Stomach cancer in transgenic mice expressing human papillomavirus type 16 early region genes from a keratin promoter

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Certain human papillomaviruses (HPVs) have been implicated as important contributory factors in the development of cervical carcinoma and other epithelial malignancies. In order to investigate the role of papillomavirus early gene expression in epithelial oncogenesis in vivo, we produced transgenic mice expressing HPV-16 early region genes from the promoter of the bovine keratin 6 gene. Spliced transcripts were detected in the tongue, forestomach, glandular stomach, female reproductive tract and tail skin of these mice. This expression was initially asymptomatic. However, at increasing frequency after approx. 100 days, solitary glands within the gastric mucosa became colonized with small dysplastic cells. Later this abnormal cell population spread within the glandular mucosa, invaded the submucosa and outer muscular wall of the stomach, and commonly metastasized to local lymph nodes and the liver. The appearance and staining characteristics of the tumours suggested their classification as malignant carcinoids, originating from the neuroendocrine enterochromaffin-like cells. Expression of HPV mRNAs was increased in the tumours, though it remained comparable to that in forestomach and tongue. The mean age at tumour presentation was 246 days in males and 352 days in females, all transgenic mice in eight independent lines were similarly susceptible. This study confirms the oncogenicity of HPV-16 early region genes, and establishes a model system in which to investigate mechanisms of malignant progression and possible therapeutic strategies for HPV-associated tumours.

Introduction

The papillomaviruses are a large group of viruses with circular DNA genomes of approximately 8 kb (Howley, 1990). They infect various cutaneous or mucosal epithelia, typically inducing hyperplasia and perturbation of keratinocyte differentiation. It is generally considered that the viruses must gain entry to a cell in the proliferative, basal layer, perhaps at a site of wounding; and that the replicative cycle of the virus is closely integrated with keratinocyte differentiation in the upper layers of the epithelium. Whereas most papillomas remain as benign tumours which may eventually be rejected by the host, it is well established in both animals and humans that some may progress to become malignant carcinomas and that the rate or frequency of this progression of this progression may be stimulated by carcinogenic agents (Shope, 1933; Rous & Kidd, 1936; Jarrett et al., 1978a, b; Orth, 1987; Shah & Howley, 1990).

Much attention in recent years has focused on those human papillomaviruses (HPVs) which infect mucosal epithelia, following the proposal that genital warts or condylomata may be precursors to anogenital carcinoma (zur Hausen, 1976; Meisels et al., 1977). Epidemiological studies support the view that infection with members of a 'high-risk' subgroup of viruses including HPV-16 and HPV-18 is associated with an increased probability of oncogenic transformation (Koutsy et al., 1988; Vosden, 1989; zur Hausen, 1989). Cell transfection experiments have shown that DNA from HPV-16 or HPV-18 but not the low-risk viruses can immortalize human keratinocytes (Schlegel et al., 1988; Woodworth et al., 1989). In combination with an activated ras gene, the same viral genes were shown to be able to transform keratinocytes to tumorigenicity (Storey et al., 1988; DiPaolo et al., 1989). Similar assays identified the E6 and E7 open reading frames (ORFs) as the transforming genes of the high-risk HPVs (Münger et al., 1989; Watanabe et al., 1989), in agreement with the observation that the E6 and E7 genes always remain intact and expressed whereas other viral genes, in particular E2, may be lost or damaged in the chromosomally integrated viral DNA often present in the tumours (Schwarz et al., 1985; Baker et al., 1987; Cullen et al.,...
1991). The E6 and E7 proteins of high-risk HPVs appear to inhibit important functions of cellular tumour-suppressor proteins. Specifically, E6 stimulates degradation of human p53 (Scheffner et al., 1990), presumably reducing its capacity to inhibit cell cycle progression in stressed cells (Hartwell, 1992; Vogelstein & Kinzler, 1992). The E7 protein binds to RB (the product of the retinoblastoma susceptibility gene), causing dissociation of the cellular transcription factor E2F (Dyson et al., 1989; Phelps et al., 1991; Pagano et al., 1992), which may contribute to the stimulation of cell cycle progression (Morris et al., 1993). The relevance of these interactions to the oncogenic process is supported by the apparently reduced dependence of HPV-positive anogenital cancers on mutations in the p53 and retinoblastoma genes (Crook et al., 1991; Scheffner et al., 1991).

