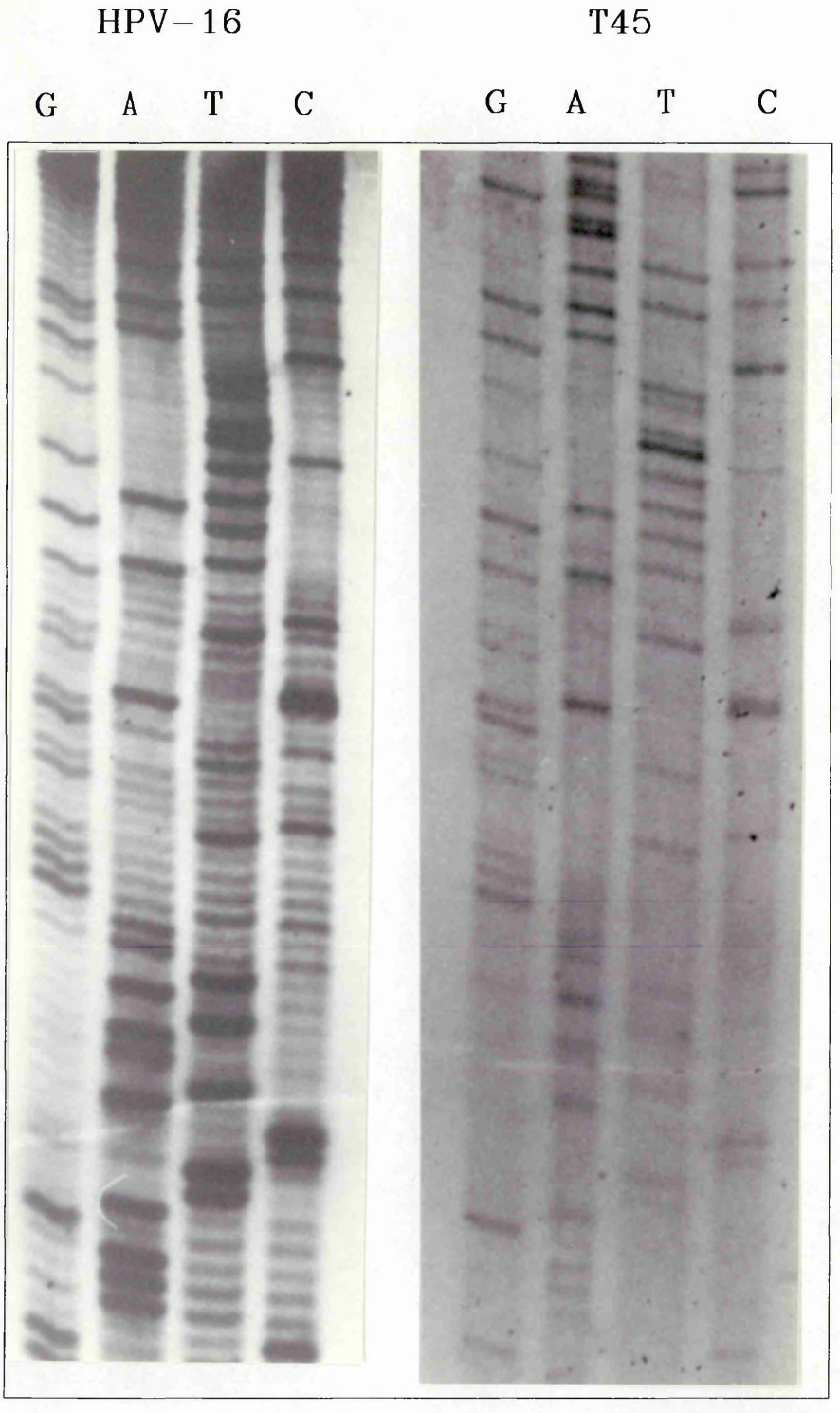


Figure 4.30(b).
Nucleotide sequence obtained by direct sequencing of the HPV-16 control and T45 PCR products in (a). No alterations in sequence were observed.

5. Discussion.

Cancer of the oral cavity accounts for three to forty percent of all human cancer, depending on geographical location. Although the incidence of the disease has decreased since the early part of this century, morbidity and mortality associated with oral malignancy remain considerable.

The aetiology of oral cancer is poorly understood; several agents of both chemical and viral origin, together with other predisposing factors, are possibly involved in the development of the disease, which is almost certainly a multistage process comprising several events over many years. Members of the human papillomavirus group are potentially oncogenic, and this study has examined their association with oral squamous cell carcinomas and dysplasias, as well as their incidence in clinically and histologically normal oral mucosa from cancer patients and from a control population free from malignancy.

5.1. Detection of Viral DNA in Oral Biopsies.

Currently, several methods are available for the detection of specific DNA sequences within heterogeneous nucleic acid samples. This study used Southern blot hybridisation (Southern, 1975) and amplification of DNA by polymerase chain reaction using a

thermostable polymerase (Saiki *et al.*, 1988) in order to detect human papillomavirus sequences in normal and malignant oral tissue biopsies.

The sensitivities of these techniques differ; using DNA probes radiolabelled with ^{32}P and a conventional Southern blot hybridisation protocol, it was possible to detect a single copy cellular gene (*c-myc*) in 10μg restriction enzyme digests of genomic DNA. Thirty picogram aliquots of complete HPV genomes (approximately equivalent to one copy of an 8kb genome per cell in 10μg of cellular DNA) could also be detected by this method. In samples C1, C2 and C4 viral DNA present in multiple copies was easily visible within several hours of exposure to high speed radiographic film.

In contrast, the use of the polymerase chain reaction enables detection of DNAs which are present at an apparent frequency of much less than one copy per cell. Although HPV-4 DNA was only visible in the cancer DNA from sample 4 when conventional Southern blot hybridisation was used, viral sequences were clearly present in the dysplastic and normal DNA preparations from the same patient when a fragment of the HPV-4 sequence was amplified using specific oligonucleotide primers, Southern blotted and hybridised to a radiolabelled homologous DNA probe. Other cancer DNA samples, previously negative for viral DNA by Southern blot hybridisation, were also shown to contain HPV-16 and HPV-18 sequences by this method.

The presence of viral DNA at less than one copy per diploid genome arises because of the nature of the samples being analysed. If the study

had utilised clonal cell populations one would expect HPV sequences to be present in all cells or in none, dependant on whether or not the original cell from which the clone was derived contained viral DNA. That is, the minimum viral copy number in an HPV-positive clone would be one HPV genome per cell. However when dealing with tissue biopsies the population is naturally heterogeneous. Malignant keratinocytes may be contaminated with normal cells or with underlying fibroblasts, muscle cells and inflammatory cells, and it is virtually impossible to prevent this completely, although as much care as possible was taken when sampling resected material, to separate normal and malignant epithelia from each other and from other tissue types. As a result, DNA prepared from carcinoma biopsies probably contained some contaminating normal DNA, providing the first dilution factor.

A second and probably more significant factor is involved in reducing the overall level of viral sequences found in biopsy DNAs. From previous studies of benign and malignant HPV-associated oral disease using *in situ* hybridisation of HPV probes on histological sections (Adler-Storthz *et al.*, 1986a,b; Syrjanen *et al.*, 1986; Milde and Loning, 1986) it is clear that not all cells in a lesion contain viral DNA. On the contrary, cells harbouring HPV DNA tend to be found clustered together throughout the epithelium. Furthermore, some HPV-positive cells contain greater or lesser amounts of viral DNA than others. A similar type of distribution of viral DNA is also seen in cervical epithelia infected with human papillomavirus types (Wells *et al.*, 1987). However the *in situ* technique is limiting in its sensitivity; the lower limit of detection is around five to twenty copies of HPV sequence per cell and, while of much use where there is a productive

viral infection, in view of the results obtained here would not have been a viable alternative method of detecting papillomavirus genomes in oral samples.

Primary cell cultures derived from oral squamous cell carcinomas also appear to harbour viral sequences in less than one hundred percent of cells, as seen in the T45 culture in this study and in those derived by other workers (Maitland *et al.*, 1989). Interestingly, these authors describe the possible selection against cells containing HPV DNA with passaging in tissue culture. This may reflect an early role for papillomaviruses in cell transformation, with cellular genes taking over the maintenance of the transformed phenotype, as is seen in BPV-4-induced transformation of bovine epithelium (Campo *et al.*, 1985) and C127 fibroblasts in culture (Smith and Campo, 1988), and viral sequences being no longer required.

Amplification of specific short regions of HPV DNA in the polymerase chain reaction is extremely useful for detection of low amounts of viral nucleic acids in a background of uninfected cells (Maitland *et al.*, 1989). However the problem of contamination of samples with viral DNA from external sources is not inconsiderable, in view of the sensitive nature of the assay. Such sources include cloned HPV plasmids present in the laboratory, products of previous amplification reactions and, probably to a lesser extent in this case, contamination from the operator.

Potential contamination was successfully overcome in this study by several means. Firstly, a facemask and disposable gloves were routinely worn; gloves were changed frequently. A separate set of

automatic pipetting devices was used for setting up PCR samples, distinct from those used for other manipulations in the laboratory, in case these had become contaminated by aerosols. The problem of aerosol-mediated contamination was further reduced by using only positive displacement pipettes, which expel the solution from the pipette tip by means of a piston in contact with the solution. Additionally, microfuge tubes containing reactants which had been incubated at 95^{o}C to denature the DNA template were centrifuged briefly to remove traces of solution from the undersurface of the tube cap, before careful opening to avoid splashing of contents. Completed reactions were similarly treated.

Where possible, the use of cloned DNA as a positive control was avoided, HPV-containing cell lines (such as SiHa and HeLa) being preferred. When HPV plasmids were used, only femtogram amounts were added to the reaction mixture, which was set up separately from the case samples. Negative controls were also included for each set of reactions performed. These included 1μg aliquots of commercially-available calf thymus or salmon sperm DNA, as well as a tube which contained each component of the reaction mix except template DNA. This was routinely pipetted last, in order to control for the possibility of contamination during the setup procedure. (target sequences consistently failed to be amplified from such negative controls, as would be expected.

Spatial separation of polymerase chain reaction components from the products of amplification reactions was also ensured in this study. Although a second room was not used for analysis of PCR products, gel samples were prepared, loaded and isolated on a separate bench,

distant from that used to prepare reaction mixtures. The combination of all these points led to successful avoidance of sample contamination in all experiments involving PCR amplification of HPV sequences.

In order to confirm that HPV sequences detected in some samples were not due to the presence of small amounts of HPV plasmids, a second amplification was performed on each using anticontamination primers situated on either side of the cloning site of the original plasmid, BamHI for HPV-16 and EcoRI in the case of HPV-18. This resulted in amplification of a short product (170bp) from all samples which had previously been shown to contain HPV-18 DNA using the E6 region PCR primers. If the E6 region product had been due to the presence of contaminating HPV plasmid, the 170bp band would not have been amplified. Instead, one would have expected to see a band of 4535bp, reflecting the length of the viral sequence plus the intervening pBR322 sequence.

However, the use of the anticontamination primers described above have several disadvantages. Firstly, as the length of the amplified fragment is increased, the efficiency of amplification appears to be reduced. The lower limit of detection of a 4.5 kb HPV-plasmid hybrid sequence was reached when attempting to amplify from cloned HPV DNA present at 10pg in a 100μl reaction. Taq polymerase is able to synthesise new DNA strands at a rate of 2000-4000 bases per minute. Although an extension time of five minutes should have been more than adequate to synthesise a DNA strand of this length, it is probable that the lack of amplification resulted from inadequate processivity of the enzyme during the early stages of the reaction, failing to provide sufficient new DNA template for priming in subsequent cycles. In

retrospect, the use of one primer within the HPV sequence and a second within the plasmid would have been a more sensible combination, and would have resulted in easy and efficient amplification of any available HPV-plasmid target sequence present in the samples. To facilitate design of a primer within the plasmid sequence, the orientation of the cloned HPV would need to be known.

Another disadvantage of the HPV-16 anticontamination primers employed in this study arises because of their location within the viral genome. Both were situated within the L1 open reading frame, a region which is frequently lost in cervical carcinomas during integration into the host genome (Wilczynski *et al.*, 1988) and which other workers have previously failed to amplify from oral tissues (Maitland *et al.*, 1989), possibly due to a variation in nucleotide sequence in this area compared to the prototype HPV-16 DNA. The hybridisation pattern seen on the Southern blot of sample C2, the reduced size of the PCR products from C3 and C5, and the variant sequences of the latter suggests that this was also a problem in the current study. It is highly likely that the lack of the expected 152bp HPV-16 band in samples previously shown to be positive for HPV-16 sequences using the E6 and E7 region PCR primers was due to the failure of the late region oligonucleotides to anneal to their intended target sequences due to absence or rearrangement. The use of a set of primers spanning the virus-plasmid junction, as described above, would have resulted in amplification of a short PCR product in the case of plasmid contamination, or no product in samples where the HPV-16 DNA had integrated or showed sequence divergence of the late region of the genome.

5.2. Frequency of HPV Sequences in Oral Tissues.

Southern blot analysis of DNA from oral squamous cell carcinoma biopsies revealed a low level of HPV DNA (7.7%), with three carcinomas out of a total of thirty nine found to harbour the DNA of HPV-18, HPV-16 and HPV-4. Compared to some previous reports (Maitland *et al.*, 1987; Chang *et al.*, 1989), which described viral DNA in 46% and 76% of oral cancers respectively using a similar screening procedure, the incidence of HPV DNA as detected by Southern blot hybridisation in this study was much reduced. The reason for this difference is unclear; it is possible that the prevalence of papillomaviruses varies between the different geographical regions from which the sample populations were derived, and that patients are infected to a different degree.

