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Progressive Squamous Epithelial Neoplasia in K14-Human Papillomavirus Type 16 Transgenic Mice

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To model human papillomavirus-induced neoplastic progression, expression of the early region of human papillomavirus type 16 (HPV16) was targeted to the basal cells of the squamous epithelium in transgenic mice, using a human keratin 14 (K14) enhancer/promoter. Twenty-one transgenic founder mice were produced, and eight lines carrying either wild-type or mutant HPV16 early regions that did not express the E1 or E2 genes were established. As is characteristic of human cancers, the E6 and E7 genes remained intact in these mutants. The absence of E1 or E2 function did not influence the severity of the phenotype that eventually developed in the transgenic mice. Hyperplasia, papillomatosis, and dysplasia appeared at multiple epidermal and squamous mucosal sites, including ear and truncal skin, face, snout and eyelids, and anus. The ears were the most consistently affected site, with pathology being present in all lines with 100% penetrance. This phenotype also progressed through discernible stages. An initial mild hyperplasia was followed by hyperplasia, which further progressed to dysplasia and papillomatosis. During histopathological progression, there was an incremental increase in cellular DNA synthesis, determined by 5-bromo-2′-deoxyuridine incorporation, and a profound perturbation in keratinocyte terminal differentiation, as revealed by immunohistochemistry to K5, K14, and K10 and filaggrin. These K14-HPV16 transgenic mice present an opportunity to study the role of the HPV16 oncogenes in the neoplastic progression of squamous epithelium and provide a model with which to identify genetic and epigenetic factors necessary for carcinogenesis.

Human papillomaviruses cause proliferative lesions in the skin and squamous mucosa which are predominantly hyperplasias, papillomas, and condylomas. Squamous mucosal lesions, particularly those of the anogenital region, will occasionally progress to carcinoma in situ, and less often to invasive cancers. The extent of neoplastic progression is viral type specific; human papillomavirus types 6 and 11 (HPV6 and HPV11) cause condyloma acuminata which generally remain benign, while HPV16, -18, -33, and -35 are associated with intraepithelial lesions that often progress to high-grade lesions and occasionally to carcinomas (53). While it is evident that HPV-associated malignancy proceeds through multiple stages, the precise sequence of events and the factors determining the rate of progression to high-grade dysplasia and invasive cancer have not been identified (33, 53).

The gene products of the HPV early region have been well characterized. The principal transforming genes of the cancer-associated HPVs are E6 and E7 (37). The E6 oncoprotein targets the proteolysis of p53 through the ubiquitination pathway (40), whereas the E7 protein binds the retinoblastoma protein (17) and related proteins p107 and p130 (16), and in so doing releases E2F, a transcription factor which transactivates several proliferation-associated genes (5). The remainder of the early region encodes the E2 transactivator/repressor, the E1 protein, which binds to the origin of replication, the E4 protein, which has been shown to dissociate actin intermediate filaments, and the E5 protein, which increases the activity of both the epidermal growth factor or platelet-derived growth factor receptors (30). Progression of HPV disease is associated with changes in the state of the viral genome and in patterns of viral transcription that may contribute to the development of malignancy. In condylomas, papillomas, and mild to moderate dysplasias, the virus is episomal (10, 12), and the entire early region is expressed (42). In high-grade dysplasias and in cancers, the viral DNA is integrated into the host genome. Integration frequently occurs in the E1/E2 open reading frame (ORF), disrupting the early region downstream of the E7 coding region and potentially leading to deregulated expression of the E6 and E7 oncoproteins, as a result of the absence of E2 transcriptional regulation (4, 41, 44). These changes in viral structure and expression patterns during clinical progression suggest that the functions of the viral early region are necessary to initiate cellular proliferative and dysplastic changes, whereas the E6 and E7 oncoproteins may be sufficient to maintain high-grade dysplasia and malignancy.

There have been several reports of HPV-induced malignancy in mice transgenic with the E6 and E7 oncogenes (2, 25, 31, 34). However, most of these models have not targeted the keratinocyte for transgene expression, and the resulting neoplastic or malignant phenotypes are dissimilar to the multistep development of squamous epithelial neoplasia seen in clinical HPV disease. Since the basal keratinocyte is the target for both HPV infection and malignant transformation, targeted expression of the HPV oncoproteins to this cell type would address the role of these oncogenes in the relevant cell in a tissue, squamous epithelium, that is the site for clinical HPV disease.