Despite these highly suggestive interactions between HPV early proteins and cellular tumour-suppressor proteins, further events beyond the initial viral infection must be required for oncogenesis. This is implied both by the cell transfection experiments referred to above, and also by epidemiological studies, which indicate that only a minority of women infected with HPV-16 eventually develop cervical cancer, and the time-scale of progression may be 20 to 50 years (Koutsy et al., 1988). In order to establish a model system in which to investigate the role of HPVs and cooperating factors in epithelial oncogenesis, we have produced transgenic mice in which viral early region genes are placed under the transcriptional control of epithelial-specific promoters from keratin genes. We described previously a line of transgenic mice in which expression of the HPV-1 early region resulted in hyperplasia of the tail skin in young mice and a switch in the pattern of keratins produced (Tinsley et al., 1992). Over several years observation, we have seen no evidence of any associated malignancy in these mice. In contrast, we report here that expression of the HPV-16 early region from the same promoter caused the development of aggressive tumours in the mucosal epithelium of the glandular stomach in all eight independent lines of transgenic mice tested.

Methods

Transgenic construct. Plasmid pHV16Bam contains a complete HPV-16 genome, linearized at the BamHI site within the viral L1 ORF at nucleotide 6150, cloned into the BamHI site of pUC18. This was digested with XhoI which cuts in the plasmid polylinker, and partially digested with Cfr101; the ends filled using Klenow fragment and religated. A plasmid containing HPV sequence from the Cfr101 site at nucleotide (nt) 56 to nt 6150, including the entire early region transcription unit, was selected and designated pHV16-56Bam. The HPV sequences were excised using Sall and Smal, recloned between these sites in pBluescriptKS+ (Stratagene), then excised using XhoI and Smal and inserted between these same sites into the plasmid pbK6-CAT (alias pbKIV-CAT, provided by J. Jorcano and M. Blessing) replacing the chloramphenicol acetyltransferase gene, to generate plasmid pbK6-HPV16e, which contains approximately 2.5 kb of 5' flanking sequence from the bK6 gene with the bK6 site of transcription initiation lying 30 bp upstream of HPV-16 sequence at nt 57. This construct is shown schematically in Fig. 1.

Transgenic mice. Plasmid pbK6-HPV16e was cut with BamHI and the 8.3 kb fragment containing the keratin promoter joined to the HPV sequence was purified by agarose gel electrophoresis. This fragment was microinjected into (CBA x C57BL/6)F_{12} zygotes, and surviving embryos were transferred to pseudopregnant foster mothers ( Hogan et al., 1986). The first session resulted in the birth of 12 mice from the manipulated embryos and dot blotting of tail DNA samples indicated that one of these (mouse 135-10) was transgenic. A further series of microinjections resulted in the birth of 32 mice, of which seven were transgenic (175-8, -9, -18, -19, -29, -30 and -32). Lines were established and maintained mainly by breeding of transgenics with (CBA x C57BL/6)F_{12} mice; homozygous transgenics were also produced in the line 135-10. Analysis of the organization of the bK6-HPV16e transgene in genomic DNA by Southern blotting was consistent with the presence of around three to five copies integrated as a tandem array at a single chromosomal site in each of the established lines (results not shown). Founder 135-10 and a few of the progeny initially had a greater number of transgene copies (around 10 to 20), however these mice died before a separate subline could be established. Mice were closely monitored for tumour development or other abnormalities and were killed for autopsy as soon as a palpable mass was detected or at the onset of other signs of morbidity. All procedures involving animals were carried out with the authority of appropriate Home Office Project and Personal Licences.

Histology. For routine histology tissues were fixed in Carnoy's fixative or formal saline, embedded in paraffin wax, sectioned at 5 to 8 μm and stained with haematoxylin and eosin.

RNA analysis. Total RNA was prepared from tissue samples using guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi, 1987), and polyadenylated mRNA was isolated using a Promega PolyATtract mRNA isolation system IV magnetic separation kit. For Northern blotting, polyadenylated RNA prepared from 250 μg of total RNA per sample (approx. 2 to 5 μg polyadenylated RNA) was separated on 1% agarose gels after glyoxal denaturation (McMaster & Carmichael, 1977), vacuum-blotted and u.v.-crosslinked to Amersham Hybond-N nylon membrane. Blots were hybridized with RNA probes labelled with [α-32P]dCTP using a random priming kit (Pharmacia), and washed, using standard procedures (Sambrook et al., 1989). Autoradiographs were obtained using preflashed Kodak XAR film, and exposed with an intensifying screen at −30 °C. The size markers were a glyoxal-denatured 1 kb DNA ladder (Gibco BRL).

