Abnormalities of epidermal differentiation associated with expression of the human papillomavirus type 1 early region in transgenic mice

J. M. Tinsley,1 C. Fisher2 and P. F. Searle1*

1Cancer Research Campaign Laboratories, Department of Cancer Studies, University of Birmingham Medical School, Birmingham B15 2TJ, U.K. and 2The Upjohn Company, Kalamazoo, Michigan 49001, U.S.A.

The promoter region of a keratin 6 (K6) gene was used to regulate expression of the early region of human papillomavirus type 1 (HPV-1e) in transgenic mice. In one line of mice the K6-HPVle transgene was transcribed in several regions of the skin, the predominant transcript being a 1.1 kb RNA including the E4 open reading frame, and E1-E4 protein was detected in the upper suprabasal layers of the skin in paws and tail. A 1.7 kb RNA corresponding to the E6/E7 transcript was also prominent in tails of homozygous transgenic animals. In young homozygous transgenic mice the epidermis of the tail showed dysplasia and hyperplasia of the suprabasal layers with both hyperkeratosis and focal parakeratosis in the stratum corneum. A similar though milder phenotype was also observed sporadically in hemizygous transgenics. Analysis of the pattern of mouse keratins present in the affected tail skin showed strong up-regulation of the endogenous keratins 6 and 16 throughout the basal and suprabasal layers, suggesting a positive feedback mechanism for the strong transgene activation. Expression of the major differentiation-specific keratins 1 and 10 was repressed. The pattern of E1-E4 expression and the perturbation of normal epithelial differentiation parallel many of the characteristics of HPV-1 warts or verrucae, suggesting that HPV transgenic mice could be useful for analysis of the interactions of HPV gene products with cellular regulatory pathways within an otherwise normal epithelium.

Introduction

The papillomaviruses are a widespread group of viruses having dsDNA genomes of around 8 kb, including six to eight ‘early’ and two ‘late’ open reading frames (ORFs) all in the same orientation. At least 60 distinct types of human papillomaviruses (HPVs) have been identified (de Villiers, 1989). They infect the various cutaneous and mucosal epithelia of the body where they cause benign tumours with a broad spectrum of morphologies from almost flat dysplasias to large, hyperplastic papillomatous lesions (Broker & Botchan, 1986; Pfister, 1984; Syrjänäen et al., 1987).

HPV-1 is associated with deep warts on the thickened palmar and plantar skin. Such warts or verrucae are typically very hyperplastic, with the proliferative basal cell layer contorted into elongated papillae, thickening of the suprabasal layers (acanthosis), marked hyperkeratosis and parakeratosis (Gross et al., 1982). HPV-1 is the most productive of the human papillomaviruses, and the virus particles form characteristic inclusion bodies in the cell nuclei in the upper layers of the wart; cytoplasmic granules containing the abundant E1-E4 protein are also present in the upper layers. The mechanism that produces the striking change in epithelial morphology is unknown, although it seems likely that expression of the viral early region genes within the lower levels of the wart is involved. Viral DNA replication is detectable only in the suprabasal layers; however it appears likely that the viral episome is present within cells of the basal layer, where early gene products might act to interfere with the normal control of cell proliferation and differentiation (Pfister, 1984; Howley et al., 1986).

Common warts on other regions of the cutaneous epithelium are generally caused by HPV-2 or -4. Neither the warts induced by these viruses nor those caused by HPV-1 have been associated with malignant progression. Other types specific for the skin, such as HPV-5 and -8, are not commonly detected in the general population but are found particularly in individuals with the rare inherited disease epidermodysplasia verruciformis; in such individuals warts associated with these viruses may progress to squamous cell carcinomas, particularly at sites exposed to the sun (Syrjänäen et al., 1987).