The accessibility and ordered architecture of the skin are features potentially of value for the characterization of a general model of multistep, neoplastic progression in epithelia. Skin is composed of both epidermal and dermal components, separated by a basement membrane. The epidermis consists of several layers of keratinocytes, basal, spinous, granular, and cornified, each identified by its location and the type of

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keratins and envelope associated proteins it expresses (18, 34). The basal keratinocytes contain keratins 5 and 14 (K5 and K14). The basal-to-spinous layer transition is marked by the appearance of K1 and K10 and the downregulation of K5 and K14 expression. K1 and K10 persist in the granular keratinocytes, which in addition produce the envelope-associated proteins filaggrin and involucrin. Thus, the extent of differentiation of the keratinocytes in normal and abnormal skin can be assessed by the types of keratins produced in cells located in distinctive strata within the epidermis. Finally, the skin, like other epithelia, also contains proliferating cells that maintain the integrity of this tissue by counterbalancing the routine shedding of cells, as well as restitution after trauma. There are two types of proliferating cells in epithelia, stem cells and transient amplifying cells (9). The former, which divide slowly but perpetually, are located in the bulge region of the hair follicle or in the deep rete ridges (9), while the latter, which rapidly proliferate for a limited time prior to terminal differentiation, are the basal cells distributed in both the interfollicular regions and outer root sheaths (9). Transient amplifying cells are the presumed targets for neoplastic conversion in epithelia that are exposed to the environmental carcinogens, e.g., murine chemical carcinogenesis models and human colon cancers. This susceptibility may be related to their rapid cell cycles, which may not allow effective repair of DNA damage (9). Thus, targeted expression of oncogenes to the basal epidermal keratinocyte offers the opportunity to examine the effects of transforming gene products on a cell that is poised for either continued proliferation or terminal differentiation and is juxtaposed to a basement membrane through which it will invade after malignant conversion.

In this study, we used the human K14 enhancer/promoter (49) to direct the expression of the early region of HPV16 to basal keratinocytes. This promoter was chosen for several reasons. The basal cells specifically express K14, and the construct used in this study has been shown to direct high-level expression of a variety of transgenes to the basal cells of many different types of squamous epithelia in transgenic mice (6, 26, 47, 48). The basal cells are mitotically active and thus may develop further mutations in response to a proliferative stimulus, and the expression of K14 has been shown to persist in well-differentiated squamous carcinomas (46). The HPV16 early region was chosen in contrast to the E6 and E7 ORFs alone because it may coordinate the early stages of clinical HPV16 disease. Moreover, when placed under control of a heterologous promoter, it is more potent than the E6 and E7 oncogenes alone in keratinocyte immortalization assays (38). In this report, we describe eight lines of K14-HPV16 early-region mice that exhibit proliferative epidermal and squamous mucosal phenotypes at multiple sites. In addition, the epidermises of the more markedly affected transgenic lines pass through multiple stages that are distinguished by histological, proliferative, and immunohistochemical criteria. This model of multistep epithelial neoplasia will facilitate the study of both the epigenetic and the genetic factors that regulate neoplastic progression and coordinate malignant conversion.

MATERIALS AND METHODS

Creation of transgenic mice. The K14 expression cassette (a gift from E. Fuchs, University of Chicago, Chicago, Ill.) contains 2 kb of the K14 promoter/enhancer and 500 bp of 3′ flanking sequence including the K14 polyadenylation signal. This construct has been shown to appropriately target expression of transgenes to the basal cells of squamous epithelium (49). Three variations of the HPV16 early region, derived from plasmids p1203, p16Nt, and p16Pt (13), were used in this study. p1203 contained wild-type HPV16 genome, p16Nt is derived from p1203 but contains a translation termination linker (TTL) in the E1 ORF at nucleotide 1311, and p16Pt contains a TTL in the E2 ORF at nucleotide 2922. Fragments encompassing the entire HPV16 early coding region from bp 97 to 6152 were excised from these plasmids and cloned into the *Bam*HI site of the K14 expression plasmid to generate plasmids pK14-1203, pK14-16Nt, and pK14-16Pt as previously described (13). For the sake of simplicity, these plasmids will be referred to as K14-wt, K14-$E1_{ttl}$, and K14-$E2_{ttl}$, respectively. The immortalization efficiency of these constructs for human keratinocytes was K14-$E2_{ttl}$ > K14-$E1_{ttl}$ > K14-wt (38). The K14-HPV early region fragments were separated from vector sequences by electrophoresis overnight in 4% polyacrylamide gels, electroeluted, and further purified by centrifugation through continuous cesium chloride gradients at 40,000 × *g* for 48 h. Fractions of 250 μl were collected from the bottom of each centrifuge tube, and samples containing DNA were pooled and then dialyzed against injection buffer (10 mM Tris, 0.1 mM EDTA [pH 8.0]) for 48 h at 4°C. The fragment concentrations were adjusted to 2 ng/μl for microinjection. Transgenic animals were created by using standard techniques by microinjecting B6D2/F2 embryos (29). Backcrosses to create transgenic lines were predominantly into C57BL/6 and BALB/c animals, and the lineages were maintained as heterozygotes.