For RNAse protection mapping (Sambrook et al., 1989), 150 μg of total RNA was hybridized with a complementary strand RNA probe for the E6/E7 region synthesized using T3 RNA polymerase and incorporating [α-32P]GTP. The template was a derivative of pBluescriptKS+ containing a 980 bp XbaI–KpnI fragment including part of the keratin promoter and viral sequences to nt 884. Samples were separated on a denaturing 6% polyacrylamide gel and autoradiographed as above. Labelled transcripts produced from the same template with restriction endonucleases at sites internal to the viral sequence were used as size markers.

For S1 nuclease mapping, polyadenylated RNA samples equivalent to 250 μg total RNA were hybridized with a denatured DNA fragment end-labelled using [α-32P]ATP at nt 163 of the viral sequence (Spst site), and extending to the XbaI site within the keratin promoter sequence; hybridization and S1 nuclease treatment followed established protocols (Sambrook et al., 1989) and the same DNA probe was used to generate the G ladder by treatment with dimethylsulphate and pipendine.
Samples were separated on a denaturing 6% polyacrylamide gel and autoradiographed as above.

**In situ hybridization.** Tissues were fixed in 4% paraformaldehyde in PBS, embedded in paraffin wax and sectioned at 6 μm. RNA probes incorporating [α-35S]thio-UTP were transcribed using T3 RNA polymerase from linearized pBluescript vectors containing either the E6/E7 region (nt 56 to 884) or the E4/E5 region (nt 3333 to 3978) of HPV-16; both were combined to increase the sensitivity of the assay. The DNA template was degraded using RNase-free DNase I; partial probe hydrolysis, pretreatment of the sections, hybridization and washing were performed essentially as described (Haffner & Willison, 1993). Slides were dipped in Ilford G5 emulsion and exposed for 5 to 10 days. Sections treated with ribonuclease prior to hybridization, or sections from non-transgenic mice, gave a uniform background level of signal.

**Results**

**The bK6-HPV16e construct and production of transgenic mice**

HPV-16 is the virus most commonly detected in advanced cervical intraepithelial neoplasia (CIN) and cervical carcinoma (Vousden, 1989; Cullen *et al.*, 1991; zur Hausen, 1991). In common with other papillomaviruses, the viral replication origin and transcriptional regulatory sequences including major early and late promoters are located within the non-coding upstream regulatory region (URR, approx. 0.8 to 1 kb), followed by about six ‘early’ and two ‘late’ ORFs, encoded unidirectionally; several mRNAs are produced by alternative splicing of the primary transcripts, and internal promoters may also contribute. Two lines of transgenic mice carrying HPV-16 constructs incorporating the URR failed to express the transgene (unpublished results); we therefore replaced the viral URR sequences from a point just upstream of the viral early region TATA sequence, with transcription regulatory sequences from the bovine keratin 6 gene (bK6, originally referred to as bovine keratin IV; Blessing *et al.*, 1987). The resulting bK6-HPV16e construct is shown in Fig. 1. Eight transgenic founder mice were produced carrying this DNA, and transgenic lines were established from each of these; see Methods section for details. The transgenic line 135-10 has been most thoroughly investigated, and all the data shown were derived from this line.

**Development of tumours in bK6-HPV16e transgenic mice**

Transgenic mice in all lines were initially healthy and normal in appearance. However commencing at around 4 to 6 months of age transgenic mice presented with tumours in the stomach. In some cases, particularly those presenting earlier, the affected mice adopted a hunched posture and lost weight; in such cases it appeared the tumour may have blocked the lumen of the pyloric region of the stomach. More commonly, the mice remained healthy in appearance and fully active but an abdominal mass was detected by palpation; on autopsy the mass was found to be either a primary tumour growth attached to the stomach, or one or more metastases in local lymph nodes or the liver. In a minority of cases, mice died before any signs of morbidity were noted, but advanced stomach tumours were detected at autopsy.