Other papillomaviruses are found specifically in lesions of mucosal epithelia. Much interest has focused on the frequent detection of HPV-16 or -18 DNA
sequences in higher grade cervical lesions and invasive carcinoma, whereas types 6 and 11, although frequently present in condylomata acuminata and low grade lesions of the cervix (CIN I), do not appear to be associated with malignant progression (zur Hausen & Schneider, 1987; Syrjänen et al., 1987; Vosden, 1989). Cellular transformation functions have been assigned to the viral E6 and E7 gene products (Münger et al., 1989; Halbert et al., 1991), and malignant progression of the initially benign lesions associated with types 16 and 18 appears to be associated with chromosomal integration of part of the HPV genome in a manner which preserves the function of the E6 and E7 genes but removes some of the normal transcriptional control over their expression (Schwarz et al., 1985). The transforming activity of these proteins is believed to be related to their ability to interact respectively with the cellular p53 and retinoblastoma gene proteins (Dyson et al., 1989; Werness et al., 1990; Scheffner et al., 1990). Although the mechanisms are currently unknown, both p53 and the retinoblastoma protein are believed to function in the regulation of the cell cycle. The E7 proteins of HPV-6 and -11 bind more weakly to the retinoblastoma gene protein, but the E6 proteins of these viruses could not be shown to interact with p53. Comparable studies of the E6 and E7 proteins of the cutaneous HPVs have not been reported.

A full understanding of the molecular function of papillomaviruses in both benign and malignant lesions must include the effects of their gene products within the context of the normally highly ordered pathway of epithelial cell differentiation, and for this reason several groups have produced transgenic mice carrying chromosomally integrated papillomavirus sequences. In the first such study, the entire genome of a bovine papillomavirus (BPV-1, which induces fibropapillomas in cattle) was introduced into the mouse genome (Lacey et al., 1986). Transgenic mice in this line initially appeared normal, but later developed patches of abnormal skin and protuberant tumours at sites of epithelial wounding. These appeared to involve primarily the fibroblasts of the dermis, and extrachromosomal copies of the viral DNA were found in the lesions. Characteristic karyotypic abnormalities were found to be associated with the locally invasive fibrosarcomas (Lindgren et al., 1989). In another study, the E6 and E7 genes of HPV-16 were introduced into transgenic mice under the control of the bovine keratin 6 (bK6) promoter, and inserted between the bovine keratin 6 (bK6) gene, and inserted between the EcoRV sites of pBluescript (Stratagene) and the (polylinker) sites of pUC18 (Blessing et al., 1989). The pUC-bK6 vector fragment was purified by electrophoresis after excising the CAT gene with XhoI and SmaI.

Plasmid pPXL-HPV1 (kindly provided by Dr T. Broker) contains an almost complete HPV-1 genome, except that the early promoter had been replaced with an XhoI linker at bp 75 of the viral genome; this had been cloned into a simian virus 40 late promoter construct (Chow et al., 1987). The promoterless HPV-1 early region from this XhoI site to the poly(A) site of the virus is shown in the lower part of the figure, together with spliced RNAs referred to in the text. We were interested in comparing the function of 'oncogenic' and 'non-oncogenic' HPV types 16 and 17 in epithelial tissues of transgenic mice. Initially we produced three lines of transgenic mice carrying the entire genome of HPV-1; however none of these produced detectable HPV transcripts, perhaps due to the observed methylation of the viral DNA (unpublished results). We therefore adapted our approach, and have produced transgenic mice carrying fusion constructs in which the early region transcription units of HPV-1 and -16 were placed under the transcriptional control of epidermis-specific promoters from keratin genes. In this paper we describe a line of transgenic mice in which high level expression of the HPV-1 early region in the tail is associated with epidermal hyperplasia, hyperkeratosis and parakeratosis, strong up-regulation of the hyperproliferation-associated keratins and down-regulation of keratins associated with normal epithelial differentiation. Similar changes are also characteristic of HPV-1-induced warts. Hence HPV transgenic mice may be useful for studying the disruption of the normal control of epidermal cell proliferation and differentiation induced by human papillomaviruses.

**Methods**

**Plasmid construction.** Plasmid pbK6-CAT (kindly provided by Dr J. L. Jorcano and Dr M. Blessing) contains approximately 2.2 kb of 5' sequence from the bovine keratin 6 (bK6) gene (formerly designated keratin IV), joined to the chloramphenicol acetyltransferase (CAT) gene, and inserted between the BamHI and SmaI sites of pUC18 (Blessing et al., 1989). The pUC-bK6 vector fragment was purified by electrophoresis after excising the CAT gene with XhoI and SmaI.

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Production and screening of transgenic mice. Transgenic mice were produced by microinjection of the bK6-HPV1e DNA fragment shown in Fig. 1 (isolated after SalI and SmaI digestion) into fertilized F2 eggs from CBA/C57Bl mice, essentially as described (Hogan et al., 1986). Transgenic mice were identified by dot and Southern blotting of DNA samples obtained from tail biopsy. All procedures involving animals were carried out under the authority of appropriate Home Office Project and Personal Licences.