When the PCR method of screening was employed, however, the frequency of papillomavirus DNA found in carcinoma DNA samples in this study increased to 48.7% (nineteen out of thirty nine samples). This may reflect an overall lower level of infection in the population studied here compared to those analysed by other workers. Alternatively, it may be a consequence of different procedures for sampling tissues by different groups, with HPV-containing cells diluted to a degree by the presence of uninfected cells.

In the three samples positive for papillomavirus sequences by Southern blot hybridisation, no viral DNA was detected in dysplastic

or normal tissues adjacent to the carcinomas by this method. Amplification of viral DNA in the polymerase chain reaction coupled with Southern blotting and hybridisation proved to be a more sensitive method of detecting viral nucleic acids in biopsy samples, as described in the previous section. This enabled the demonstration of HPV DNA in the non-malignant tissues adjacent to the HPV positive cancers, present at a greatly reduced level compared to the corresponding carcinomas. Furthermore, the same type of viral DNA was present in carcinomas, dysplasias and normals in all three cases. No evidence of multiple papillomavirus infection was observed in any of the samples studied. It is unclear why this should be so. One could argue that increased levels of viral DNA might be directly responsible for the transition of normal epithelium to a malignant state. Increased viral copy number, perhaps resulting in higher overall levels of the HPV-16 and HPV-18 oncoproteins might be one way of forcing previously initiated epithelial cells towards malignancy. Although the E6 and E7 gene products of cervical isolates of HPV-16 are recognised as performing immortalising functions (Hawley-Nelson *et al.*, 1989; Bedell *et al.*, 1989), HPV-16 DNA is also capable of inducing malignant transformation of previously immortalised cell lines such as NIH3T3 (Yasumoto *et al.*, 1986). However this does not explain why the remaining sixteen HPV-positive carcinomas were found to harbour viral sequences at low levels, similar to those found in the corresponding dysplastic and normal biopsies.

Using this approach to rescreen the available material forty eight percent of samples from cancer patients were found to harbour papillomavirus sequences, a similar overall incidence to that reported by Maitland *et al.* (1987). However these authors found only HPV-16

DNA, whereas in this study the prevalence of HPV-16 was only marginally greater than that of HPV-18 DNA (26% and 20.5% respectively), with HPV-4 sequences being an isolated finding. Other workers in the USA have found HPV-18 DNA more frequently than HPV-16 in oral cancers (K. Storthz, personal communication).

In all cases, except for two containing HPV-18 DNA, viral sequences were detected in cellular DNA from both normal and malignant samples, and in DNA prepared from associated lymph nodes (present in three cases). This is the first study to examine the distribution of HPV DNA in tissues adjacent to oral carcinomas, and strongly suggests that not only carcinoma cells harbour viral nucleic acids. It is possible that, due to the sensitivity of the PCR technique, a small amount of contamination of normal cells may have resulted from the nearby carcinoma. However there is no reason to suspect that the tissues adjacent to HPV-infected carcinoma cells should be free from viral sequences, as several reports have documented HPV DNA in normal genital and oral mucosa (MacNab *et al.*, 1986; Maitland *et al.*, 1987). From the data presented here, it is likely that equivalent amounts of papillomavirus DNA were present in normal and malignant oral epithelium in the majority of cases in the present study, as viral sequences were amplified to the same degree from both tissue types and no viral sequences were detected in either by conventional Southern blotting.

An accurate estimation of copy number and comparative levels of HPV infection between normal and malignant tissues could be achieved by using a co-amplification strategy, such as that described by Frye *et al.* (1989). A standard PCR protocol using HPV primers

together with a pair of primers for a single copy cellular gene would control for unequal levels of amplification between samples, thus allowing a meaningful comparison to be made.

The majority of investigations of papillomaviruses in human cancers have concentrated on the association of the so-called oncogenic genital viruses, mainly HPV-16 and HPV-18, with squamous cell carcinoma of the uterine cervix. The frequency of HPV infection in human genital cancers is high. Around 90% of biopsies from such lesions harbour HPV sequences (reviewed by zur Hausen, 1988); 50% contain HPV-16 DNA, 20% contain HPV-18 DNA and a further 20% other HPV types. Compared to this, the frequency of papillomavirus infection in oral carcinomas is low, according to the data available thus far.

One explanation for this is that new HPV types exist in oral epithelium, which either do not cross-hybridise to the probes which are currently available, or are present at such low levels that they are outwith the detection limits of Southern blot hybridisation. This may indeed be so, if consideration is given to the amounts of HPV DNA found in this study. If polymerase chain reaction technology is required to render oral HPV infections detectable, then this will present a problem for the isolation of new virus types, unless there is some degree of redundancy of oligonucleotides primers used which could then anneal to a region of the new HPV sequence. PCR primers with several base mismatches have been used by other workers to amplify several different HPV types (Snijders *et al.*, 1990). If regions of the genome showing high degrees of homology between papillomavirus types were used as amplification targets, together with

a reduced stringency of primer annealing, the PCR could be a useful tool for isolation of novel HPV types present at low abundance in oral and other tissues.

Another possibility which might account for the different frequencies of HPV DNA in cervical and oral epithelia is that the viral DNA has a different role to play at each site. In cervical carcinoma biopsies and cell lines studied to date, continued transcription of the E6 and E7 ORFs has been shown to occur (Yee *et al.*, 1985; Schwarz *et al.*, 1985; Schneider-Gadicke and Schwarz, 1986). This is entirely consistent with the *in vitro* evidence, which demonstrates that the E6 and E7 gene products of HPV-16 and HPV-18 are competent in the transformation of primary cells together with a second oncogene (Storey *et al.*, 1988; Barbosa and Schlegel, 1989), and that continued expression of the E7 ORF is required for maintenance of the transformed state of these cells (Crook *et al.*, 1989b). In contrast, no data have been published so far on the expression of these viral gene products in either oral carcinoma biopsies or cell lines. If E6-E7 expression does not occur in oral carcinomas, one might be tempted to speculate that the virus acts during the earlier stages of malignant progression, perhaps switching on a cellular target sequence or acting as an insertional mutagen.

Continued presence and expression of papillomavirus transforming genes may not be necessary for oral keratinocytes to retain their malignant phenotype, as seems to be the case in the HPV model of cervical carcinogenesis. Other *in vitro* systems have been described where papillomavirus DNA has been shown to transform cells in culture where, in spite of the loss of viral sequences with passaging of

the cells, the transformed phenotype is maintained. Transfection of C127 mouse fibroblasts with the DNA of HPV-6b produced transformed cells which, after several passages, exhibited tumorigenicity *in vivo* (Morgan *et al.*, 1990). These cells contained little or no viral DNA, suggesting that the transformed phenotype did not require the continued presence of HPV-6b sequences. To exclude the possibility that viral sequences were suppressing tumorigenicity, these authors also demonstrated that the transformed nature of the cells was maintained even when virus-containing cells were selected for. This strongly suggests that, firstly, HPV-6b DNA has transforming activity and, secondly, it acts as an initiator of transformation in these cells, after which it is no longer required. Maintenance functions might then be attributable to activity of specific cellular genes which had perhaps been subject to *cis*- or *trans*-activation by the papillomavirus DNA. However, Morgan and coworkers (1990) were unable to detect any increase in the transcriptional activity of a variety of cellular oncogenes, or any transforming mutations of *ras* oncogenes.

A similar situation has also been shown to exist where C127 fibroblasts are transfected with BPV-4 DNA (Smith and Campo, 1988). After several passages viral DNA is lost from the vast majority of cells, although they remain transformed. This mirrors the situation *in vivo*, where BPV-4 DNA can be isolated from benign neoplasms of the upper alimentary canal of cattle, but is rarely present in malignant tumours (Campo *et al.*, 1985). Furthermore, in the *in vitro* situation, it has been demonstrated that the introduction of viral DNA results in amplification of specific cellular sequences, which in subsequent

transfection experiments are capable of inducing cell transformation (Smith and Campo, 1989).

It is therefore clear that papillomaviruses are capable of inducing cellular transformation by more than one means, dependant on the type of virus involved and the nature of the target cell. Thus HPV DNA might not be required to be present in transformed oral keratinocytes for the malignant phenotype to be maintained. This would help to account for the differing incidence of HPV DNA in oral and cervical squamous cell carcinomas reported to date.

The possibility exists, however, that the presence of HPV DNA in oral cancer tissues is unrelated to the development of malignancy. Chemical carcinogens alone might be capable of initiation of oral epithelium and, subsequently, tumour promotion, or they could act together with other environmental or genetic factors (such as allelic deletion of a tumour suppressor gene). Papillomavirus infection might then be secondary to tumour development, with the virus taking advantage of an impaired host immune system to opportunistically infect the oral mucosa. Papillomavirus infection is a well-recognised feature of several diseases where there is a defect of host immunity. Patients suffering from epidermodysplasia verruciformis become infected with a variety of HPV types, some of which are associated with malignant progression of skin warts to frank carcinomas at sites exposed to UV radiation. Renal transplant recipients who are chemically immunosuppressed also frequently develop skin warts, and HIV infection predisposes sufferers to HPV-associated disease, frequently in the oral cavity (Greenspan *et al.*, 1988). Such infections may be the result of a defective cell-mediated host immune response

where cells infected with virus (or indeed transformed) are unable to be recognised as foreign, thus escaping the normal immune surveillance mechanism.

Alteration of immunological recognition mechanisms as a result of HLA class I gene rearrangements has previously been shown to occur in several human tumours (Bar-Eli *et al.*, 1988; Natali *et al.*, 1989; Lopez-Nevot *et al.*, 1989), and although no data are available at present regarding HLA gene loss or altered expression in oral cancers, this work is currently being carried out. It would be interesting to compare host immune status in papillomavirus-positive and negative oral carcinomas. This may give an indication of whether HPV infection is likely to be involved in malignant transformation of oral epithelial cells, or is merely an opportunistic infection secondary to tumour development.

A further reason why papillomavirus DNA has not been reported in one hundred per cent of oral cancers might be that the oral cavity is serving solely as a reservoir for these viruses, which are not involved in development of malignancy but are able to infect epithelial cells at other body sites. This might explain why similar HPV types are found in both the oral cavity and the genital tract, with transmission between the two sites occurring during oro-genital contact. If the frequency of papillomavirus infection was the same in both normal and malignant oral epithelium, as reported by Maitland *et al.* (1987), it would be tempting to speculate that the oral cavity was merely acting as a reservoir for HPVs. However the incidence of papillomavirus DNA in normal mucosa from cancer-free individuals in this study was much lower than that found in either frank cancers or in clinically normal

tissues from cancer patients (eight per cent compared to forty nine per cent) and does not support this hypothesis.

5.3. HPV-18 DNA in Oral Samples.

Southern blot analysis revealed HPV-18 DNA to be present in one oral carcinoma at high copy number, not an unexpected finding in view of its potential oncogenicity, its association with cancers at other sites and its propensity to infect the epithelium of mucous membranes, similar to HPV-16. When the polymerase chain reaction was used to increase the sensitivity of detection, HPV-18 DNA was found to be present in around twenty per cent of oral cancer biopsies and adjacent normal tissues from patients in this study; this is a similar incidence to that quoted previously for HPV-18 in cervical carcinomas (zur Hausen, 1988). This suggests that HPV-18 may be involved to an equal extent in both oral and cervical carcinogenesis, although one can not rule out the possibility of the oral cavity acting as a reservoir of HPVs.

The HPV-18 detected in carcinoma C1 showed aberrant restriction digestion patterns for both PstI and HpaII, but in the form of additional sites for these enzymes in the E1 ORF instead of absence of restriction fragments as was the case for HPV-16 DNA described above. A different polymorphism of PstI restriction sites of HPV-18 DNA in genital lesions in the west of Scotland has been reported by other workers (Millan *et al.*, 1986), suggesting that a degree of heterogeneity of this viral type exists in the area. HPV-18 DNA has only been described in association with oral malignancy in one

previous study (Syrjanen *et al.*, 1988). In that case, though, detection was by *in situ* hybridisation and therefore no detail of restriction digestion patterns was available.

An interesting finding was the detection of HPV-18 DNA in two samples of normal oral epithelium from cancer-free individuals. Maitland and co-workers (1987) previously reported HPV-16 DNA in biopsies of normal oral tissues, but this is the first demonstration of HPV-18 in a similar situation. Overall, this study found a much lower level of papillomavirus DNA in normal oral epithelium (8% of samples) than these authors (42%), probably reflecting different levels of exposure of each population to HPV infection, as discussed previously. The type of viral DNA found also differs between these two studies; exclusively HPV-16 DNA was detected in the south of England population, whereas this study found only HPV-18 sequences. This result reflects the viral types found in oral carcinomas in both studies, and mirrors the incidence of HPV-16 and HPV-18 DNA in cervical lesions in different parts of the UK (Millan *et al.*, 1986).

The significance of finding HPV-18 DNA in histologically normal oral mucosa obtained from cancer-free subjects in at present unclear. This may represent a latent infection of oral mucosa with this viral type, with the viral life cycle being suppressed by normal cellular control mechanisms. In a recent study on control of HPV-16 gene expression in cultured human foreskin or cervical epithelial cells immortalised with HPV-16, Woodworth *et al.* (1990) demonstrated that transcription of early viral genes was modulated by the action of the growth factors TGF-β1 and 2, although cell growth was not

reduced. This may be a means by which the host is able to down-regulate the transforming genes of papillomaviruses in normal, non-transformed keratinocytes.