The incidence of tumour development appears to be 100% of transgenic mice in each of the lines; Fig. 2 shows survival curves for transgenic mice in line 135-10. In this line, male transgenics presented with stomach tumours at a mean age of 246 days (s.d. = 62, n = 86); the average age at presentation in females was significantly later (mean age 352 days, s.d. = 88, n = 73). Transgenic mice in the other seven lines have developed stomach tumours on a similar timescale (data based on between 14 and 60 transgenic mice in the different lines), and interestingly the sex difference in average age at presentation was also maintained.
The stomachs of young adult transgenic mice appeared both grossly and histologically normal (not shown). The mouse stomach is divided into a very distensible forestomach lined with squamous epithelium, and a glandular region lined with columnar, mucus-secreting cells and invaginated into pits, into which the gastric glands open. An abrupt transition between the types of epithelium runs equatorially around the stomach. At a gross level the tumours appeared to arise within the glandular region of the stomach, causing thickening and loss of elasticity of the stomach wall as well as more prominent attached tumour masses in this region. This was confirmed histologically. In mice killed before the appearance of palpable tumours or other signs of morbidity, microscopic foci of tumour cells were detected in the glandular stomach in slightly over half of the mice examined between 100 and 150 days old and in an increasing proportion of older mice. Fig. 3(a) shows a region of the glandular stomach from an asymptomatic transgenic mouse at 132 days old; an early stage of tumour development is seen, in which a solitary gastric gland has been populated by dysplastic cells with large nuclei and relatively little cytoplasm, while the surrounding tissue remains normal in appearance. Later, more extensive colonization of glands with dysplastic cells occurs as shown in Fig. 3(b). In more advanced stages, similar poorly differentiated cells spread laterally in the glandular mucosa, invade through the muscularis mucosa into the submucosa, and eventually through the outer muscular wall of the stomach (Fig. 3(e)).
of tumour presentation, metastases are frequently present in the liver (Fig. 3d) and/or the local lymph nodes and very rarely in the lung. The histological appearance of the tumours is similar to that of human carcinoid tumours, which arise from neuroendocrine cells, such as the enterochromaffin-like cells of the gut. This identity was confirmed by staining sections with Grimelius’ stain; as shown in Fig. 3(e) the invading tumour cells stain more darkly than the normal glands which are seen in cross-section. At higher magnification it can be seen that the silver stain is concentrated particularly to perinuclear granules (Fig. 3f), which is characteristic of carcinoid tumours. The malignant nature of the transgenic tumours was further indicated by their ability to grow when transplanted subcutaneously in non-transgenic (CBA x C57BL/6) F1 hybrid mice (P. F. Searle, D. P. Thomas, K. B. Faulkner & J. M. Tinsley, unpublished results).

Expression of HPV-16 mRNAs in tissues and tumours

The development of the same tumours in multiple independent lines of transgenic mice indicates that their development must be due to expression of HPV-16 genes rather than to the chance effects of insertional mutagenesis. Expression of HPV-16 early region mRNAs in normal tissues and tumours of transgenic mice was analysed by Northern blotting of polyadenylated RNA. Using a probe corresponding to the HPV-16 early region, three predominant RNA species approximately...
4.5, 1.8 and 1.3 kb in size were detected (Fig. 4a). The highest levels were detected in the tongue and forestomach with lower levels in the glandular region of the stomach, tail skin and female reproductive tract (vagina, cervix and uterus). Later experiments in which the reproductive tract was subdivided showed that the transcripts were associated with the vagina and cervix and not with the uterus (see Fig. 6b). The integrity of the mRNA was confirmed by rehybridizing the stripped membrane with a β-actin probe (lower panel).

The promoter used in the transgene was from the bovine keratin 6 gene. Keratin 6 is a hyperproliferation-associated keratin, expressed transiently e.g. during epithelial wound healing, but constitutively in foot sole epidermis, in the outer root sheath of hair follicles, in several mucosal epithelia including tongue and cervix and in well differentiated squamous cell carcinomas (Moll et al., 1982; Stotler et al., 1988). We have observed high level expression of mouse keratin 6 in the cervix, vagina and forestomach (results not shown). In cattle, expression of bovine keratin 6 was reported in tongue, snout, heel pad and a mammary epithelial cell line (Blessing et al., 1987). The tissue distribution of transgene expression was therefore broadly as expected, though expression in the reproductive tract appeared relatively low and expression in the glandular stomach was not anticipated. It is interesting that expression of the HPV-1 early region from the same promoter in a single line of transgenic mice was biased towards expression in cutaneous epithelia (Tinsley et al., 1992) whereas the pattern of expression of the HPV-16 transgene in all of the lines described here was considerably higher in mucosal epithelia, suggesting that viral sequences within the constructs might contribute to the tissue specificity of expression.

The same predominant HPV-16 mRNA species were observed in RNA extracted from primary tumours (Fig. 4b), as well as in metastases and transplanted tumours (results not shown). Expression was greater in tumour tissue than in premalignant glandular stomach, but comparable to levels seen in the tongue and forestomach of transgenic mice, and also to that in the HPV-16-expressing human cervical carcinoma cell line, CaSki, shown in the last lanes of Fig. 4(a and b). Note however that these mRNAs in CaSki cells are not indicative of the sizes of normal HPV-16 mRNAs because they are fusion transcripts also containing cellular sequences (Smotkin & Wettstein, 1986).