RNA isolation and analysis. Standard procedures were followed (Maniatis et al., 1982); briefly, RNA was prepared from freshly excised tissue by homogenization in guanidine thiocyanate and ultracentrifugation through a cushion of CsCl. For Northern blotting, an estimated 15 μg RNA (unless otherwise stated) was denatured using glyoxal, electrophoresed on 1.5 % agarose gels in phosphate buffer, transferred to nitrocellulose and hybridized using probes (usually the entire HPV-1 early region), labelled using [α-32P]dCTP and a random primer labelling kit (Pharmacia).

For mapping the site of transcription initiation directed by the keratin promoter, a 297 bp probe was prepared by end-labelling at the BclI site (nucleotide 226 of the viral sequence) within the HPV-1 E6 ORF using [α-32P]ATP and T4 polynucleotide kinase, after treatment of the BclI-linearized plasmid with calf alkaline phosphatase. Following digestion with XbaI which cuts within the bK6 promoter region, the 297 bp BclI-XbaI DNA fragment spanning the junction between the bK6 promoter region and the HPV-1 early region was isolated by gel electrophoresis. The hybridization reactions containing a molar excess of the denatured radioactive probe and 30 μg of total RNA were incubated for 16 h at 53 °C in 80% formamide, 0.4 M NaCl, 40 mM-PIPES pH 6.4. After treatment with S1 nuclease (250 units, 30 min at 37 °C), the products were separated by electrophoresis on a denaturing 5 % polyacrylamide gel. A portion of the probe was treated with dimethyl sulphate followed by piperidine cleavage (Maniatis et al., 1982) to generate a G ladder.

Histology and immunofluorescence. Tissues for histology were fixed in Carnoy's fixative, embedded in paraffin wax and 8 μm sections were stained with haematoxylin and eosin.

For immunofluorescence, tissues were frozen in liquid nitrogen. Keratins were detected in 4 μm frozen sections using monospecific rabbit sera (kindly provided by Dr S. Yuspa; Roop et al., 1984); the HPV-1 E1-E4 protein was detected using a mouse monoclonal antibody (Doorbar et al., 1988), after post-fixation of the sections with 4% paraformaldehyde. Antigens were visualized using appropriate fluorescein isothiocyanate-conjugated secondary antibodies (Harlow & Lane, 1988).

Results

Production of transgenic mice and transgene expression

The DNA construct introduced into transgenic mice in this study contained 2.2 kb of upstream regulatory sequences from the bK6 gene joined to 4.5 kb of HPV-1 DNA, encompassing the entire early region transcription unit (Fig. 1). Five founder transgenic mice were obtained carrying the bK6-HPV1e transgene, and
Southern blots confirmed the presence of the intact gene in each of the mice (data not shown), at one or two copies per cell (mice 27-5 and 48-16), at approximately 10 copies (mice 48-10 and 48-11) and at 15 to 20 copies (mouse 27-7). Each of these founder animals was mated to provide transgenic progeny for further analysis.

To look for papillomavirus gene expression in the skin of transgenic mice descended from each of the founder animals, RNA was extracted from the tails and analysed by Northern blotting for the presence of HPV early region transcripts. As shown in Fig. 2(a), low levels of transcripts were detected in the sample from line 27-7; the most prominent of these was approximately 1.1 kb in length; faint bands at about 1.7 and 4 kb were just visible on the original autoradiograph. No transgene expression could be detected in any of the other lines, and we therefore concentrated on line 27-7 in our subsequent studies.

Fig. 2(b) shows a Northern blot of RNA from tissues of a 27-7 hemizygous mouse. The tail contained the highest levels of the 1.1 kb transcript, but it was also detectable in paws, ears, dorsal skin and tongue. No HPV transcripts were detected in the intestine (the high Mr smear in this and other lanes was attributed to DNA contamination), skeletal muscle or brain. The transgenic mouse used as the source of these RNA samples was normal in appearance. We have occasionally observed the development of a very flaky appearance of the tail skin of young hemizygous transgenic mice, apparently associated with epidermal hyperproliferation (see below). A similar though more pronounced phenotype was reproducibly observed in homozygous transgenic mice in this line.