Alternatively, the cells containing viral DNA may be initiated while still exhibiting a normal phenotype. These may remain dormant for a considerable time, during which they might be subject to the action of other carcinogenic agents, such as several constituents of tobacco smoke, eventually resulting in a fully malignant phenotype.

In order to compare oral isolates of HPV-18 with the original genital isolate, nucleotide sequencing of HPV-18 E6 PCR products amplified from one carcinoma and two normal samples was carried out. This did not reveal any alterations in base composition over the region of DNA amplified from oral tissues which was sequenced compared to that of wild-type HPV-18. This is not surprising, considering that the E6 and E7 ORFs are retained intact in cervical carcinoma tissues and cell lines (Seedorf *et al.*, 1987; Smotkin and Wettstein, 1987) and are required for viral transformation functions *in vitro* (Bedell *et al.*, 1989). In view of the altered restriction pattern of the HPV-18 DNA in carcinoma C1, it would be interesting to amplify and sequence the E1 ORFs of these oral isolates to discover if there is a consistent difference in nucleotide sequence resulting in the creation of additional restriction sites in this region, and allowing identification of a specific geographical variant.

Transformation of cells by HPV-18 DNA has been shown to occur with around a ten-fold higher efficiency than transformation by HPV-16 DNA (Barbosa and Schlegel, 1989). This difference has now

been mapped to the viral LCR-E6-E7 region (Villa and Schlegel, 1991), who also demonstrated that the difference was consistent between separate isolates of HPV-18 and HPV-16 from cervical, vulvar and penile epithelium. Thus it is likely that similar differences in transformation efficiency between virus types will be inherent in HPV-18 and HPV-16 isolated from oral epithelium. As Villa and Schlegel (1991) rightly note, the presence of HPV-18 DNA rather than HPV-16 sequences in carcinomas may give more cause for concern, in view of the former viral type being associated with a more aggressive tumour phenotype. Comparison of survival rates for oral cancer patients with HPV-18 and HPV-16 positive lesions might help to substantiate this theory.

Foreskin keratinocytes immortalised *in vitro* by HPV-18 DNA have recently been shown to progress to a fully malignant phenotype with continued passaging in culture (Hurlin *et al.*, 1991). No alteration in expression of E6 or E7 genes was noted in tumorigenic compared with non-tumorigenic cells; however a deletion of a region of the long arm of chromosome 5 was consistently noted. It is likely that this chromosome encodes a tumour suppressor element, in view of the finding that complementation of colon carcinoma cells with a normal chromosome 5 results in loss of tumorigenic activity (Tanaka *et al.*, 1991). Thus the initial step in HPV-18-induced oral or cervical epithelial carcinogenesis might involve the E6 and E7 oncoproteins acting on cellular p53 and $p105^{Rb}$, with additional genetic events further reducing cellular control.

5.4. HPV-4 DNA in Oral Lesions.

An unusual finding was the presence of HPV-4 DNA in one oral cancer sample (C4). This viral type was originally isolated from a benign skin lesion (Heilman *et al.*, 1980) and is not generally considered to have a high oncogenic potential or to be associated with mucosal epithelium. Although one previous study has described HPV-4 DNA in benign oral warts (Adler-Storthz *et al.*, 1986a), the present work is the first demonstration of this viral type in a malignant lesion at any site. In order to facilitate comparison of the oral isolate of HPV-4 with the prototype from epidermis, viral DNA was molecularly cloned from cancer C4. Two inserts positive for HPV-4 sequences were obtained; the 8kb insert co-electrophoresed with the prototype HPV-4 DNA and represents the full-length viral genome. Comparative restriction enzymes analysis of both viral DNAs revealed no differences between the two isolates at this level, but nucleotide changes which do not create or delete a restriction site for one of the enzymes used would not be apparent.

Further analysis by sequencing a region of both HPV-4 clones again failed to show any differences over the three hundred and eighty nucleotides sequenced. Again, this does not prove identity between the clones; it is possible that the sequenced region is one of functional significance, and is therefore conserved. Homology comparison of the available HPV-4 sequence with those of other papillomaviruses revealed only poor homology with the HPV genomes sequenced to date at the nucleotide level. This reflects and confirms the isolated

position of HPV-4 in the phylogenetic tree of papillomaviruses (Pfister, 1984).

Using the TRANSLATE programme contained in the UWGCG sequence analysis software package, a region coding for a putative protein greater than seventy seven amino acids in length was identified, starting upsteam to nucleotide 1 of the sequence and reading through to a TAA termination codon at nt 232-234. Protein homology comparison of the potential coding region was performed with the previously identified proteins of HPV-6b, HPV-16 and HPV-18 using the programme BESTFIT. From the data obtained, it is likely that the region sequenced is the 3' portion of the HPV-4 E2 ORF. Further sequencing and homology comparison will confirm this.

To exclude the possibility that the region sequenced was the LCR, the nucleotide sequence was searched for potential regulatory elements present in other papillomavirus control regions. Using BESTFIT, no binding sites were identified for the papillomavirus E2 protein, the AP1 complex, or NF1. In view of the lack of regulatory sites and the potential to code for a protein, it is unlikely that the region of HPV-4 sequence obtained is the LCR.

From the limited restriction analysis carried out on the shorter 6kb fragment, cloned at the same time as the full-length HPV-4, it would appear that approximately 2kb of sequence upstream of the BamHI site of the prototype virus (Heilman *et al.*, 1980) is absent. Nucleotide sequencing of a region of the 6kb DNA 3' to the cloning site using the same sequencing primer as for the 8kb DNA revealed identity with the previously obtained sequences of both full-length

HPV-4 genomes over the 150 bases sequenced. It is likely that this shorter clone is an artefact of the cloning procedure, either by the creation of an additional BamHI site internally in the HPV-4 molecule or by an illegitimate recombination between vector and viral sequences. The possibility exists that the 6kb fragment represents a second HPV-4 genome present in the cancer and which harboured an internal deletion, or one containing an additional BamHI site. This is unlikely as there was no evidence of a second HPV-4 genome in the carcinoma from which the clone was derived by Southern blot analysis, although it might have been present at a very low level undetectable by this method. Comparison of the complete nucleotide sequences of the 8.0kb and 6.0kb DNAs would resolve this point.

The HPV-4 DNAs described above may be worthy of a more detailed analysis than was performed here, such as co-transformation experiments similar to those carried out for HPV-6, 11, 16 and 18 (Storey *et al.*, 1988; Storey *et al.*, 1990). The DNAs of HPV-6 and HPV-11, commonly found in benign genital mucosal lesions, have low transformation efficiencies *in vitro* (Storey *et al.*, 1990) when used in a co-transformation assays together with an activated Ha-*ras* oncogene, but the cells transformed by these viral DNAs are extremely tumorigenic when transplanted into immunocompetent animals. These authors were also able to show that the ability of HPV-11 DNA to transactivate a viral promoter was similar to that of HPV-16 DNA. If HPV-4 possessed similar transactivating potential, it might be able to upregulate transcription of other cellular genes involved in growth regulation.

To strengthen the case for a transforming role for HPV-4 in the oral carcinoma described in this study, other reports exist of papillomavirus genomes generally associated with benign lesions being isolated from frank cancers *in vivo*. HPV-6vc and HPV-6b DNA have been cloned from invasive vulvar carcinomas (Rando *et al.*, 1986; Kasher and Roman, 1988) and shown to harbour nucleotide sequence alterations in the viral LCR. It is feasible that HPV-4 DNA may have a similarly low efficiency of transformation of primary cells, and analysis of the viral DNA function in a co-transformation assay would confirm or deny this. Additionally, nucleotide sequence comparison of the LCRs of the prototype HPV-4 DNA, isolated from a benign lesion, and the oral virus described here might reveal some rearrangement or enhanced activity which could have a bearing on the transforming potential of HPV-4 and help to explain its presence in a malignant oral tumour.

5.5. HPV-16 Sequences in Oral Tissue Biopsies.

One cancer positive for viral sequences by Southern blot analysis contained the DNA of HPV-16, frequently found to be associated with genital carcinomas (Durst *et al.*, 1983) and cell lines derived from such lesions (Yee *et al.*, 1985), and capable of transforming primary cells *in vitro* together with co-factors (Storey *et al.*, 1988). The presence of this viral type in oral carcinomas in the current series was not surprising, in view of the oncogenic potential of HPV-16 and the similarity of oral and genital mucosa. Indeed, of the 46% of oral

cancers found to contain viral DNA by Maitland *et al.* (1987), HPV-16 sequences were found exclusively. This was also the case in the study of Chang and co-workers (1989), and de Villiers *et al.* (1985) found HPV-16 DNA in two out of three tongue carcinomas.

One of these groups (Maitland *et al.*, 1987) reported that the HPV-16 sequences present in oral carcinoma DNAs differed from the prototype genital isolate in the PstI digestion pattern, with the expected 1.55kb 'C' fragment being absent. The PstI digestion pattern of the HPV-16 DNA detected in this study showed a similar feature, and digestion with a second restriction enzyme confirmed that a deletion or rearrangement event had occurred in the oral variant virus compared to the original HPV-16. As this alteration in viral DNA structure involves the late region open reading frames (which encode the viral capsid proteins) it is debatable whether this variant of HPV-16 would be able to complete the viral life cycle and form progeny virus, if indeed a large proportion of the L2 and L1 genes were lost. Alternatively, new sequences might be present which lack any cross-reactivity with the genital viral DNA, thus remaining undetectable with the probe used.

When the PCR data are taken into account, the overall incidence of HPV-16 DNA in oral cancers in this study is approximately half that reported for similar lesions of genital mucosa (zur Hausen, 1988). This most likely reflects a geographic variation in the incidence of HPV-16 as discussed previously, as other workers studying oral tissues have reported up to seventy six per cent of oral carcinomas to harbour HPV-16 sequences (Chang *et al.*, 1989). Other as yet unidentified HPVs may be present in the remaining negative samples

in this study. It could, however, be a reflection of different mechanisms of HPV-16-mediated transformation at these sites. In the majority of cervical carcinoma tissues and cell lines analysed so far, the viral genome becomes integrated into the host chromosomal DNA to a greater or lesser extent, compared to largely episomal HPV DNA in normal and premalignant cervical epithelium (Fuchs *et al.*, 1988). Furthermore, the viral DNA was shown to persist in lymph node metastases derived from the primary tumours. This is consistent with data obtained from *in vitro* transformation experiments using prototype HPV-16 cloned from a cervical carcinoma, where continued expression of the E7 protein was found to be necessary for maintenance of the transformed state of the cells (Crook *et al.*, 1989a). In contrast, the level of viral DNA detected in nine of the ten oral cancers positive for HPV-16 in this study is more consistent with the maintenance role being assumed by cellular genes rather than papillomavirus proteins. It may be another example of a "hit-and-run" mechanism for viral transformation of cells, similar to that observed with BPV-4 in bovine alimentary tract epithelium (Campo *et al.*, 1985) and C127 fibroblasts (Smith and Campo, 1988), or HSV-2 in cervical carcinomas (reviewed by Galloway and McDougall, 1984). Further evidence for this might be gained by studying papillomavirus presence and expression in premalignant oral lesions such as some leukoplakias, erythroplakias and carcinomas-in-situ.

The lack of the prototype PstI 'C' fragment from the HPV-16 genome found in sample C2 by Southern blot analysis was interesting, if not totally unexpected; a similar phenomenon has previously been documented in oral samples containing HPV-16 DNA (Maitland *et*

al., 1987). HpaII digestion confirmed that the L2-L1 region of this viral genome was altered in some way, as only the 3' 0.8kb of the expected 2.6kb HpaII fragment could be detected by hybridisation to a prototype HPV-16 probe.

Although no DNA from this sample remained from which to clone the viral DNA, a PCR-based strategy using HPV-16 oligonucleotide primers situated on either side of the putative deletion or rearrangement enabled detection of aberrant sized amplification products in two further samples, C3 and C5, which had been shown to contain HPV-16 early region sequences by PCR. In both cases the amplified fragment was shorter than that amplified from a control of religated HPV-16 DNA by approximately 0.8kb. It is unlikely that these amplification products are artefacts of the polymerase chain reaction caused by random priming by the oligonucleotides in the cellular DNA, as the amplification was specific for these two samples, was not detected in the DNAs of other samples, and was reproducible.

Use of these amplified fragments as hybridisation probes revealed hybridisation with specific regions of the prototype HPV-16 genome at high stringency, namely the 1.78kb PstI fragment, which is situated 3' to the 1.55kb fragment absent in sample C2, and the 1.81kb BamHI-HpaII band, which runs from position 6150 to 57 of the HPV-16 genome and encompasses the 3' end of the 1.55kb PstI fragment. Experiments performed at reduced stringency did not reveal additional hybridising bands; attempts to map the hybridising regions more finely by probing the 2.6kb HpaII band after more extensive restriction enzyme digestion did not provide any further information. These data suggest that the 5' oligonucleotide primer was situated

close to the area of rearrangement, otherwise hybridisation to the 0.48kb band would have been observed on Southern blot analysis.

Probing Southern blots of restriction enzyme digested cellular DNAs from a variety of oral samples, both normal and malignant, resulted in hybridisation to the whole smear of genomic DNA. No reduction in intensity of signal was observed when the blots were washed at high stringency, and this data suggests that the fragments amplified using the HPV-specific primers were hybridising to some highly repetitive sequences present in the human genome.