To examine the cellular distribution of HPV-16 mRNAs within transgenic tissues, in situ hybridization was performed using 35S-labelled RNA probes corresponding to the E6/E7 and E4/E5 regions. Fig. 5(a) and (b) show respectively the darkfield image of the autoradiographic silver grains over a section of forestomach, and the brightfield image of the same section stained with haematoxylin and eosin. It is clear that the highest level of expression is localized to suprabasal keratinocytes. A similar pattern of hybridization has been seen in the oesophagus, the lining of the buccal cavity and the tongue (results not shown). Fig. 5(c) and (d) show a region of the glandular stomach mucosa with invading tumour; the pattern of high-level expression indicated by the silver grains corresponds precisely to the tumour tissue. The question arises whether the progenitor cells which give rise to the tumours constitutively express the HPV transgene at this relatively high level, or whether an increase in HPV gene expression occurs as an early step in tumour progression. The latter appears more likely, as we have not been able to detect foci of high-level expression in the glandular stomach except in association with histologically recognizable tumour.

**Structure of HPV-16 mRNAs expressed**

The early region transcription unit of HPV-16 contained in the bK6-HPV16e construct contains multiple ORFs downstream of the keratin promoter (as indicated in Fig. 1 and 6d). Which of these may actually be translated will depend on the pattern of splicing of the primary transcript, which we have therefore analysed in greater detail. Northern blots of tumour mRNA were hybridized separately with probes corresponding to the E6/E7, E1 and E4/E5 regions, or the entire early region as indicated (Fig. 6a). The 4.5 kb mRNA was detected with each of the probes, suggesting that this is a full-length transcript initiating at the keratin promoter and terminating at the early region polyadenylation site. The 1.3 and 1.8 kb bands were both detected using either the E6/E7 or E4/E5 probes but not the intervening E1 probe, suggesting that these mRNAs are produced by splicing of the full-length early region transcript. The size of the 1.8 kb mRNA is consistent with that of a full-length primary transcript of the early region spliced between the known splice donor and acceptor sites at viral nt 880 and 3358 (splice 880-3358; Vormwald-Dogan et al., 1992). The E4/E5 probe hybridizes equivalently with both the 1.8 and 1.3 kb mRNAs, whereas the E6/E7 probe gives a stronger signal with the 1.8 kb mRNA than with the smaller species, suggesting that both mRNAs contain the same sequences from the E4/E5 region but that the 1.3 kb mRNA has a shorter region of homology with the E6/E7 probe. This might result from splicing of the same primary transcript between nt 226 and 3358 (see below). Additional faint bands present on the Northern blots in Fig. 4 and 6(a) may represent additional minor RNA species with different splicing patterns, or sites of initiation or termination.

Efficient translation of E7 is thought to require internal
splicing within the E6 gene (Smotkin et al., 1989). We therefore performed ribonuclease protection assays on RNA from several tissues from the transgenic mice, using an E6/E7 probe. As indicated in Fig. 6(b), a full-length transcript of this region initiating at the keratin promoter would protect 854 nt of probe; this was seen as a minor species in the forestomach, tongue and tail skin. The resolution of this assay does not distinguish whether or not the RNAs are spliced at the nt 880 splice donor site, as this is too close to the end of the homologous region of the probe. The predominant bands seen in these tissues and also in the glandular stomach and vagina/cervix, though not in the duodenum or uterus, correspond to the sizes predicted for mRNA spliced 226-409. This mRNA includes a truncated E6*I open reading frame (Smotkin et al., 1989) and the intact E7 coding sequence; this was previously found to be the predominant splicing pattern of the E6/E7 region in CIN and invasive carcinomas (Smotkin et al., 1989; Sherman et al., 1992). Equimolar amounts of the 200 and 471 nt protected bands would give a > twofold greater intensity of the upper band; we interpret the approximately equal intensities, together with the results shown in Fig. 6(a), to indicate that approximately 50% of transcripts are spliced directly from nt 226 within the E6 coding sequence to the E4/E5 region to produce the 1.3 kb