A comparison of HPV early region transcripts in paws, ears, dorsal skin and tail of a young homozygous transgenic mouse from line 27-7 is shown in Fig. 2(d). The intensity of the bands suggested that the level of RNA expression was at least an order of magnitude higher than that seen in samples from hemizygotes. Highest expression was again observed in the tail sample, followed by paws, skin, then ears (taking the intensity of the β-actin signal as an internal control for RNA loading). In addition to the major 1.1 kb mRNA, the 1.7 kb RNA was clearly visible in the paw sample, and was particularly prominent in the tail. The 4 kb RNA visible in ears, paws and particularly tail is the expected length for unspliced, full-length early region transcripts. A direct comparison between the levels of HPV RNA in a hemizygous animal (of normal appearance) and a homozygous 27-7 mouse, both 14 days old, is shown in Fig. 2(e). The 1.1 kb RNA was at least 10-fold higher in the homozygous tail compared with the hemizygote, and the 1.7 kb RNA was increased by a greater factor in the homozygote, being barely detectable in the hemizygote. Such an increase in the level of transgene expression in the homozygous mice was considerably greater than expected from the twofold difference in transgene copy number. As discussed further below it appears likely that the keratin promoter of the transgene was up-regulated as a specific response to epithelial hyperproliferation.

The 1.1 kb HPV mRNA detected in the transgenic mouse (Fig. 2) corresponded in size to the spliced Ei=E4,E5a mRNA detected as a major species in HPV-1 warts as indicated in the lower part of Fig. 1 (Chow et al., 1987). This is believed to initiate at a promoter towards the end of the E7 ORF. We found that our 1.1 kb RNA can be detected with an E4 sequence probe; furthermore use of the polymerase chain reaction and primers designed to detect possible novel splices between the E6/E7 region and E4 provided evidence for use only of the known splice sites indicated in the lower part of Fig. 1 (results not shown). It therefore seemed most likely that the 1.1 kb RNA in the transgenics resulted from initiation not at the bovine keratin promoter, but at a natural viral site of mRNA initiation within the HPV E7 sequence, with appropriate splicing shortly downstream of the beginning of E1 to the E4 ORF.

The 1.7 kb RNA corresponds in size to the E6,E7,Ei=E4,E5a detected in warts (Chow et al., 1987). Although the normal viral initiation site for this mRNA is included in the construct, the viral promoter has been replaced by that of the bovine keratin gene. S1 nuclease mapping using a probe end-labelled at nucleotide 226 of the viral sequence (within the E6 ORF) produced a cluster of three strong and five faint bands 21 to 28 bp downstream of the sequence TATATAA within the keratin promoter region (Fig. 3a, lane 2; bold arrow). As indicated in Fig. 3(b), the most abundant RNA transcript therefore contained a total of 18 nucleotides corresponding to the keratin gene and linker sequences before entering the HPV-1 sequence at nucleotide 75 of the viral genome. A very faint band (indicated by the small arrow in Fig. 3a, lane 2) was also observed, corresponding to a low level of initiation at viral nucleotide 76; this is about 20 bp upstream of the probable start site within the viral DNA. The major RNA species initiating from the keratin promoter in the transgenic mice are therefore approximately 38 nucleotides longer at their 5' end relative to the corresponding viral transcripts; this extra sequence does not contain an AUG initiation codon.

**Epithelial abnormalities**

Homozygous transgenic mice in line 27-7 appeared normal at birth; however by day 7 the skin of the tail
became abnormally hard; a few days later the surface broke up into large flakes, often forming a series of rings around the tail (Fig. 4b; compare with normal in Fig. 4a). At later times the distal region of the tail generally failed to grow normally and atrophied, leaving a tail stump usually between 30 to 70% of the normal length by the time the mice were 3 weeks old. At the same time, the skin on the remaining region of the tail reverted to a normal appearance. The appearance of hemizygous transgenic animals was identical to that of control animals at this age.