Limited DNA sequence analysis was carried out on the 1.0kb PCR fragments amplified from C3 and C5. Comparison of the sequences with the HPV-16 genome showed poor homology with different areas of the L2-L1 region of the viral genome; however if both fragments were sequenced completely perhaps more homology to HPV sequences would be observed. Unfortunately, technical problems prevented this work from being accomplished.

Hybridisation of the amplified fragments from C3 and C5 to both papillomavirus and cellular sequences implies that at least one recombination event has occurred between viral and host DNA in these two carcinomas. However, if the viral episome had become integrated into the cellular genome by opening within the region spanned by the two primers and then recombining, amplification of DNA using the 16La/b primers would not have been possible. For PCR amplification to occur following such a scenario, the viral DNA would have to excise from the host DNA and religate, thus involving

two recombination events. The excision of HPV sequences would be imprecise, removing some cellular DNA at the same time.

Alternatively, a form of "crossing over" of viral and cellular sequences may have occurred, without the actual integration of HPV DNA into the host genome. It is possible that such a recombination might form the first step of a sequence of events by which the episomal HPV genome integrates into the cellular DNA. A primary illegitimate recombination would result in incorporation of cellular sequences into the viral episome. If these sequences were highly repeated in the human genome, or contained specific sequence motifs or recognition sequences, a subsequent recombination resulting in integration of the viral DNA might occur more readily.

Recombination of DNA tumour virus genomes with host DNA sequences has been previously reported. Defective variants of SV40 have been isolated (Wakamiya *et al.*, 1979; McCutchan *et al.*, 1979), two of which harboured deletions of regions of the wild-type SV40 genome adjacent to the sites of recombination with cellular sequences, inversions within the remaining DNA, and incorporation of highly repeated monkey α-sequences as well as sequences present in the monkey genome at much lower frequency. These authors noted that the monkey DNA sequences present in the recombinant viruses bore no significant homology to the viral sequences that had been lost during recombination. At the sites involved in the deletion and inversion of wild-type viral sequences, again no good regions of homology were present. In both variant genomes described by these authors the low reiteration frequency host DNA is very A-T rich, although different in each case. The host-viral joins in the SV40

variants were also rich in A-T base pairs, and McCutchan *et al.* (1979) speculated that such regions of DNA duplex instability might facilitate recombination, without the need for long regions of sequence homology, although short tetra-, hexa- and heptanucleotide homologies were observed adjacent to the sequence joints. With regard to recombination of HPV-16 DNA with cellular sequences in samples C3 and C5 in this study, no sequences involved in the recombination mechanism can as yet be identified, although the regions of nucleotide sequence determined to date are indeed rich in A-T base pairs. This would require the entire DNA sequence of both PCR fragments to be determined, and the human DNA sequence involved to be cloned and sequenced. Both of these aims must be regarded as high priority.

This is not the first association of papillomavirus sequences with repetitive human DNA. Schneider-Gadicke and Schwarz (1986) reported HPV-16 DNA integrated adjacent to cellular sequences which showed homology to the *Alu* family of repeats. However screening of the available sequences from C3 and C5 against *Alu* sequences present in the Genbank and EMBL databases did not reveal any homology, and the repeated sequences remain to be characterised.

Recently, Choo *et al.* (1990) analysed the site of integration of HPV-16 DNA in a primary cervical carcinoma, where the viral DNA had undergone sequence duplication and internal deletion events (Choo *et al.*, 1988). Nucleotide sequencing of the upstream viral-cellular integration junction enabled identification of two regions of homology between the HPV-16 and host cell DNAs, one of which

was situated in an A-T rich region of the viral genome. This information enabled the authors to propose a model for the integration mechanism at the 5' junction involving these short homologies in an illegitimate recombination, while analysis of the viral and cellular sequences present at the other junction suggested that after the initial integration occured a secondary event took place resulting in deletion of both viral and cellular sequences. It is possible, though speculative, that the PCR products amplified from samples C3 and C5 reflect products of illegitimate recombination between HPV-16 and cellular DNA, involving an A-T rich region.

5.6. Oral Keratinocyte Cultures Harbour HPV Sequences.

PCR analysis of DNA from the T45 oral keratinocyte cell line enabled detection of HPV-16 E6-E7 sequences in low amounts, which were not apparent by conventional Southern blot hybridisation. This suggests that not all of the cells contain HPV sequences, as the cell population is not clonal. No late region amplification products were observed using the 16La/b primer pair, implying that this region has been deleted, or become rearranged such that the primers used were unable to anneal to the DNA template, perhaps in a way similar to that observed for HPV-16 DNA in sample C2. Attempted amplification of HPV-16 late genes in oral keratinocyte cultures derived from squamous cell carcinomas has previously been unsuccessful (Maitland *et al.*, 1989), although, as seen in this case, the

E6-E7 genes were retained. This may reflect a function of the products of these genes in cell immortalisation.

Other workers studying oral keratinocyte cultures have observed loss of viral sequences with increased passage of cells (N. Maitland, personal communication). Whether this is due to loss of HPV DNA from individual cells as a result of redundancy following establishment in culture or to selective pressure against cells harbouring papillomavirus sequences is at present unclear. Examination of the mechanism by which this phenomenon occurs would then allow possible reasons for the loss of viral DNA to be elucidated. The T45 cell culture described here would be a reasonable starting point for such studies, as HPV sequences are present in only a few cells. Single cell cloning performed on T45 cells would allow establishment of cell lines which were positive or negative for viral sequences. The behaviour of these clonal populations in culture could then be examined, and changes in viral DNA content, growth characteristics and *in vivo* tumorigenicity monitored.

The availability of clonal oral carcinoma cell lines containing HPV genomes would also be conducive to the study of viral transcription and regulation in oral tissues. No data concerning these aspects of HPV infection of oral epithelium have so far been documented; this would help to clarify the role of such viruses in oral carcinogenesis.

The interaction of viral gene products with host proteins and the effects of such on the phenotype of the cell could also be examined. For example, nothing is known about possible modulation of normal cellular tumour suppressor function in oral keratinocytes or

transactivation of cellular genes by viral proteins, both of which could be important for malignant progression.

If recombination between viral and cellular sequences does occur, as the data presented here suggests, HPV-containing cell lines derived from the oral keratinocyte culture described would provide a good model for the study of such events. Integration or recombination events could be monitored as the time in culture increased and any genotypic changes noted, such as amplification of specific cellular sequences similar to those described by Smith and Campo in BPV-4 transformed fibroblasts (1989), or induction of cellular regulators of transcription. Such events could then be related to changes in the phenotype of the cells, in particular their tumorigenicity *in vivo* and their ability to differentiate.

The T45 cell culture derived in the course of this work has several advantages over other available *in vitro* systems.The majority of cervical carcinoma cell lines which have been used previously to study the biology of HPV-16 and HPV-18 and their role in epithelial carcinogenesis, such as C-41, SiHa, CaSki and HeLa, have been in culture for many years (HeLa since 1951). During their considerable *in vitro* lifespans, these cell lines will most likely have lost many of the properties of the cervical epithelial cells from which they originated, due to selective pressure on cells in culture. For instance, they may have undergone mutational events or chromosome loss, if these would impart a growth advantage to the cells. The use of a newly-explanted cell line largely overcomes such difficulties, being temporally closer to the original tissue phenotype and being a more representative system

for studying malignant progression of epithelial cells and the involvement of papillomavirus genomes.

A further advantage of the T45 keratinocytes described here is their apparent lack of *in vivo* tumorigenicity, despite the source being a malignant oral tumour. As reviewed in sections 1 and 2, the development of oral squamous cell carcinoma is undoubtedly a multistage process involving several genetic events. The availability of an immortal, though non-tumorigenic, oral epithelial cell line provides a suitable *in vitro* system for investigating the multifactorial nature of oral cancer by using, for example, cloned viral and cellular oncogenes and tumour suppressor genes in transfection experiments to progress immortal cells to overt malignancy, and to regress malignant cells to a non-tumorigenic phenotype. This could be performed in parallel with experiments to study the response of cells to growth factor-mediated regulation of proliferation, the factors important for production of distinct *in vitro* phenotypes identified, and correlation made with gene mutations and altered expression *in vivo*.

5.7. Conclusions.

This study has succeeded in identifying human papillomavirus sequences in both normal and malignant tissues from patients suffering from oral squamous cell carcinoma in around fifty per cent of cases. This was six times greater than the frequency of HPV infection in an age-matched control population of cancer-free patients, and is suggestive of an aetiological role for certain HPV types in at least some oral carcinomas. In view of the low levels of viral DNA present, it is unlikely that viral sequences are required for maintaining the transformed state of the oral epithelium. Alternatively, the oral cancer patients may be more susceptible to HPV infection due to reduced immune function, although the two are not necessarily mutually exclusive. Analysis of premalignant oral lesions for viral DNA and gene expression may shed further light on the importance of papillomaviruses in the aetiology of oral cancer.

The failure to identify HPV DNA in the remaining fifty per cent of oral cancer cases may reflect the multifactorial nature of the disease, with additional agents playing an aetiological role. It should be stressed that, when using the most sensitive procedure available, only three HPV types were screened for. Use of specific oligonucleotide primers for other HPVs, or degenerative primers at reduced stringency, may reveal viral DNA to be present in further samples, or indeed allow the isolation of new viral types from oral mucosa.

During the course of this work, HPV-4 DNA was found in one oral cancer, the first report of this papillomavirus, normally associated

with benign lesions, in a malignancy. Its role in the aetiology of oral cancer is, at present, unclear. Transfection experiments using the cloned viral DNA to assess its transforming potential, together with sequencing of prototype and oral isolates may provide an answer to this question.

In two cancer cases containing HPV-16 DNA the evidence suggests that recombination has occurred between viral and cellular sequences. Although this has previously been demonstrated in mammalian cells infected with a DNA tumour virus, the significance is unclear. Molecular cloning and nucleotide sequence analysis of the viral DNA and the associated repetitive human sequences may provide clues to the mechanism of recombination and perhaps the significance of such events.

Finally, a keratinocyte population has been derived from malignant oral epithelium which will provide an excellent system for the study of the factors involved in malignant progression of oral epithelium, including the role of papillomavirus sequences together with cellular oncogenes and tumour suppressors.

The information gained from such studies will help to shed some light on the involvement of human papillomavirus genomes and their interaction with cellular factors in the development of oral squamous cell carcinoma.

REFERENCES.

Adler-Storthz, K, Newland, JR, Tessin, BA, Yeudall, WA and Shillitoe, EJ. (1986a). Human papillomavirus types in oral verruca vulgaris. J. Oral Pathol. *15*, 230.

Adler-Storthz, K, Newland, JR, Tessin, BA, Yeudall, WA and Shillitoe, EJ. (1986b). Human papillomavirus type 2 DNA in oral verrucous carcinoma. J. Oral Pathol. *15*, 472.

Ahlbom, HE. (1936). Simple achlorhydric anaemia, Plummer-Vinson, syndrome and cancer of the mouth, pharynx and oesophagus in women. Br. Med. J. *2*, 331.

Albrecht, T and Rapp, F. (1973). Malignant transformation of hamster embryo fibroblasts following exposure to ultraviolet-irradiated human cytomegalovirus. Virology *55*, 53.

Banoczy, J and Sugar, L. (1972). Longitudinal studies in oral leukoplakias. J. Oral Pathol. *1*, 265.

Barbosa, MS and Schlegel, R. (1989). The E6 and E7 genes of HPV-18 are sufficient for inducing two-stage *in vitro* transformation of human keratinocytes. Oncogene *4*, 1529.

Barbosa, MS, Lowy, DR and Schlegel, R. (1987). Papillomavirus polypeptides E6 and E7 are zinc-binding proteins. J. Virol. *63*, 1404.

Barbosa, MS, Edmonds, C, Fisher, C, Schiller, JT, Lowy, DR and Vousden, KH. (1990). The region of the HPV E7 oncoprotein homologous to adenovirus E1a and SV40 large T antigen contains separate domains for Rb binding and casein kinase II phosphorylation. EMBO J. *9*, 153.

Bar-Eli, M, Battifora, H and Cline, MJ. (1988). Alterations of class I HLA genes in human colon cancers. Hum. Genet. *78*, 86.

Beasley, RP, Lin, C-C, Hwang, LY and Chen, C-S. (1981). Hepatocellular carcinoma and hepatitis B virus: a prospective study of 22,707 men in Taiwan. Lancet *ii*, 1129.

Beaudenon, S, Kremsdorf, D, Croissant, O, Jablonska, S, Wain-Hobson, S and Orth, G. (1986). A novel type of human papillomavirus associated with genital neoplasias. Nature *321*, 246.

Beaudenon, S, Kremsdorf, D, Obalek, S, Jablonska, S, Pehau-Arnaudet, G, Croissant, O and Orth, G. (1987a). Plurality of genital papillomaviruses: characterisation of two new types with distinct biological properties. Virology *161*, 374.

Beaudenon, S, Praetorius, F, Kremsdorf, D, Lutzner, M, Worsaae, N, Pehau-Arnaudet, G and Orth, G. (1987b). A new type of human papillomavirus associated with oral focal epithelial hyperplasia. J. Invest. Dermatol. *88*, 130.