Fig. 5. In situ hybridization of transgenic stomach sections with E6/E7 and E4/E5 probes. (a, b) Forestomach sections; note that the tissue has pulled apart in the submucosa; the cornified layer facing the lumen of the stomach is towards the top, arrows indicate the basal cell layer. (c, d) Glandular stomach mucosa; note colocalization of silver grains with clusters of tumour cells; the muscularis mucosa is near the right of the photographs. (a, c) Darkfield view showing autoradiographic silver grains; (b, d) brightfield view of the same area of the sections, stained with haematoxylin and eosin. Bar marker represents 200 μm.
Fig. 6. Mapping of bK6-HPV16e transcripts. (a) Northern blot of polyadenylated RNA from a tumour electrophoresed and blotted in four identical lanes; the nylon membrane with the cross-linked RNA was cut into strips and hybridized with probes as indicated (left to right) corresponding to the E6/E7 region (nt 56 to 884), the E1 region (nt 884 to 3210), the E4/E5 region (nt 3210 to 4335) or the intact early region. (b) Ribonuclease protection mapping of total RNA from tissues, using a 980 nt E6/E7 complementary strand RNA probe. The probe was initiated in the plasmid vector, and was complementary to the bK6-HPV16e transgene from HPV-16 nt 884 to an XbaI site within the keratin promoter. Tissues are (lane 1) forestomach, (lane 2) glandular stomach, (lane 3) duodenum, (lane 4) vagina/cervix, (lane 5) uterus, (lane 6) tongue and (lane 7) tail skin. m, Markers, fragment lengths are shown in nt. The diagram to the left explains the origin of the protected fragments; the hatched boxes indicate the E6 and E7 ORFs, the angled arrow indicates the major site of initiation in the keratin promoter and the dotted line indicates the intron within E6. (c) S1 nuclease mapping, using polyadenylated RNA from transgenic forestomach, glandular stomach, tumour and non-transgenic glandular stomach (lanes 1 to 4). The probe was end-labelled at nt 163 of the viral sequence and extended upstream into the bK6 promoter sequence. A G-ladder generated from the probe fragment is shown on the left. The positions of TATA sequences in the keratin and HPV sequences are indicated, as are the major sites of transcription initiation within the keratin promoter and the minor site of initiation detected in the tumour, at viral nt 92 (angled arrows). (d) Summary of viral mRNAs in bK6-HPV16e transgenic mice. The open boxes represent HPV-16 ORFs; thick lines with arrowheads indicate viral sequence present in mRNAs, the dotted lines indicate splicing; the coding potential of the mRNAs is indicated on the left, and the symbols to the right indicate the relative abundance of the different mRNA species, and their approximate sizes.

mRNA. An mRNA with this structure has previously been detected in CINs, a derived keratinocyte line, and invasive cervical carcinomas (Doorbar et al., 1990; Sherman et al., 1992). The absence of major bands other than those at 200 and 471 nt discussed above also argues against significant levels of transcription initiation within the E6/E7 region.

Additionally, to confirm the site of transcription initiation, we performed S1 nuclease mapping (Fig. 6c). The 5' ends of the majority of mRNAs from transgenic
forestomach, glandular stomach and tumour mapped to the expected site of transcription initiation within the bK6 sequence. Interestingly in the tumour RNA sample, approximately 20% of the RNAs appeared to initiate further downstream at viral nucleotide 92. This probably corresponds to the normal start site of HPV-16 early region transcription ("p97"; Smotkin & Wettstein, 1986). The deduced structure of the predominant mRNAs produced from the bK6-HPV16e transgene, their coding potential and relative abundance are summarized in Fig. 6(d). Note that our present results do not indicate whether any HPV mRNAs which are not spliced 226-409 have removed the 880-3358 intron; either of these has the potential to produce the intact E6 oncoprotein. The two most abundant mRNAs are spliced within the E6 ORF. It is thought that the truncated E6* proteins are never produced in significant amounts; it therefore appears most likely that the major 1.8 and 1.3 kb mRNAs produce on translation the E7 and E5 proteins, respectively.

Sporadic phenotypic effects in squamous epithelial tissues

Despite the presence in the tongue and forestomach of considerably higher levels of HPV-16 mRNAs than in the glandular stomach, the forestomachs of the transgenic mice generally appeared normal and, with one exception noted below, no malignant tumours arising in the squamous epithelium of the forestomach have been detected. Occasional small focal forestomach abnormalities have been observed in mice presenting with glandular stomach tumours, typically showing localized hyperplasia/acanthosis (Fig. 7a). No abnormalities have been observed in the tongue. Transgenic mice in line 135-10 have occasionally developed skin lesions with a grey, warty appearance, either on the upper back where they reach a diameter up to 5 to 10 mm, or on the snout or eyelids. Histologically, these show epithelial hyperplasia, with hyperkeratosis and parakeratosis (Fig. 7b). They appear to be similar to the abnormal skin which
developed more widely on the body of other recently
described transgenic mice also expressing the HPV-16
E6/E7 region in skin (Lambert et al., 1993). However to
date malignant progression of these lesions has not been
observed, and some have regressed spontaneously after
several weeks. Older transgenic mice (approx. 1 year or
more) occasionally develop nodules in the tail caused by
accumulation of cornified keratinocyte ‘pearls’ within
hair follicles (Fig. 7c); the outer root sheath of hair
follicles corresponds to a site of constitutive keratin 6
expression, and hence expected transgene expression.