Histologically the abnormalities of 27-7 homozygote tails were most conspicuous between about days 7 and 14; longitudinal sections through the tails of 7 day old mice are shown in Fig. 5. In a normal tail (Fig. 5a), the epithelium had a well ordered appearance; the replicative basal layer of the epidermis followed a regular ‘saw-tooth’ profile, which would lead later to the characteristic scale pattern of the adult mouse tail. The tail epithelium of a homozygous transgenic mouse (Fig. 5c) appeared disorganized by comparison; the basal layer followed a more highly indented, less regular course and the thickness of the suprabasal layer was increased around twofold, due to an increase in the number of cell layers and also to the fact that many of the cells retained a cuboidal shape rather than flattening out shortly after leaving the basal layer. The nucleus to cytoplasm ratio also appears higher than in the normal tail. Whereas the normal tail at this age showed a fairly uniform granular
Fig. 5. Histology of skin from tail and lower back. (a), (b) and (c) show longitudinal sections through tail skin; (d), (e) and (f) show sections through dorsal skin. All tissues were collected from 7 day old animals, and 8 μm sections were stained with haematoxylin and eosin. (a) and (d) are from normal (non-transgenic) mice; (b) and (e), hemizygous 27-7 transgenic; (c) and (f), homozygous 27-7 transgenic (see text). The opposed open and filled arrowheads indicate respectively the transition between the granular (suprabasal) layer of the epidermis and the dead stratum corneum (\(\triangleright\)), and the position of the basal membrane separating the basal layer of the epidermis (above) from the dermis (\(\blacktriangle\)). All are shown at the same magnification; the bar marker represents 100 μm.

layer about two cells thick between the living suprabasal cells and the dead stratum corneum, the granular layer in the affected transgenics in some regions could be up to four cells thick, whereas elsewhere it was not apparent. Finally, the cornified layer in the affected tails was hyperkeratotic, generally two to three times the thickness of that in normal tails and also rather more compact or dense in appearance. Regions of parakeratosis in which the terminal differentiation of the keratinocytes was abnormal leading to the persistence of densely staining cell nuclei in the outer layers were also commonly observed in the affected transgenic animals' tails (not shown).

Most hemizygous 27-7 transgenics were indistinguishable from normal animals; however, occasionally the tail skin of an entire litter of hemizygous 27-7 transgenics developed a flaky appearance at around 2 weeks of age (not shown). This lasted only a few days, and was always milder than the homozygous phenotype. This sporadic phenotype has been seen in litters from several crosses between a homozygous 27-7 transgenic mouse of either sex, mated to a normal animal; both previous and subsequent litters born to the same parents were generally normal in appearance. The appearance of the hemizygous phenotype appeared to correlate with increased levels of the HPV RNAs presumably induced by an unidentified environmental cofactor (data not shown). Fig. 5(b) shows a stained section through the tail skin of a day 7 hemizygous mouse in which hyperplasia of the suprabasal layers was apparent, although the organization is clearly less abnormal than in the homozygote (Fig. 5c). This section probably represents the development of the sporadic 'flaky' phenotype, since the tail skin of the surviving hemizygous littermates of this animal became very flaky for a few days around days 12 to 14 after birth.

In homozygous 27-7 transgenics a transient flakiness of the skin on other regions of the body, particularly
Fig. 6. Detection of E1–E4, K10 and K6 proteins on longitudinal sections of tail skin. The antigens visualized by immunofluorescence are HPV-1 E1–E4 (a, b and c; the fluorescence of the dermis in these sections is non-specific, see text); K10 (d, e and f) and K6 (g, h and i). Sections (a), (d) and (g) show normal (non-transgenic) tissue; (b), (e) and (h) are from a hemizygous 27-7 transgenic mouse, and (c), (f) and (i) are from a homozygous 27-7 transgenic mouse. All tissues are from 7 day old animals, and all panels show comparable sections at the same magnification; the bar marker represents 100 μm.

El-E4 protein expression

The El-E4 protein is a major protein in HPV-1 warts, where it may contribute up to 30% of the total protein content (Doorbar et al., 1986). As discussed above, the 1.1 kb transcript detected in the 27-7 transgenic mice appears to correspond to the viral 1.1 kb transcript which encodes the E1–E4 protein. Using a monoclonal antibody against E4 (Doorbar et al., 1988), the viral protein was detected in the upper suprabasal layers of the skin in both hemizygous and homozygous mouse tails (Fig. 6b, c), whereas no specific reactivity was seen in controls (Fig. 6a; the relatively high background fluorescence of the underlying dermal tissue was also seen when the E4 primary antibody was omitted, and is attributed to the use of anti-mouse secondary antibody). The layer of cells expressing E1–E4 is appreciably thicker in the homozygous than the hemizygous mice; assuming this to be a reflection of the distribution of the 1.1 kb mRNA, this greater thickness may contribute to the greater abundance of the 1.1 kb mRNA in homozygous mice, since the RNA was prepared from the whole tails. Weaker E4 immunofluorescence was also observed in the footpads of both hemizygous and homozygous 27-7 transgenic mice (results not shown).