Histology. Tissues were immersion fixed overnight at 4°C in fresh 3.75% paraformaldehyde in calcium- and magnesium-free phosphate-buffered saline (PBS). They were processed through graded alcohols and three changes of xylene and embedded in paraffin. Sections of 5 μm were stained with hematoxylin and eosin.

BrdU incorporation. Animals were injected intraperitoneally (100 μg/g of body weight) with a solution (5 mg/ml) of 5-bromo-2′-deoxyuridine (BrdU; Sigma, St. Louis, Mo.) in a 10 mM Tris–0.9% saline–1 mM EDTA (pH 8.0) buffer. After 2 h, the animals were sacrificed, tissues were fixed, processed, embedded in paraffin, and 5-μm sections were obtained. After deparaffinization and rehydration, the slides were immersed in 2 N HCl for 1 h, extensively rinsed in tap water, and equilibrated in PBS. The sections were then treated for 60 s with 0.1% bacterial protease (Sigma type XXIV), rinsed extensively in tap water, equilibrated in PBS, and blocked in 3% normal goat serum. A 1:50 dilution of a biotinylated mouse monoclonal anti-BrdU antibody (Br-3; Cal-Tag, Burlingame, Calif.) was applied, and the sections were incubated overnight at 4°C. Antibody binding was detected by using a peroxidase-avidin-biotin complex (ABC; Vector Elite, Burlingame, Calif.), with 3,3′-diaminobenzidine (Sigma) as the chromogen.

Immunohistochemistry. Paraffin sections of 5 μm were stained with the following types and titers of specific antisera or affinity-purified antibodies: rabbit anti-human K14, 1:1,000; rabbit anti-human K5, 1:1,000 (both obtained from E. Fuchs, Chicago, Ill.); affinity-purified rabbit anti-mouse K10, 1:1,000 (obtained from S. Yuspa, Bethesda, Md.); and rabbit anti-mouse filaggrin, 1:1,000 (obtained from B. Dale, Seattle, Wash.). A 1:200 dilution of a peroxidase-conjugated goat anti-rabbit antibody was used as the secondary antibody (Vector) and specific binding was detected by using the reagents described above.

Southern blotting. Five micrograms of DNA was digested with *Eco*RV, electrophoresed through 0.6% agarose gels, and transferred to nylon (Magnagraph; MSI, Westborough, Mass.). Prehybridization and hybridization were performed according to the manufacturer's instructions. The probe was an 800-bp fragment encompassing the E6 and E7 ORFs. Plasmid recon-

structions corresponding to 1, 5, 10, and 25 copies were run concurrently to determine copy number (2).

RNA-PCR. RNA was isolated from pieces of tail clipping (100 mg), using a variation of the acid-phenol-guanidinium technique (7). One microgram of total RNA was reverse transcribed, and the cDNA was amplified by using primers that spanned the E6 ORF to encompass the E6* splice site (2). PCR products were visualized by ethidium bromide staining of 2.0% agarose gels. The identities of the reaction products with HPV16 sequences have been previously confirmed by Southern blotting (2).

HPV16 E7 protein expression. HPV16 E7 protein was detected by using a combined immunoprecipitation-Western blotting (immunoblotting) technique previously described (2). Two-day-old mice were sacrificed, and the entire full thickness skin extending from the hind limbs to fore limbs was circumferentially removed from the torso, cleaned of adipose tissue, and snap-frozen in liquid nitrogen. The samples were pulverized over solid CO_2, and the powder was rapidly homogenized in a radioimmunoprecipitation assay buffer containing protease inhibitors (2). After preclearing at 15,000 × *g*, the total protein content was determined in the supernatant (Bio-Rad, Richmond, Calif.), and a volume corresponding to 500 μg of total protein was cleared with protein A-Sepharose-rabbit immunoglobulin G and used for immunoprecipitation with a rabbit polyclonal E7 antibody (a gift from D. Galloway, University of Washington, Seattle). After electrophoresis and Western transfer to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, Mass.), a monoclonal E7 antibody (Triton, Alameda, Calif.) was used for detection, and the signal was developed by using an enhanced chemiluminescence system (Amersham, Arlington Heights, Ill.).

RESULTS

Transgenic founders and lineages. Three forms of HPV16 DNA were used in this study: wild type, and two early-region variants containing mutations, $E1_{ttl}$ and $E2_{ttl}$, that abolished the functions of the E1 and E2 genes. Data from keratinocyte transfections comparing these three constructs showed that both mutant early regions were both more efficient than the wild type in producing immortalization (38). Twenty-one founder mice were created, and eight of these founders were bred to generate lineages designated wt (wild type) 1 and 3, $E1_{ttl}$ 1, 4, and 6, and $E2_{ttl}$ 1, 5, and 9. The transgenic lines were backcrossed with both C57 and BALB/c mice. During the course of this study 35 to 60 mice in each line were evaluated.