Bedell, MA, Jones, KH, Grossman, SR and Laimins, LA. (1989). Identification of human papillomavirus type 18 transforming genes in immortalized and primary cells. J. Virol. *63*, 1247.

Berk, AJ, Lee, F, Harrison, T, Williams, J and Sharp, PA. (1979). Pre-early Ad5 gene product regulates synthesis of early viral mRNAs. Cell *17*, 935.

Binnie, WH. (1976). Epidemiology and aetiology of oral cancer in Britain. Proc. Royal Soc. Med. *69*, 737.

Binnie, WH, Cawson, RA, Hill, GB and Soaper, AE. (1972). Oral cancer in England and Wales. A national study of morbidity, mortality, curability and related factors. HMSO.

Birnboim, HC and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. *7*, 1513.

Blot, WJ and Fraumeni, JF Jr. (1977). Geographic patterns of oral cancer in the United States: etiologic implications. J. Chron. Dis. *30*, 745.

Bolivar, F, Rodriguez, RL, Greene, PJ, Betlach, MC, Heynecker, HL, Boyer, HW, Crosa, JH and Falkow, S. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene *2*, 95.

Boshart, M, Gissmann, L, Ikenberg, H, Kleinheinz, A, Scheurlen, W and zur Hausen, H. (1984). A new type of papillomavirus DNA, its presence in genital cancer and in cell lines derived from cervical carcinoma. EMBO J. *3*, 1151.

Brandsma, JL, Steinberg, BM, Abramson, AL and Winkler, B. (1986). Presence of human papillomavirus type 16 related sequences in verrucous carcinoma of the larynx. Cancer Res. *46*, 2185.

Broker, TR and Botchan, M. (1986). Papillomaviruses: retrospectives and prospectives. Cancer Cells 4: DNA Tumor Viruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 17.

Brown-Kelly, A. (1919). Spasm at the entrance of the oesophagus. J. Laryngol. *34*, 285.

Camacho, A and Spear, PG. (1978). Transformation of hamster embryo fibroblasts by a specific fragment of the herpes simplex virus genome. Cell *15*, 993.

Campo, MS, Moar, MH, Sartirana, ML, Kennedy, IM and Jarrett, WFH. (1985). The presence of bovine papillomavirus type 4 DNA is not required for the progression to, or the maintenance of, the malignant state in cancers of the alimentary canal in cattle. EMBO J. *4*, 1819.

Campo, MS and Jarrett, WFH. (1986). Papillomavirus infection in cattle: Viral and chemical cofactors in naturally-occurring and experimentally induced tumours. Ciba Found. Symp. *120*, 114.

Cawson, RA. (1969). Leukoplakia and oral cancer. Proc. Royal Soc. Med. *62*, 610.

Cawson, RA. (1984). Essentials of dental surgery and pathology. 4th ed. Churchill Livingston. Edinburgh.

Chang, K-W, Chang, C-S, Lai, K-S, Chou, M-J and Choo, K-B. (1989). High prevalence of human papillomavirus infection and possible association with betel quid chewing and smoking in oral epidermoid carcinomas in Taiwan. J. Med. Virol. *28*, 57.

Chirgwin, JM, Przbyla, AE, McDonald, RJ and Rutter, WJ. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. Biochemistry *18*, 5294.

Chisari, FV, Klopchin, K, Moriyama, T, Pasquinelli, C, Dunsford, HA, Sell, S, Pinkert, CA, Brinster, RL and Palmiter, RD. (1989). Molecular pathogenesis of hepatocellular carcinoma in hepatitis B virus transgenic mice. Cell *59*, 1145.

Chisholm, D. (1989). A convenient moderate-scale procedure for obtaining DNA from bacteriophage lambda. Biotechniques *7*, 21.

Chong, T, Chan, W-K and Bernard, H-U. (1990). Transcriptional activation of human papillomavirus 16 by nuclear factor I, AP1, steroid receptors and a possibly novel transcription factor, PVF: a model for the composition of genital papillomavirus enhancers. Nucl. Acids Res. *18*, 465.

Choo, K-B, Lee, H-H, Liew, L-N, Chong, K-Y and Chou, H-F. (1990). Analysis of the unoccupied site of an integrated human papillomavirus 16 sequence in a cervical carcinoma. Virology *178*, 621.

Choo, K-B, Lee, H-H, Pan, C-C, Wu, S-M, Liew, L-N, Cheung, W-F and Han, S-H. (1988). Sequence duplication and internal deletion in the integrated human papillomavirus type 16 genome cloned from a cervical carcinoma. J. Virol. *62*, 1659.

Ciuffo, G. (1907). Innesto positivo con filtrado di verrucae volgare. G. Ital. Mol. Venereol. *48*, 12.

Coggin, JR Jr. and zur Hausen, H. (1979). Workshop on papillomaviruses and cancer. Cancer Res. *39*, 545.

Copple, CD and McDougall, JK. (1976). Clonal derivatives of a herpes type-2 transformed hamster cell line (339-8-9): cytogenetic analysis, tumorigenicity and virus sequence detection. Int. J. Cancer *17*, 501.

Courtneidge, SA. (1985). Activation of the pp60$^{c\text{-}src}$ kinase by middle T antigen binding or by dephosphorylation. EMBO J. *4*, 1471.

Cowley, GP, Smith, JA and Gusterson, BA. (1986). Increased EGF receptors on human squamous carcinoma cell lines. Br. J. Cancer *53*, 223.

Cox, MF, Meanwell, CA, Maitland, NJ, Blackledge, G, Scully, C and Jordan, JA. (1986). Human papillomavirus type 16 DNA in normal human ectocervix. Lancet *ii*, 157.

Crook, T, Almond, N, Murray, A, Stanley, M and Crawford, L. (1989a). Constitutive expression of c-myc oncogene confers hormone independence and enhanced growth factor responsiveness to mouse cells transformed by human papilloma virus type 16. Proc. Natl. Acad. Sci. USA. *86*, 5713.

Crook, T, Banks, L, Smith, K and Crawford, L. (1989b). Amplification and overexpression of c-*myc* proto-oncogene correlate with the loss of glucocorticoid dependence in rodent cells transformed by human papillomavirus type 16. Mol. Carcinogen. *2*, 308.

Crook, T, Morgenstern, JP, Crawford, L and Banks, L. (1989c). Continued expression of HPV-16 E7 protein is required for maintenance of the transformed phenotype of cells co-transformed by HPV-16 plus EJ-*ras*. EMBO J. *8*, 513.

Crook, T, Storey, A, Almond, N, Osborn, K and Crawford, L. (1988). Human papillomavirus type 16 cooperates with activated *ras* and *fos* oncogenes in the hormone-dependent transformation of primary mouse cells. Proc. Natl. Acad. Sci. USA. *85*, 8820.

Dagert, M and Ehrlich, SD. (1979). Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene *6*, 23.

de Caprio, JA, Ludlow, JW, Figge, J, Shew, J-Y, Huang, C-M, Lee, W-H, Marsilio, E, Paucha, E and Livingston, DM. (1988). SV40 large tumour antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell *54*, 275.

Deininger, P, Esty, A, LaPorte, P and Friedmann, T. (1979). Nucleotide sequence and genetic organization of the polyoma late region: Features common to the polyoma early region and SV40. Cell *18*, 771.

Devereux, J, Haeberli, P and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res. *12*, 387.

de Villiers, EM. (1989). Heterogeneity of the human papillomavirus group. J. Virol. *63*, 4898.

de Villiers, EM, Weidauer, Otto, H and zur Hausen, H. (1985). Papillomavirus DNA in human tongue carcinomas. Int. J. Cancer, *36*, 575.

de Villiers, EM, Hirsch-Behnan, A, von Knebel-Doeberitz, C, Neumann, C and zur Hausen, H. (1989). Two newly identified human papillomavirus types (HPV 40 and 57) isolated from mucosal lesions. Virology *171*, 248.

de Villiers, EM, Gissmann, L and zur Hausen, H. (1981). Molecular cloning of viral DNA from human genital warts. J. Virol. *40*, 932.

diLuca, D, Rotola, A, Pilotti, S, Monini, P, Caselli, E, Rilke, F and Cassai, E. (1987). Simultaneous presence of herpes simplex and human papilloma virus sequences in human genital tumors. Int. J. Cancer *40*, 763.

di Paolo, JA, Woodworth, D, Popescu, NC, Notario, V and Doniger, J. (1989). Induction of human squamous cell carcinoma by sequential transfection with human papillomavirus type 16 DNA and viral Harvey *ras*. Oncogene *4*, 395.

Doerfler, W. (1968). The fate of the DNA of adenovirus type 12 in baby hamster kidney cells. Proc. Natl. Acad. Sci. USA. *60*, 636.

Douglass, CW, Gammon, MD and Horgan, WJ. (1984). *In* Oral Cancer. Ed. G. Shklar. WB Saunders Co., Philadelphia.

Duff, R and Rapp, F. (1971). Oncogenic transformation of hamster cells after exposure to herpes simplex virus type 2. Nature *233*, 48.

Duff, R and Rapp, F. (1973). Oncogenic transformation of hamster embryo cells after exposure to inactivated herpes simplex virus type 1. J. Virol. *12*, 209.

Dunn, AE and Ogilvie, MM. (1968). Intranuclear virus particles in human genital wart tissue: observations on the ultrastructure of the epidermal layer. J. Ultrastruct. Res. *22*, 282.

Durst, M, Gissmann, L, Ikenberg, H and zur Hausen, H. (1983). A papillomavirus DNA from a cervical carcinoma and its prevalence in

cancer biopsy samples from different geographic regions. Proc. Natl. Acad. Sci. USA. *80*, 3812.

Dyson, N, Howley, PM, Munger, K and Harlow, E. (1989). The human papillomavirus 16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science *243*, 934.

Edmonds, C and Vousden, KH. (1989). A point mutational analysis of human papillomavirus type 16 E7 protein. J. Virol. *63*, 2650.

Eglin, RP, Sharp, F, MacLean, AB, MacNab, JCM, Clements, JB and Wilkie, NM. (1981). Detection of RNA complementary to herpes simplex virus DNA in human cervical squamous cell neoplasms. Cancer Res. *41*, 3597.

Eglin, RP, Scully, C, Lehner, T, Ward-Booth, P and McGregor, IA. (1983). Detection of RNA complementary to herpes simplex virus in human oral squamous cell carcinoma. Lancet *ii*, 766.

Einhorn, J and Wersall, J. (1967). Incidence of oral carcinoma in patients with leukoplakia of the oral mucosa. Cancer *20*, 2189.

Eisenberg, E, Rosenberg, B and Krutchkoff, DJ. (1985). Verrucous carcinoma: a possible viral pathogenesis. Oral Surg. *59*, 52.

Epstein, MA, Achong, BG and Barr, YG. (1964). Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet *i*, 702.

Esche, H, Mathews, MB and Lewis, JB. (1980). Proteins and messenger RNAs of the transforming region of wild type and mutant adenoviruses. J. Mol. Biol. *142*, 399.

Feinberg, AP and Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. *132*, 6.

Field, JK, Lamothe, A and Spandidos, DA. (1986). Clinical relevance of oncogene expression in head and neck tumours. Anticancer Res. *6*, 595.

Fiers, W, Contreras, R, Haegeman, G, Regiers, R, van der Voorde, A, van Heuverswyn, H, van Herreseghe, J, Volkaert, G and Ysebaert, M. (1978). The complete nucleotide sequence of SV40 DNA. Nature *273*, 113.

Francheschi, S, Talamini, R, Barra, S, Baron, AE Negri, E, Bidoli, E, Serraino, D and La Vecchia, C. (1990). Smoking and drinking in relation to cancers of the oral cavity, pharynx, larynx and esophagus in Northern Italy. Cancer Res. *50*, 6502.

Freeman, AE, Black, PH, Vanderpool, EA, Henry, PH, Austin, JB and Huebner, RJ. (1967). Transformation of primary rat embryo cells by adenovirus type 2. Proc. Natl. Acad. Sci. USA. *58*, 1205.

Frenkel, N, Locker, H, Batterson, W, Hayward, G and Roizman, B. (1976). Anatomy of herpes simplex virus DNA. VI. Defective DNA originates from the 'S' component. J. Virol. *20*, 527.

Freshney, RI. (1987). Culture of animal cells. A manual of basic technique. 2nd ed. Alan R. Liss Inc. New York.

Friedmann, T, Esty, A, LaPorte, P and Deininger, P. (1979). Nucleotide sequence and genetic organization of the polyoma early region: Features common to the polyoma late region and SV40. Cell *17*, 751.

Frischauf, A-M, Lehrach, H, Poustka, A and Murray, N. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. *170*, 827.

Frye, RA, Benz, CC and Liu, E. (1989). Detection of amplified oncogenes by differential polymerase chain reaction. Oncogene *4*, 1153.

Fuchs, PG, Girardi, F and Pfister, H. (1988). Human papillomavirus 16 DNA in cervical cancers and in lymph nodes of cervical cancer patients: a diagnostic marker for early metastases? Int. J. Cancer *43*, 41.

Fujinaga, K and Green, M. (1970). Mechanism of viral carcinogenesis by DNA mammalian viruses. VII. Viral genes transcribed in adenovirus type 2 infected and transformed cells. Proc. Natl. Acad. Sci. USA. *65*, 375.

Galloway, DA, Copple, CD and McDougall, JK. (1980). Analysis of viral DNA sequences in hamster cells transformed by herpes simplex type 2. Proc. Natl. Acad. Sci. USA. *77*, 880.