The squamous epithelium lining the vagina and
exocervix of the female mouse undergoes extreme
fluctuations associated with the 4-day oestrus cycle.
Starting from a non-cornified epithelium only about
two cells deep at dioestrus, it undergoes very rapid cell
proliferation during prooestrus to give a very thick and
highly cornified epithelium at oestrus. The basal layer becomes more convo-
lated in older mice, and the overall appearance becomes
more irregular. Despite high level expression of en-
dogenous keratin 6, expression of the transgene was
considerably lower than in the squamous epithelia of the
aerodigestive tract. We have not observed any ab-
normalities clearly attributable to expression of papil-
lovirus genes, and there is no apparent predisposition
among the transgenics to develop vaginal or cervical
tumours.

To investigate whether increasing the transgene dosage
might lead to further phenotypic effects as a result of
increased expression, we produced homozygous bK6-
HPV16e transgenic mice in line 135-10. Eyelid opening
was generally delayed by several days in homozygous
transgenics relative to hemizygous or non-transgenics.
The timing of stomach tumour development appeared
similar to that in hemizygotes; however, one small,
though clearly malignant, tumour of foreshomach epi-
thelium has been seen in a mouse presenting with the
usual glandular stomach tumour (Fig. 7d). The incidence
of the abnormal ‘warty’ skin appears to be higher in the
homozygous mice.

Discussion
A high proportion of human cervical carcinomas contain
DNA sequences from a high-risk subset of HPVs, of
which HPV-16 is the most prevalent (Vousden, 1989; zur
Hausen, 1989). In most cases viral sequences are
integrated into chromosomal DNA, in a manner which
allows deregulated expression of the E6 and E7 ORFs
(Schwarz et al., 1985; Baker et al., 1987; Cullen et al.,
1991). It is now generally accepted that infection with
these viruses is an important risk factor for the
development of cervical cancer, however the relative
infrequency of oncogenic progression from the initially
benign infections and the extended timescale have
hindered detailed studies of the precise role of the virus
(Koutsky et al., 1988). As described in this paper, we
have now demonstrated using transgenic mice that the
presence of HPV-16 early region genes under the
transcriptional control of a heterologous promoter can
provide an overwhelming predisposition to develop
invasive, metastatic tumours. In this model system the
chromosomally integrated papillomavirus DNA is pres-
cent in every cell of the mouse, and replacement of the
viral URR with keratin gene regulatory sequences allows
epithelial expression while removing the potential for
down-regulation of expression proposed to act via the
URR. The status of the papillomavirus genes in the
transgensics is thus to some extent analogous to that
within human cervical cancers, and in this configuration
they caused a 100% incidence of stomach tumours at
around 200 to 400 days of age, in multiple independent
lines of transgenic mice.

The cellular morphology and staining characteristics
of the tumours suggested their classification as car-
cinoids. Carcinoid tumours arise from neuroendocrine
cells in a variety of tissues; they are relatively rare, and
in man occur more frequently in the lower gut. Those in
the stomach can be associated with pernicious anaemia
and hypergastrinaemia. In the transgenic mice however,
expression of HPV-16 mRNAs in the tumours suggests a
direct role for the viral proteins in the tumorigenic
process. Interestingly, HPV-16 and, more frequently,
HPV-18 have been detected in small-cell neuroendocrine
carcinomas of the human cervix (Stoler et al., 1991);
though aggressive, these are relatively rare cervical
cancers accounting for < 5% of the total at that site, the
majority being squamous cell carcinomas, with an
intermediate proportion of adenocarcinomas.

We do not know why the average age at tumour
presentation in male bK6-HPV16e transgenic mice is
consistently lower than in females, in each of the lines.
Possible explanations include hormonal influences on the
oncogenic pathway, increased numbers or turnover of the
precursor cells in males, or an increased rate of
mutagenesis in male mice. Further experiments will be
required to distinguish between these possibilities. It is
interesting that in humans, males have a 1-5-fold higher
incidence of gastric cancer than females, however
carcinoid tumours make up a small proportion of these;
for malignant carcinoids as a whole, an incidence 1-5-
fold higher in women than in men has been reported
(Brennan & Macdonald, 1982).