Changes in expression of mouse keratins

The keratins are characteristic markers of the state of epithelial cell differentiation, and we therefore examined the 27-7 bK6-HPV1e transgenic animals' tails for
abnormalities of keratin expression using a panel of monospecific antibodies (Roop et al., 1984). Several changes in keratin expression were observed in the tails of homozygous transgenic mice as shown in Fig. 6(d) to (i); these changes were most clearly defined at around day 7 after birth.

Keratin K10 is generally associated with normal differentiation of squamous epithelium (Moll et al., 1982; Stoler et al., 1988), and as expected normal mouse tail showed strong expression of K10 throughout the suprabasal layers (Fig. 6d). The epitope appears to be less accessible in the outer cornified layers. This same pattern of expression was seen in the 27-7 hemizygote (Fig. 6e). In homozygous animals K10 was strongly down-regulated; this appeared to be somewhat asynchronous in different regions of the tail, as in some regions cells still containing K10 could be seen in the upper layers of the skin. However as shown in Fig. 6(f), by day 7 K10 was virtually undetectable throughout much of the hyperplastic epithelium of the homozygous animal. Keratin K1, which is normally coexpressed with K10, showed a similar distribution (results not shown).

Expression of keratins K6 and K16 has been linked with epithelial hyperproliferation (Weiss et al., 1984; Stoler et al., 1988), although as shown in Fig. 6(g), K6 is expressed in the outer root sheath of the hair follicles in normal epidermis and low levels of K16 were detected in the basal layer (not shown). Strikingly, both K6 (Fig. 6i) and K16 (result not shown) were strongly expressed throughout the basal and suprabasal layers of the hyperplastic tail epithelium of the 27-7 homozygote. In the hemizygote, K16 was not detectable above control levels (results not shown) and only occasional clusters of cells expressing K6 were observed in the epidermis, generally around the join between the outer root sheath of the hair follicles and the interfollicular epidermis (Fig. 6h). The hemizygous sections shown in this figure were from a litter of normal appearance, thus despite the E1–E4 expression epidermal hyperplasia was not observed. The dramatic induction of the endogenous mouse K6 expression in the homozygous 27-7 transgenics correlated with the strong increase in expression of the transgene observed in these animals from the bovine K6 promoter used in this construct.

Discussion

Young mice homozygous for the bK6-HPV1e transgene locus in line 27-7 have been shown to develop a striking transient disturbance of normal epithelial differentiation. The effect was greatest on the tail, where the epithelium became hyperproliferative in appearance, with several layers of irregular or cuboidal cells above the basal layer, an increase in the total number of cell layers, and abnormal cornification. A similar transient flaky appearance also associated with epidermal hyperplasia was observed on other regions of the skin around 7 days of age. The hyperproliferation-associated keratins K6 and K16 were shown to be highly induced in the tail, while the high Mr keratins K1 and K10 were down-regulated to virtually undetectable levels.

The effects described were observed in only one line of transgenic mice (the only line in which transcripts of the HPV transgene were detectable), and were consistently observed only in mice homozygous for the transgene. It could therefore be argued that the phenotype may be due to the mutation of a host gene affecting the control of epithelial growth and differentiation, associated with the essentially random process of transgene integration. However for several reasons discussed below, it appears considerably more likely that the phenotype is induced by HPV gene products. First, it is clear that the capacity for normal epithelial differentiation has not been irretrievably lost, because although in most cases the epithelial abnormality led to the loss of a significant length of the tail, the remainder of the tail reverted to a normal appearance, as did the skin on the rest of the mouse. Second, there was strong evidence linking expression of HPV early region transcripts with the abnormality. For example the highest level of HPV transcripts was found in the tail coincident with the greatest disturbance of epidermal differentiation, and the strong up-regulation of expression in the homozygous transgenics both correlated with the abnormality and provided a rationale for the difference in phenotype between hemizygotes and homozygotes. Furthermore, although the appearance of the hemizygotes was generally normal, we have occasionally observed a flakiness of the tail of young hemizygous animals also, which was associated with a similar epidermal hyperplasia. This is believed to be due to up-regulation of the transgene by an unidentified environmental factor, since this flakiness of hemizygous animals' tails has been seen only occasionally but tended to affect an entire hemizygous litter when it was observed. Previous and subsequent litters born to the same parents were unaffected. Although this sporadic hemizygous phenotype has not been observed to develop to the same extent as is usual in the homozygotes, it is consistent with the interpretation of the difference between hemizygotes and homozygotes being related to the level of transgene expression, rather than to insertional mutagenesis of a host gene.