Galloway, DA and McDougall, JK. (1981). Transformation of rodent cells by a cloned DNA fragment of herpes simplex virus type 2. J. Virol. *38*, 749.

Galloway, DA and McDougall, JK. (1983). The oncogenic potential of herpes simplex viruses: evidence for a hit-and-run mechanism. Nature *302*, 21.

Galloway, DA, Nelson, JA and McDougall, JK. (1984). Small fragments of herpesvirus DNA with transforming activity contain insertion sequence-like structures. Proc. Natl. Acad. Sci. USA. *81*, 4736.

Garlick, JA, Calderon, S, Buchner, A and Mitrani-Rosenbaum, S. (1989). Detection of human papillomavirus (HPV) DNA in focal epithelial hyperplasia. J. Oral Pathol. *18*, 172.

Gelb, LD, Huang, JJ and Wellinghoff, WJ. (1980). Varicella-zoster virus transformation of hamster embryo cells. J. Gen. Virol. *51*, 171.

Giri, I and Danos, O. (1986). Papillomavirus genomes: from sequence data to biological properties. Trends Genet. *2*, 227.

Gissmann, L. (1984). Papillomaviruses and their association with cancer in animals and in man. Cancer Surveys *3*, 161.

Gissmann, L and zur Hausen, H. (1980). Partial characterization of viral DNA from human genital warts (condyloma acuminata). Int. J. Cancer *29*, 143.

Gissmann, L, Diehl, V, Schulz-Coulon, H and zur Hausen, H. (1982). Molecular cloning and characterisation of human papillomavirus DNA derived from a laryngeal papilloma. J. Virol. *44*, 393.

Gissmann, L, Boshart, M, Durst, M, Ikenberg, H, Wagner, D and zur Hausen, H. (1983). Presence of human papillomavirus in genital tumours. J. Invest. Dermatol. *83*, 26s.

Gloss, B, Bernard, H-U, Seedorf, K and Klock, G. (1987). The upstream regulatory region of the human papillomavirus-16 contains an E2 protein-independent enhancer which is specific for cervical carcinoma cells and regulated by glucocorticoid hormones. EMBO J. *6*, 3735.

Graham, FL, Abrahams, PS, Mulder, C, Heijneker, HL, Warnaar, SO, deVries, FAJ, Fiers, W and van der Eb, AJ. (1975). Studies on in vitro transformation by DNA and DNA fragments of human adenoviruses and simian virus 40. Cold Spring Harbor Symp. Quant. Biol. *39*, 637.

Graham, GJ and Rennie, JS. (1987). Biochemical abnormalities of rat lingual epithelium following chronic alcohol intake. Arch. Oral Biol. *32*, 1.

Graham, S, Dayal, H, Rohrer, T, Swanson, M, Sultz, H, Shedd, D and Fischman, S. (1977). Dentition, diet, tobacco and alcohol in the epidemiology of oral cancer. J. Natl. Cancer Inst. *59*, 1611.

Greene, WC, Leonard, WJ, Wano, Y, Svetlik, PB, Peffer, NJ, Sodroski, JG, Rosen, CA, Goh, WC and Haseltine, WA. (1986). *Trans*-activator gene of HTLV-II induces IL-2 receptor and IL-2 cellular gene expression. Science *232*, 877.

Greenspan, D, de Villiers, EM, Greenspan, JS, de Souza, YG and zur Hausen, H. (1988). Unusual HPV types in oral warts in association with HIV infection. J. Oral Pathol. *17*, 482.

Grunstein, M and Hogness, DS. (1975). Colony hybridisation: A method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA *72*, 3961.

Hampar, B, Boyd, AL, Derge, JG, Zweig, M, Eader, L and Showalter, SD. (1980). Cancer Res. *40*, 2213.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. *166*, 557.

Hawley-Nelson, P, Vousden, KH, Hubbert, NL, Lowy, DR and Schiller, JT. (1989). HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. EMBO J. *8*, 3905.

Heilman, CA, Law, M-F, Israel, MA and Howley, PM. (1980). Cloning of human papilloma virus genomic DNAs and analysis of homologous polynucleotide sequences. J. Virol. *36*, 395.

Hinds, MW, Kolonel, LN, Lee, J and Hirohata, T. (1980). Association between cancer incidence and alcohol/cigarette consumption among five ethnic groups in Hawaii. Br. J. Cancer *41*, 929.

Hirayama, T. (1966). An epidemiological study of oral and pharyngeal cancer in central and southeast Asia. Bull. World Hlth. Org. *34*, 41.

Houweling, A, van den Elsen, PJ and van der Eb, AJ. (1980). Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. Virology *105*, 537.

Hsu, T, Moroy, T, Etiemble, J, Louise, A, Trepo, C, Tiollais, P and Buendia, M-A. (1986). Activation of c-*myc* by woodchuck hepatitis virus insertion in hepatocellular carcinoma. Cell *55*, 627.

Huebner, RJ. (1967). Adenovirus-directed tumor and T antigens. Perspect. Virol. *5*, 147.

Hurlin, PJ, Kaur, P, Smith, PP, Perez-Reyes, N, Blanton, RA and McDougall, JK. (1991). Progression of human papillomavirus type 18-immortalized human keratinocytes to a malignant phenotype. Proc. Natl. Acad. Sci. USA *88*, 570.

Inoue, J, Seiki, M, Taniguchi, T, Tsuru, S and Yoshida, M. (1986). Induction of interleukin 2 receptor gene expression by $p40^{x}$ encoded by human T-cell leukemia virus type 1. EMBO J. *5*, 2883.

Ishitoya, J, Toriyama, M, Oguchi, N, Kitamura, K, Ohshima, M, Asano, K and Yamamoto, T. (1989). Gene amplification and overexpression of EGF receptor in squamous cell carcinomas of the head and neck. Br. J. Cancer *59*, 559.

Jarrett, WFH, Campo, MS, O'Neil, BW, Laird, HM and Coggins, LW. (1984). A novel bovine papillomavirus (BPV-6) causing true epithelial papillomas of the mammary gland skin: a member of a proposed new BPV subgroup. Virology *136*, 255.

Jarrett, WFH, McNeil, PE, Grimshaw, WTR, Selman, IE and McIntyre, WIM. (1978).High incidence area of cattle cancer with a possible interaction between an environmental carcinogen and a papilloma virus. Nature *274*, 215.

Jarriwalla, RJ, Aurelian, L and Ts'o, POP. (1980). Tumorigenic transformation induced by a specific fragment of DNA from herpes simplex virus type 2. Proc. Natl. Acad. Sci. USA. *77*, 2279.

Jenson, AB, Link, CC and Lancaster, WD. (1982). Papillomavirus etiology of oral cavity papillomas. *In* Viral infections in oral medicine. Ed. J. Hooks and G. Jordan. Elsevier. New York. 133.

Jin, Y-T and Toto, PD. (1984). Detection of human papovavirus antigen in oral papillary lesions. Oral Surg. *58*, 702.

Johnson, NW. (1977). Histological and histochemical studies of oral cancer. Int. Dental J. *27*, 25.

Kahn, T, Schwarz, E and zur Hausen, H. (1986). Molecular cloning and characterisation of the DNA of a new human papillomavirus (HPV-30) from a laryngeal carcinoma. Int. J. Cancer *37*, 61.

Kao, H-T and Nevins, JR. (1983). Transcriptional activation and subsequent control of the human heat shock gene during adenovirus infection. Mol Cell. Biol. *3*, 2058.

Kasher, MS and Roman, A. (1988). Characterization of human papillomavirus type 6b DNA isolated from an invasive squamous carcinoma of the vulva. Virology *165*, 225.

Kaur, P and McDougall, JK. (1988). Characterization of primary human keratinocytes transformed by human papillomavirus type 18. J. Virol. *62*, 1917.

Keller, AZ. (1969). Survivorship with mouth and pharynx cancers and their association with cirrhosis of the liver, marital status, and residence. Am. J. Pub. Hlth. *59*, 1139.

Keller, AZ. (1977). Alcohol, tobacco and age factors in the relative frequency of cancer among males with and without liver cirrhosis. Am. J. Epidemiol. *106*, 194.

Kidd, JG and Rous, P. (1940). Cancers deriving from the virus papillomas of wild rabbits under natural conditions. J. Exp. Med. *71*, 469.

Kleinsasser, O and Oliveira e Cruz, G. (1973). "Juvenile" und "adulte" Kehlkopfpapillome. HNO *21*, 97.

Kloster, BE, Manias, DA, Ostrow, RA, Shaver, MK, McPherson, SW, Rangen, SR, Uno, H and Faras, AJ. (1988). Molecular cloning and characterization of the DNA of two papillomaviruses from monkeys. Virology *166*, 30.

Kramer, IRH. (1969). Precancerous conditions of the oral mucosa. Ann. Royal Coll. Surg. Engl. *45*, 340.

Kreider, JW. (1980). Neoplastic progression of the Shope rabbit papilloma. Cold Spring Harbor Conf. Cell Prolif. *7*, 283.

Kremsdorf, D, Favre, M, Jablonska, S, Obalek, S, Rueda, LA, Lutzner, MA, Blanchet-Bardon, C, Van Voorst Vader, PC and Orth, G. (1984). Molecular cloning and characterisation of the genomes of nine newly recognised human papillomavirus types associated with epidermodysplasia verruciformis. J. Virol. *52*, 1013.

Kremsdorf, D, Jablonska, S, Favre, M and Orth, G. (1982). Biochemical characterization of two types of human papillomaviruses associated with epidermodysplasia verruciformis. J. Virol. *43*, 436.

Kremsdorf, D, Jablonska, S, Favre, M and Orth, G. (1983). Human papillomaviruses associated with epidermodysplasia verruciformis. J. Virol. *48*, 340.

Krieg, P, Amtmann, E and Sauer, G. (1983). The simultaneous extraction of high-molecular-weight DNA and of RNA from solid tumors. Anal. Biochem. *134*, 288.

Kwok, S and Higuchi, R. (1989). Avoiding false positives with PCR. Nature *339*, 237.

Landgraf-Leurs, M and Green, M. (1973). DNA strand selection during the transcription of the adenovirus 2 genome in infected and transformed cells. Biochim. Biophys. Acta *312*, 667.

Lane, DP and Crawford, LV. (1979). T antigen is bound to a host protein in SV40-transformed cells. Nature *278*, 261.

Lane, EB. (1982). Monoclonal antibodies provide specific molecular markers for the study of epithelial tonofilament organisation. J. Cell Biol. *92*, 665.

Langdon, JD. (1985). *In* Malignant tumours of the oral cavity. Ed. JM Henk and JD Langdon. Edward Arnold, London.

Langdon, JD, Harvey, PW, Rapidis, AW, Patel, MF, Johnson, NW and Hopps, R. (1977). Oral cancer: the behaviour and response to treatment of 194 cases. J. Maxillofac. Surg. *5*, 221.

Larsson, LG, Sandstrom, A and Westling, P. (1975). Relationship of Plummer-Vinson disease to cancer of the upper alimentary tract in Sweden. Cancer Res. *35*, 3308.

Le, J-Y and Defendi, V. (1988). A viral-cellular junction fragment from a human papillomavirus type 16-positive tumor is competent in transformation of NIH3T3 cells. J. Virol. *62*, 4420.

Lees, E, Osborn, K, Banks, L and Crawford, L. (1990). Transformation of primary BRK cells by human papillomavirus type 16 and EJ-*ras* is increased by overexpression of the viral E2 protein. J. Gen. Virol. *71*, 183.

Lehn, H, Krieg, P and Sauer, G. (1985). Papillomavirus genomes in human cervical tumors: analysis of their transcriptional activity. Proc. Natl. Acad. Sci. USA *82*, 5540.

Lehner, T, Shillitoe, EJ, Wilton, JMA and Ivanyi, L. (1973). Cell mediated immunity to herpesvirus type 1 in carcinoma and pre-cancerous lesions. Br. J. Cancer *28*, 128.

Levin, ML, Kress, LC and Goldstein, H. (1942). Syphilis and cancer. New York State Med. J. *42*, 1737.

Lopez-Nevot, MA, Esteban, F, Ferron, A, Gutierrez, J, Oliva, MR, Romero, C, Huelin, C, Ruiz-Cabello, F and Garrido, F. (1989). HLA class I gene expression on human primary tumours and autologous

metastases: demonstration of selective losses of HLA antigens on colorectal, gastric and laryngeal carcinomas. Br. J. Cancer *59*, 221.

Lorincz, AT, Lancaster, WD and Temple, GF. (1986). Cloning and characterization of the DNA of a new human papillomavirus from a woman with dysplasia of the uterine cervix. J. Virol. *58*, 225.

Lorincz, AT, Quinn, AP, Lancaster, WD and Temple, GF. (1987). A new type of papillomavirus associated with cancer of the uterine cervix. Virology *159*, 187.

Lucas, RB. (1984). Pathology of tumours of the oral tissues. 4th ed. Churchill Livingston, Edinburgh.

Lutzner, MA. (1978). Epidermodysplasia verruciformis. Bull. Cancer *65*, 169.

MacDonald, DG. (1975). Premalignant lesions of oral epithelium. *In* Oral mucosa in health and disease. Ed. AE Dolby. Blackwell Scientific Publications, Oxford.

Martin, RG and Chou, JY. (1975). Simian virus 40 functions required for the establishment and maintenance of the transformed state. J. Virol. *15*, 599.