The occurrence of identical tumours in all eight
independent lines of transgenic mice provides strong
evidence that expression of HPV-16 gene products is ultimately responsible. The delayed appearance of the tumours and their focal origin suggest however that additional, stochastic events are required for the neoplasia. One such event could cause the apparent increase in HPV-16 transgene expression in the tumours, presumably thereby conferring a growth advantage. Although enterochromaffin-like cells, the presumed precursors of the carcinoid tumours, are a minor, rather inconspicuous component of the mucosa, in situ hybridization showed no evidence of a cell population expressing the transgene at elevated levels within the glandular stomach except when histologically identifiable tumour was already present, indicating that the tumour cells do indeed express more HPV-16 mRNA than their precursors. Evidence from Northern and Southern blots of samples from a number of tumours suggests that DNA rearrangements involving the transgene are rare or absent (P. F. Searle, D. P. Thomas, K. B. Faulkner & J. M. Tinsley, unpublished results), so other mechanisms must be responsible for the increased expression. It appears probable that mutations in cellular oncogenes or tumour-suppressor genes could be involved in tumour progression, and this is currently being investigated.

The tissue specificity of tumorigenesis caused by the bK6-HPV16e transgene was unexpected. It cannot be explained solely on the basis of expression levels, since the level in the squamous epithelia of the tongue and forestomach appeared as high as that found in advanced tumours by Northern blotting, and on a per cell basis as judged by in situ hybridization, the tumours did not exceed the levels seen in the squamous mucosal epithelia. One possible explanation for the relative resistance of the squamous epithelia is that expression of the transgene was at least largely restricted to suprabasal cells; these cells are in transit between the proliferative basal layer and the outer, terminally differentiated cornified layer and it may be too late in the differentiation pathway for viral gene expression to have much effect. Although in principle cooperating mutations might occur prior to activation of HPV transcription, the tissue dynamics reduce the opportunity for clonal expansion and persistence of suprabasal cells expressing the transgene. The occasional observation of forestomach epithelial hyperplasia suggests this natural barrier may occasionally be overcome, which might then afford the possibility to progress to malignancy as suggested by the single malignant squamous epithelial tumour observed. It would be of interest to determine whether expression of the HPV-16 early region genes specifically in basal keratinocytes would provide a greater predisposition to tumours of squamous epithelia.

Several other papers describing transgenic mice carrying HPV-16 genes have been published while this work was in progress. The first reported expression of the HPV-16 E6/E7 region in the submandibular gland and testis, using the mouse mammary tumour virus long terminal repeat to direct transcription; a variable incidence of tumours was reported in three lines of mice, occurring preferentially in the germ cells of the testes causing seminomas (Kondoh et al., 1991). More recently, expression of a similar E6/E7 fragment of HPV-16 was targeted to the eye lens by use of α-crystallin gene regulatory sequences (Griep et al., 1993). This resulted in disorganization and dysplasia of the eye lens epithelium, impairment of terminal differentiation and, in up to 40% of mice from the most highly expressing line, development of lens tumours. In two of these lines with ectopic epidermal expression of the E6 and E7 genes, skin tumours developed from areas of abnormal skin similar to those observed occasionally in old bK6-HPV16e transgenics (Lambert et al., 1993). Another E6/E7 construct incorporating the human β-actin promoter resulted in brain tumours expressing the transgene in a single line of mice (Arbeit et al., 1993). The several lines of transgenic mice now described expressing the HPV-16 E6/E7 region confirm the ability of E6 and E7 to predispose to oncogenesis in diverse tissues, consistent with the general importance of p53 and RB, known cellular targets of these oncoproteins. We have produced a single line of transgenic mice expressing just the E6/E7 region of HPV-16 from the bK6 promoter; these mice did not develop stomach tumours (P. F. Searle, D. P. Thomas, K. B. Faulkner & J. M. Tinsley, unpublished results). It is therefore possible that additional early region genes may contribute to tumorigenesis in our bK6-HPV16e mice. The most likely candidate is E5, which has been reported to enhance signalling pathways involving growth factor receptors (Leechanachai et al., 1992; Pim et al., 1992).

In conclusion, the transgenic mice described here provide dramatic confirmation of the ability of HPV-16 early region gene expression to cause an aggressive, metastatic cancer. The apparent neuroendocrine origin of the tumours suggests they may be most directly relevant to the relatively rare small-cell neuroendocrine carcinomas of the human cervix, nevertheless the absolute predisposition to malignancy provided by this transgene further emphasizes the significance of the virus in the more common HPV-associated cancers. These mice provide an in vivo system in which to investigate events associated with malignant progression in an HPV-16-dependent tumour. They may also be useful in the development of immunological or other therapies for HPV-associated cancers.

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