The great increase in transgene expression in homozygous 27-7 transgenic mice was initially unexpected; normally (and in the absence of negative feedback mechanisms) a transgenic mouse with two copies of a transgene locus on the two homologous chromosomes
would be expected to express twice the level of transcripts as a mouse hemizygous for that transgene locus. However in this particular case we suggest that the transgene expression can induce a hyperproliferative state within the cell, and that the transgene expression is further induced as a response to this state. For reasons discussed above, we believe it was HPV-1 proteins encoded by the transgene which induced the epidermal keratinocytes of the tail to a state of hyperproliferation, as evidenced by the histological appearance and up-regulation of keratins 6 and 16. It is most likely that this state was induced by the E6 and/or E7 proteins, encoded by the 1.7 kb mRNA. The up-regulation of the mouse K6 (and K16) may have occurred either as a direct response to viral proteins, or perhaps more probably as a secondary consequence of the hyperproliferation. Because of the use of a (bovine) keratin 6 promoter to control the transgene, it is understandable that transgene expression should have been increased in parallel to the observed expression of mouse K6, completing a positive feedback loop to increase the transgene expression. The question remains why this positive feedback loop was usually only activated in homoyzogous animals. We suggest that the establishment of the hyperproliferative state requires a certain threshold level of the HPV proteins, and that hemizygous transgenics in this line generally fail to reach this threshold (but may do so if there is an initial environmental stimulus, to account for the sporadic appearance of the flaky phenotype in hemizyogotes). In homoyzogous animals on the other hand we propose that an initial level of transgene expression twice that in hemizyogotes exceeds the threshold that leads to hyperproliferation, thus activating the positive feedback loop. Analysis of the histology and keratin expression in younger mice suggests that the cells become hyperproliferative with up-regulation of K6 at around 2 to 3 days after birth (unpublished data).

It is striking that the epithelial abnormalities described are transient, with the skin reverting to a normal appearance by about 3 weeks after birth. This could indicate the existence of a negative feedback pathway which operates to limit the hyperproliferative response. Alternatively, the tails and skin of normal mice undergo rapid growth and differentiation during the period when the abnormalities were observed in the transgenic mice; thus the scale pattern is established in the tail with up-regulation of further differentiation-specific keratins (Schweizer et al., 1987), and the fur develops over the body. It could be that the HPV-1 proteins can induce the effects described only in skin undergoing these changes, or in conjunction with internal factors present for a limited time while such changes are occurring. A similar effect might underlie the specific association of HPV-1 with the thickened epithelium of the hands and feet in man. Investigation of these questions may provide interesting insight into the interactions of papillomaviruses with the cellular and tissue environment. It is also not clear at present what causes the distal region of the tail to degenerate in the homozygous transgenics; it is possible that this is a side effect not directly related to the cellular effects of the viral proteins; for example the physical restraint imposed by the hard hyperkeratinized rings around the tail at a time when it is growing, rapidly may restrict the proper development of the blood supply to the distal region.

The histological appearance of the affected mouse tails strongly suggests that the epidermal cells are hyperproliferative, and expression of keratins 6 and 16 as seen in the tails is generally associated with epidermal hyperproliferation (Weiss et al., 1984; Stoler et al., 1988). Concomitantly with the expression of keratins 6 and 16, the differentiation-specific keratins 1 and 10 were down-regulated to undetectable levels, as observed in the productively infected cells within HPV-1 warts (Breitburd et al., 1987). The E1–E4 protein was expressed in the upper layers of the transgenic skin, mimicking its distribution within warts. The ability to reproduce these characteristic disturbances in the control of epithelial cell proliferation and differentiation in HPV transgenic mice indicates that this may be a useful model system to study the mechanism of action of HPV proteins within an otherwise normal epithelium.

We thank Dr J. L. Jorcano and Dr M. Blessing for the kind gift of the plasmid pbK6-CAT, and Dr T. Broker for the plasmid pPXL-HPV-1. Monospecific anti-mouse keratin anti sera were the generous gift of Dr S. H. Yuspa, and the E4 monoclonal antibody was kindly provided by Dr J. Doorbar. We thank Professor P. H. Gallimore, Dr J. L. Jorcano, Dr J. Schweizer and Dr S. H. Yuspa for helpful discussions. This work was supported by the Cancer Research Campaign.

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