MacNab, JCM, Stephen, A, Walkinshaw, MB, Cordiner, JW and Clements, JB. (1986). Human papillomavirus in clinically and histologically normal tissue of patients with genital cancer. New Eng. J. Med. *315*, 1052.

Maitland, NJ, Cox, MF, Lynas, C, Prime, SS, Meanwell, CA and Scully, C. (1987). Detection of human papillomavirus DNA in biopsies of human oral tissue. Br. J. Cancer *56*, 245.

Maitland, NJ, Bromidge, T, Cox, MF, Crane, IJ, Prime, SS and Scully, C. (1989). Detection of human papillomavirus genes in human oral tissue biopsies and cultures by polymerase chain reaction. Br. J. Cancer *59*, 698.

Mandel, M and Higa, A. (1970). Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. *53*, 154.

Maniatis, T, Fritsch, EF and Sambrook, J. (1982). Molecular cloning - a laboratory manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.

Mascres, C, Ming-Wen, F and Joly, JC. (1984). Morphologic changes of esophageal mucosa in the rat after chronic alcohol ingestion. Expl. Path. *25*, 147.

Matlashewski, G, Schneider, J, Banks, L, Jones, N, Murray, A and Crawford, L. (1987). Human papillomavirus type 16 DNA cooperates with activated *ras* in transforming primary cells. EMBO J. *6*, 1741.

Matlashewski, G, Osborn, K, Banks, L, Stanley, M and Crawford, L. (1988). Transformation of primary human fibroblast cells with human papillomavirus type 16 DNA and EJ-*ras*. Int. J. Cancer *42*, 232.

Matsukuru, T, Kanda, T, Furuno, A, Yoshikawa, H, Kawana, T and Yoshiike, K. (1986). Cloning of monomeric human papillomavirus type 16 DNA integrated within cell DNA from a cervical carcinoma. J. Virol. *58*, 979.

McBride, WD and Weiner, A. (1964). In vitro transformation of hamster kidney cells by human adenovirus type 12. Proc. Soc. Exp. Biol. Med. *115*, 870.

McCarthy, PL. (1984). *In* Oral Cancer. Ed. G. Shklar. WB Saunders Co., Philadelphia.

McDougall, JK, Galloway, DA and Fenoglio, CM. (1980). Cervical carcinoma: detection of herpes simplex virus RNA in cells undergoing neoplastic change. Int. J. Cancer *25*, 1.

McCutchan, T, Singer, M and Rosenberg, M. (1979). Structure of simian virus 40 recombinants that contain both host and viral DNA sequences. II. The structure of variant 1103 and its comparison to variant CVP8/1/P2 (EcoRI res). J. Biol. Chem. *254*, 3592.

Meanwell, CA, Cox, MF, Blackledge, G and Maitland, NJ. (1987). HPV 16 DNA in normal and malignant cervical epithelium: implications for the aetiology and behaviour of cervical neoplasia. Lancet *i*, 703.

Mehta, FS, Pindborg, JJ and Hamner, JE. (1971). Oral cancer and precancerous conditions in India. Munksgaard, Copenhagen.

Melbye, M, Palefsky, J, Gonzales, J, Ryder, LP, Nielsen, H, Bergmann, O, Pindborg, J and Biggar, RJ. (1990). Immune status as a determinant of human papillomavirus and its association with anal epithelial abnormalities. Int. J. Cancer *46*, 203.

Milde, K and Loning, T. (1986). Detection of papillomavirus DNA in oral papillomas and carcinomas: application of in situ hybridization with biotinylated HPV16 probes. J. Oral Pathol. *15*, 292.

Millan, DWM, Davis, JA, Torbet, TE and Campo, MS. (1986). DNA sequences of human papillomavirus types 11, 16 and 18 in lesions of the uterine cervix in the west of Scotland. Br. Med. J. *293*, 93.

Miller, AB. (1974). The epidemiology of oral cancer. J. Canad. Dent. Assn. *40*, 211.

Moore, C. (1965). Smoking and cancer of the mouth, pharynx and larynx. J. Am. Med. Assn. *191*, 283.

Moore, AE, Sabachewsky, L and Toolan, HW. (1955). Culture characteristics of four permanent lines of human cancer cells. Cancer Res. *15*, 598.

Morgan, D, Pecoraro, G, Rosenberg, I and Defendi, V. (1990). Human papillomavirus type 6b DNA required for initiation but not maintenance of transformation of C127 mouse cells. J. Virol. *64*, 969.

Moroy, T, Marchio, A, Etiemble, J, Trepo, C, Tiollais, P and Buendia, M-A. (1986). Rearrangement and enhanced expression of c-*myc* in hepatocellular carcinoma of hepatitis virus infected woodchucks. Nature *324*, 276.

Moss, E and Lee, WR. (1974). Occurrence of oral and pharyngeal cancers in textile workers. Br. J. Ind. Med. *31*, 224.

Mounts, P, Shah, KV and Kashima, H. (1982). Viral etiology of juvenile and adult onset squamous papilloma of the larynx. Proc. Natl. Acad. Sci. USA *79*, 5425.

Munoz, N, Bosch, X and Kaldor, JM. (1988). Does human papillomavirus cause cervical cancer? The state of the epidemiological evidence. Br. J. Cancer *57*, 1.

Naghashfar, Z, Sawada, E, Kutcher, MJ, Swancar, J, Gupta, J, Daniel, R, Kashima, H, Woodruff, JD and Shah, K. (1985). Identification of genital tract papillomaviruses HPV-6 and HPV-16 in warts of the oral cavity. J. Med. Virol. *17*, 313.

Natali, PG, Nicotra, MR, Bigotti, A, Venturo, I, Marcenaro, L, Giaconini, P and Russo, C. (1989). Selective changes in expression of HLA class I polymorphic determinants in human solid tumours. Proc. Natl. Acad. Sci. USA *86*, 6719.

Neel, BG and Hayward, WS. (1981). Avian leukosis virus-induced tumours have common proviral integration sites and synthesize discrete new RNAs: oncogenesis by promoter insertion. Cell *23*, 323.

Nilsen, TW, Maroney, PA, Goodwin, RG, Rottman, FM, Crittenden, LB, Raines, MA and Kung, H-J. (1985). c-*erb*B activation in ALV-induced erythroblastosis: novel RNA processing and promoter insertion result in expression of an amino-truncated EGF receptor. Cell *41*, 719.

Novak, Y, Dilworth, SM and Griffin, BE. (1980). Coding capacity of a 35% fragment of the polyoma virus genome is sufficient to initiate and maintain cellular transformation. Proc. Natl. Acad. Sci. USA. *77*, 3278.

Nusse, R. (1986). The activation of cellular oncogenes by retroviral insertion. Trends Genet. *2*, 244.

Okazaki, W, Purchase, HG and Burmester, BR. (1970). Protection against Marek's disease by vaccination with a herpesvirus of turkeys. Avian Diseases *14*, 413.

Oltersdorf, T, Campo, MS, Favre, M, Dartmann, K and Gissmann, L. (1986). Molecular cloning and characterization of human papillomavirus type 7 DNA. Virology *149*, 247.

Orth, G, Favre, M and Croissant, O. (1977). Characterisation of a new type of human papillomavirus that causes skin warts. J. Virol. *24*, 108.

Orth, G, Favre, M, Breitburd, F, Croissant, O, Jablonska, S, Obalek, S, Jarzabek-Chorzelska, M and Rzesa, G. (1980). Epidermodysplasia verruciformis: a model for the role of papillomaviruses in human cancer. *In* Viruses in naturally ocurring cancers. Ed. M. Essex. p 259. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Osborn, M and Weber, K. (1975). Simian virus 40 gene A function and maintenance of transformation. J. Virol. *15*, 636.

Ostrow, RS, Bender, M, Niimura, M, Seki, T, Kawashima, M, Pass, F and Faras, AJ. (1982). Human papillomavirus DNA yin cutaneous primary and metastasized squamous cell carcinomas from patients with epidermodysplasia verruciformis. Proc. Natl. Acad. Sci. USA *79*, 1634.

Ostrow, R, Manias, DA, Fong, WJ, Zachow, KR and Faras, AJ. (1987). A survey of human cancers for HPV DNA by filter hybridisation. Cancer *59*, 429.

Ozanne, B, Richards, CS, Hendler, F, Burns, D and Gusterson, B. (1986). Over-expression of the EGF receptor is a hallmark of squamous cell carcinomas. J. Pathol. *149*, 9.

Pagano, JS. (1975). Disease and mechanisms of persistent DNA virus infection: latency and cellular transformation. J. Infect. Dis. *132*, 209.

Parkinson, EK and Yeudall, WA. (in press). The culture of primary tumours from human epidermis. *In* Primary cultures from human tumour biopsies: A Handbook. Ed. J. Masters. Kluwer.

Paterson, DR. (1919). A clinical type of dysphagia. J. Laryngol. *34*, 285.

Pettersson, U and Roberts, RJ. (1986). Adenovirus gene expression and replication: a historical review. Cancer Cells Vol. 4. DNA Tumor Viruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 37.

Pfister, H. (1984). Biology and biochemistry of papillomaviruses. Rev. Physiol. Biochem. Pharmacol. *99*, 111.

Pfister, H, Gassenmeier, A, Nurnberger, F and Stuttgen, G. (1983a). HPV-5 DNA in a carcinoma of an epidermodysplasia verruciformis patient infected with various human papillomavirus types. Cancer Res. *43*, 1436.

Pfister, H, Hettich, I, Runne, U, Gissmann, L and Chilf, N. (1983b). Characterisation of human papillomavirus type 13 from lesions of focal epithelial hyperplasia Heck. J. Virol. *47*, 363.

Phelps, WC, Yee, CL, Munger, K and Howley, PM. (1988). The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1a. Cell *53*, 539.

Pindborg, JJ. (1972). Is submucous fibrosis a precancerous condition in the oral cavity? Int. Dent. J. *22*, 474.

Pindborg, JJ. (1980). Oral cancer and precancer. John Wright and Sons Ltd., Bristol.

Pindborg, JJ, Mehta, FS, Gupta, PC, Daftary, DK and Smith, CJ. (1971). Reverse smoking in Andhra Pradesh, India: a study of palatal lesions among 10,169 villagers. Br. J. Cancer *25*, 10.

Pindborg, JJ, Poulsen, HE and Zachariach, J. (1967). Oral epithelial changes in thirty Indians with oral cancer and submucous fibrosis. Cancer *20*, 1141.

Pirisi, L, Yasumoto, S, Feller, M, Doninger, JK and DiPaolo, JA. (1987). Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. J. Virol. *61*, 1061.

Praetorius-Clausen, F. (1972). Rare oral viral disorders (molluscum contagiosum, localized keratoacanthoma, verrucae, condyloma acuminatum, and focal epithelial hyperplasia). Oral Surg. *34*, 604.

Praetorius-Clausen, F and Willis, JM. (1971). Papova virus-like particles in focal epithelial hyperplasia. Scand. J. Dent. Res. *79*, 362.

Prime, SS, McDonald, DG and Rennie, JS. (1983). The effect of iron deficiency on experimental oral carcinogenesis in the rat. Br. J. Cancer *47*, 413.

Raab-Traub, N, Flynn, K, Pearson, G, Huang, A, Lanier, A and Pagano, JS. (1987). The differentiated form of nasopharyngeal carcinoma contains Epstein-Barr virus DNA. Int. J. Cancer *39*, 25.

Rando, RF, Groff, DE, Chirikjian, JG and Lancaster, WD. (1986). Isolation and characterization of a novel human papillomavirus type 6 DNA from an invasive vulvar carcinoma. J. Virol. *57*, 353.

Rassoulzadegan, M, Naghashfar, Z, Cowie, A, Carr, A, Grisoni, M, Kamen, R and Cuzin, F. (1983). Expression of the large T protein of polyoma virus promotes the establishment in culture of "normal" rodent fibroblast cell lines. Proc. Natl. Acad. Sci. USA *80*, 4354.

Rawls, WE, Tompkins, WA and Melnick, JL. (1969). The association of herpes virus type 2 and carcinoma of the uterine cervix. Am. J. Epidemiol. *89*, 547.

Reyes, GR, LaFemina, R, Hayward, SD and Hayward, GS. (1979). Morphological transformation by DNA fragments of human herpesviruses: evidence for two distinct transforming regions in herpes simplex virus types 1 and 2 and lack of correlation with biochemical transfer of the thymidine kinase gene. Cold Spring Harbor Symp. Quant. Biol. *44*, 629.

Rheinwald, JG and Beckett, MA. (1981). Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinomas. Cancer Res. *41*, 1657.

Rheinwald, JG and Green, H. (1975). Serial cultivation of strains of human epidermal keratinocytes: The formation of keratinizing colonies from single cells. Cell *6*, 331.

Rigby, PWJ, Dieckmann, M, Rhodes, C and Berg, P. (1977). Labelling deoxyryibonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. *113*, 237.

Rothman, K and Keller, AZ. (1972). The effect of joint exposure to alcohol and tobacco on risk of cancer of the mouth and pharynx. J. Chron. Dis. *25*, 711.

Rotkin, ID. (1973). A comparison review of key epidemiological studies in cervical cancer related to current searches for transmissible agents. Cancer Res. *33*, 1353.

Rous, P. (1911). A sarcoma of the fowl transmissible by an agent separable from the tumour cells. J. Exp. Med. *13*, 397.

Rous, P and Kidd, JG. (1938). The carcinogenic effect of a papilloma virus on the tarred skin of rabbits. J. Exp. Med. *67*, 399.

Ruley, HE. (1983). Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature *304*, 602.

Saiki, RK, Gelfand, DH, Stoffel, S, Scharf, SJ, Higuchi, R, Horn, GT, Mullis, KB and Ehrlich, HA. (1988). Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science *239*, 487.

Sambrook, J, Fritsch, EF and Maniatis, T. (1989). Molecular cloning. A laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Sanger, F, Nicklen, S and Coulsen, AR. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA *74*, 5463.

Santos, E, Tronick, SR, Aaronson, SA, Pulciani, S and Barbacid, M. (1982). T24 human bladder carcinoma oncogene is an activated form of the normal homologue of BALB- and Harvey-MSV transforming genes. Nature *298*, 343.

Sarnow, P, Ho, YS, Williams, J and Levine, AJ. (1982). The adenovirus E1b-55Kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54K cellular protein. Cell *28*, 387.

Schneider-Gadicke, A and Schwarz, E. (1986). Different cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J. *5*, 2285.

Schneider-Maunoury, S, Croissant, O and Orth, G. (1987). Integration of human papillomavirus type 16 DNA sequences: a possible early event in the progression of genital tumors. J. Virol. *61*, 3295.

Schwarz, E, Freese, UK, Gissmann, L, Mayer, W, Roggenbuck, B, Stremlau, A and zur Hausen, H. (1985). Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature *314*, 111.

Scully, C, Cox, MF, Prime, SS and Maitland, NJ. (1988). Papillomaviruses : the current status in relation to oral disease. Oral Surg. *65*, 526.

Seedorf, K, Krammer, G, Durst, M, Suhal, S and Rowekamp, WG. (1985). Human papillomavirus type 16 DNA sequence. Virology *145*, 181.

Seiki, M, Inoue, J, Takeda, T and Yoshida, M. (1986). Direct evidence that $p40^{X}$ of human T-cell leukemia virus type I is a *trans*-acting transcription factor. EMBO J. *5*, 561.

Sharp, PA, Pettersson, U and Sambrook, J. Viral DNA in transformed cells. I. A study of the sequences of adenovirus 2 DNA in a line of transformed rat cells using specific fragments of the viral genome. J. Mol. Biol. *86*, 709.

Shillitoe, EJ, Greenspan, D, Greenspan, JS and Silverman, S Jr. (1983). Immunoglobulin class of antibody to herpes simplex virus in patients with oral cancer. Cancer *51*, 73.

Shillitoe, EJ, Greenspan, D, Greenspan, JS and Silverman, S Jr. (1984). Antibody to early and late antigens of herpes simplex virus type 1 in patients with oral cancer. Cancer *54*, 266.

Shillitoe, EJ, Tarro, G and Lehner, T. (1976). Cell-mediated immunity to herpes simplex virus types 1 and 2 antigens in leukoplakia and carcinoma in man. Oncology *33*, 192.

Shope, RE and Hurst, EW. (1933). Infectious papillomatosis of rabbits; with a note on the histopathology. J. Exp. Med. *58*, 607.

Sibbet, GJ and Campo, MS. (1990). Multiple interactions between cellular factors and the non-coding region of human papillomavirus type 16. J. Gen. Virol. *71*, 2699.

Smith, CJ. (1973). Global epidemiology and aetiology of oral cancer. Int. Dental J. *23*, 82.

Smith, EM. (1979). Epidemiology of oral and pharyngeal cancers in the United States: review of the recent literature. J. Natl. Cancer Inst. *63*, 1189.

Smith, KT and Campo, MS. (1988). "Hit and run" transformation of mouse C127 cells by bovine papillomavirus type 4: the viral DNA is required for the initiation but not for maintenance of the transformed phenotype. Virology *164*, 39.

Smith, HG, Horowitz, N, Silverman, NA, Henson, DE and Chretien, PB. (1976). Humoral immunity to herpes simplex viral-induced antigens in smokers. Cancer *38*, 1155.

Smotkin, D and Wettstein, FO. (1987). The major human papillomavirus protein in cervical cancers is a cytoplasmic phosphoprotein. J. Virol. *61*, 1686.

Smotkin, D, Prokoph, H and Wettstein, FO. (1989). Oncogenic and nononcogenic human genital papillomaviruses generate the E7 mRNA by different mechanisms. J. Virol. *63*, 1441.

Snijders, PJF, van den Brule, AJC, Schrijnemakers, HFJ, Snow, G, Meijer, CJLM and Walboomers, JMM. (1990). The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. J. Gen. Virol. *71*, 173.

Southern, EM. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. *98*, 503.

Spandidos, DA, Lamothe, A and Field, JK. (1985). Multiple transcriptional activation of cellular oncogenes in human head and neck solid tumours. Anticancer Res. *5*, 221.

Steinberg, B, Pollack, R, Topp, W and Botchan, M. (1978). Isolation and characterisation of T-antigen revertants from a line of transformed rat cells containing one copy of the SV40 genome. Cell *13*, 19.

Storey, A, Pim, D, Murray, A, Osborn, K, Banks, L and Crawford, L. (1988). Comparison of the *in vitro* transforming activities of human papillomavirus types. EMBO J. *7*, 1815.

Storey, A, Osborn, K and Crawford, L. (1990). Co-transformation by human papillomavirus types 6 and 11. J. Gen. Virol. *71*, 165.

Sundberg, JP, Schmidt, E, O'Banion, K and Olson, C. (1985). Squamous cell carcinoma associated with canine oral papillomavirus. J. Cell. Biochem. *9*, 75.

Sutcliffe, JG. (1979). Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. *43*, 77.

Syrjanen, K, Syrjanen, S, Lamberg, Pyrhonen, S and Nuutinen, J. (1983). Morphological and immunohistochemical evidence suggesting human papillomavirus (HPV) involvement in oral squamous cell carcinogenesis. Int. J. Oral Surg. *12*, 418.

Syrjanen, S, Syrjanen, K, Ikenberg, H and Gissmann, L. (1984). A human papillomavirus closely related to HPV13 found in a focal epithelial hyperplasia lesion (Heck's disease). Arch. Dermatol. Res. *276*, 199.

Syrjanen, S, Syrjanen, KJ and Happonen, R-P. (1988). Human papillomavirus (HPV) DNA sequences in oral precancerous lesions and squamous cell carcinoma demonstrated by *in situ* hybridisation. J. Oral Pathol. *17*, 273.

Syverton, JT. (1952). The pathogenesis of the rabbit papilloma-to - carcinoma sequence. Ann. NY Acad. Sci. *54*, 1126.

Tadokoro, K, Ueda, M, Ohshima, T, Fujita, K, Rikimaru, K, Takahashi, N, Enomoto, S and Tsuchida, N. (1989). Activation of oncogenes in human oral cancer cells: a novel codon 13 mutation of c-H-*ras*-1 and concurrent amplifications of c-*erb*B-1 and c-*myc*. Oncogene *4*, 499.

Tanaka, K, Oshimura, M, Kikuchi, R, Seki, M, Hayashi, T and Miyaka, M. (1991). Suppression of tumorigenicity in human colon carcinoma cells by introduction of normal chromosome 5 or 18. Nature *349*, 340.

Todaro, GJ and Green, H. (1963). Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol. *17*, 299.

Treisman, RH, Novack, V, Favoloro, J and Kamen, R. (1981). Transformation of rat cells by an altered polyoma virus genome expressing only the middle T protein. Nature *292*, 595.

Tyldesley, WR. (1981). Oral medicine. Oxford University Press.

Ullrich, A, Coussens, L, Hayflick, JS, Dull, TJ, Gray, A, Tam, AW, Lee, J, Liberman, TA, Schlessinger, J, Downward, J, Mayes, EL, Whittle, N, Waterfield. MD and Seeburg, PH. (1984). Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature *309*, 418.

Villa, LL and Schlegel, R. (1991). Differences in transformation activity between HPV-18 and HPV-16 map to the viral LCR-E6-E7 region. Virology *181*, 374.

Vincent, RG, Marchetta, FC and Nigogosyan, G. (1964). Incidence of cirrhosis in oral cancer. N. Y. State J. Med. *64*, 2174.

Vogler, WR, Bross, IJ and Milmore, BK. (1962). A retrospective study of etiological factors in cancer of the mouth, pharynx and larynx. Cancer *15*, 246.

Vousden, KH and Jat, PS. (1989). Functional similarity between HPV16 E7, SV40 large T and adenovirus E1a proteins. Oncogene *4*, 153.

Wakamiya, T, McCutchan, T, Rosenberg, M and Singer, M. (1979). Structure of simian virus recombinants that contain both host and viral DNA sequences. I. The structure of variant CVP8/1/P2 (EcoRI res). J. Biol. Chem. *254*, 3584.

Watanabe, S, Kanda, T, Sato, H, Furuno, A and Yoshiike, K. (1990). Mutational analysis of human papillomavirus type 16 E7 functions. J. Virol. *64*, 207.

Wells, M, Griffiths, S, Lewis, FA and Bird, CC. (1987). Demonstration of human papillomavirus types in paraffin processed tissue from human ano-genital lesions by *in-situ* DNA hybridization. J. Pathol. *152*, 77.

Werness, BA, Levine, AJ and Howley, PM (1990). Association of human papillomavirus type 16 and 18 E6 proteins with p53. Science *248*, 76.

Westaway, D, Payne, G and Varmus, H. (1984). Proviral deletions and oncogene base-substitutions in insertionally mutagenized c-myc alleles may contribute to the progression of avian bursal tumors. Proc. Natl. Acad. Sci. USA *81*, 843.

Whyte, P, Buchkovich, KJ, Horowitz, JM, Friend, SH, Raybuck, M, Weinberg, RA and Harlow, E. (1988). Association between an oncogene and an anti-oncogene: the adenovirus E1a proteins bind to the retinoblastoma gene product. Nature, *334*, 124.

Wilczynski, SP, Pearlman, L and Walker, J. (1988). Identification of HPV 16 early genes retained in cervical carcinomas. Virology *166*, 624.

Winn, DM, Blot, WJ, Shy, CM, Pickle, LW, Toledo, A and Fraumeni, JF Jr. (1981). Snuff-dipping and oral cancer among women in the southern United States. New Eng. J. Med. *304*, 745.

Winship, PR. (1989). An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. Nucl. Acids. Res. *17*, 1266.

Wong-Staal, F and Gallo, RC. (1985). Human T-lymphotropic retroviruses. Nature *317*, 395.

Woodruff, JD, Braun, L, Cavallieri, R, Gupta, P, Pass, F and Shah, KV. (1980). Immunological identification of papillomavirus antigen in paraffin-processed condyloma tissues from the female genital tract. Obst. Gynecol. *56*, 727.

Woodworth, CD, Doniger, J and DiPaolo, JA. (1989). Immortalization of human foreskin keratinocytes by various human papillomavirus DNAs corresponds to their association with cervical carcinoma. J. Virol. *63*, 159.

Woodworth, CD, Notario, V and DiPaolo, JA. (1990). Transforming growth factors beta 1 and 2 transcriptionally regulate human papillomavirus (HPV) type 16 early gene expression in HPV-immortalized human genital epithelial cells. J. Virol. *64*, 4767.

World Health Organization. (1980) Oral mucosa manual. WHO. Geneva.

Wynder, EL and Stellman, SD. (1977). Comparative epidemiology of tobacco-related cancers. Cancer Res. *37*, 4608.

Wynder, EL, Bross, IJ and Feldman, RM. (1957a). A study of the etiological factors in cancer of the mouth. Cancer *10*, 1300.

Wynder, EL, Hultberg, S, Jacobson, F and Bross, IJ. (1957b). Environmental factors in cancer of the upper alimentary tract. A Swedish study with special reference to Plummer-Vinson's (Paterson-Kelly) syndrome. Cancer *10*, 470.

Addendum.

Sambrook, J, Westphal, H, Srinivasan, PR and Dulbecco, R. (1968). The integrated state of viral DNA in SV40-transformed cells. Proc. Natl. Acad. Sci. USA *60*, 1288.

Smith, KT and Campo, MS. (1989). Amplification of specific DNA sequences in C127 mouse cells transformed by bovine papillomavirus type 4. Oncogene *4*, 409.

Vogelstein, B and Gillespie, D. (1979). Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA *76*, 615.

Walter, JB and Israel, MS. (1974). General Pathology 4th ed. Churchill Livingston, Edinburgh.

Yasumoto, S, Burkhardt, AL, Doniger, J and DiPaolo, JA. (1986). Human papillomavirus type 16 DNA-induced malignant transformation of NIH 3T3 cells. J. Virol. *57*, 572.

Yee, C, Krishnan-Hewlett, I, Baker, CC, Schlegel, R and Howley, PM. (1985). Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. Am. J. Pathol. *119*, 361.

zur Hausen, H. (1976). Condylomata acuminata and human genital cancer. Cancer Res. *36*, 794.

zur Hausen, H. (1977). Human papillomaviruses and their possible role in squamous cell carcinomas. Curr. Top. Microbiol. Immunol. *78*, 1.

zur Hausen, H. (1982). Human genital cancer: synergism between two virus infections or synergism between a virus infection and initiating events? Lancet *ii*, 1370.

zur Hausen, H. (1988). Papillomaviruses in human cancers. Mol. Carcinog. *1*, 147.

zur Hausen, H, Schulte-Holthausen, H, Wolf, H, Dorries, K and Egger, H. (1974). Attempts to detect virus-specific DNA sequences in human tumors: II. Nucleic acid hybridizations with complementary RNA of human herpes group viruses. Int. J. Cancer *13*, 657.