Pathology and penetrance of phenotypes. A spectrum of pathologies arose in the K14-HPV16 transgenic mice, most of which originated in squamous epithelium (Table 1). While there were lineage-specific differences in phenotype severity, the differences were not correlated with the type of early-region DNA used in the K14-HPV transgenes. These data suggested that expression of the HPV early region behind a strong heterologous promoter was not influenced by E1 or E2 in terms of the severity or the extent of progression of the hyperplastic/dysplastic phenotype.

The epidermis of the ear was consistently affected in all eight lines, and some degree of hyperplasia arose in every mouse (Table 1). Papillomatosis of the snout and eyelids developed in six of the lines, with a 100% penetrance (Table 1). Truncal ulcers appeared in areas of preexisting focal epidermal hyperplasia on the thorax and lower neck in two lines, with a 50% penetrance. Diffuse epidermal hyperplasia occurred in three lines (Table 1) and was associated with growth retardation and inability to gain weight despite a greater than normal food intake when normalized for total body weight (data not shown). Cataracts developed in three lines, with a penetrance of approximately 30% (Table 1). These cataracts were the result of circumferential lenticular hyperplasia (data not shown).

TABLE 1. Spectrum of pathologies and their penetrance range

Type of pathology	No. of lines[a]	Penetrance range (%)[b]
Ear epidermal hyperplasia/dysplasia	8	100
Facial epidermal hyperplasia/papillomatosis	6	100
Anal papillomas	5	43–82
Truncal ulcers	2	42
Diffuse epidermal hyperplasia	3	45–71
Cataracts	2	25–30

[a] Since there was no correlation between lesional distribution or severity and type of HPV16 transgenic DNA, either wild type or with mutations abolishing the E1 or E2 functions, the results are presented for the entire population of K14-HPV16 mice.

[b] (Number of affected mice/total number of mice in each line) × 100. The phenotypes were analyzed when the mice were 6 to 8 months old, although some changes such as ear thickening and acanthosis appeared at a younger age.

Since HPVs are a frequent cause of anal and perineal lesions in humans, it was of interest that anal papillomas developed in five lines, with a penetrance ranging from 45 to 80% (Table 1). These papillomas first appeared between 2 and 3 months, and all of the affected mice evidenced these lesions by 5 months. Grossly, the anal papillomas were grape-like, 0.3- to 0.5-mm masses, circumferentially distributed in the anal squamous mucosa (Fig. 1D). Histological analysis of an anal canal from an affected transgenic animal demonstrated a typical papilloma with frond-like epidermal projections, covered by parakeratotic caps and surrounding a central vascularized stalk (Fig. 1B and C). A detailed examination of one of the papillary fronds of this lesion revealed cells resembling koilocytes, which are frequently seen in clinical HPV infections (arrowheads in Fig. 1C).

Classification of transgenic lines into phenotype severity groups. The eight transgenic lines were categorized by the severity of phenotype into groups based on the number of involved epidermal and squamous epithelial sites and on the ultimate degree of ear and facial acanthosis, hyperkeratosis, and papillomatosis (Table 2). Six transgenic lines were severely affected: $E1_{ttl}$ 4 and 6; $E2_{ttl}$ 1, 5, and 9; and wt 3. Mice in the severely affected lines developed four to five different types of lesions. All of these lines except $E2_{ttl}$ 1 displayed extensive acanthotic and hyperkeratotic ear and facial skin changes by 5 months of age. Despite extensive squamous involvement, the average life span of these mice ranged from 8 to 15 months, which is in contrast to the 2- to 6-month life span of a line of mice transgenic with a keratin-promoted c-Ha-*ras* that also displays severe epidermal involvement (3). The difference between the wt 1 and $E1_{ttl}$ 1 lines, both of which developed three types of lesions (Table 2), was that an intermediate degree of ear and facial involvement developed in the wt 1 line, while both of these phenotypes were barely detectable in the $E1_{ttl}$ 1 line.

Progressive histopathological changes leading to dysplasia and papillomatosis. The development of severe ear lesions was progressive in that affected animals had detectable hyperkeratosis by 1 month of age, which progressed to marked acanthosis, hyperkeratosis, and papillomatosis by 5 months. Detailed histological analysis was undertaken in one severely affected line, $E1_{ttl}$ 4, in order to further characterize these progressive changes. Subsequent biopsies and autopsy material

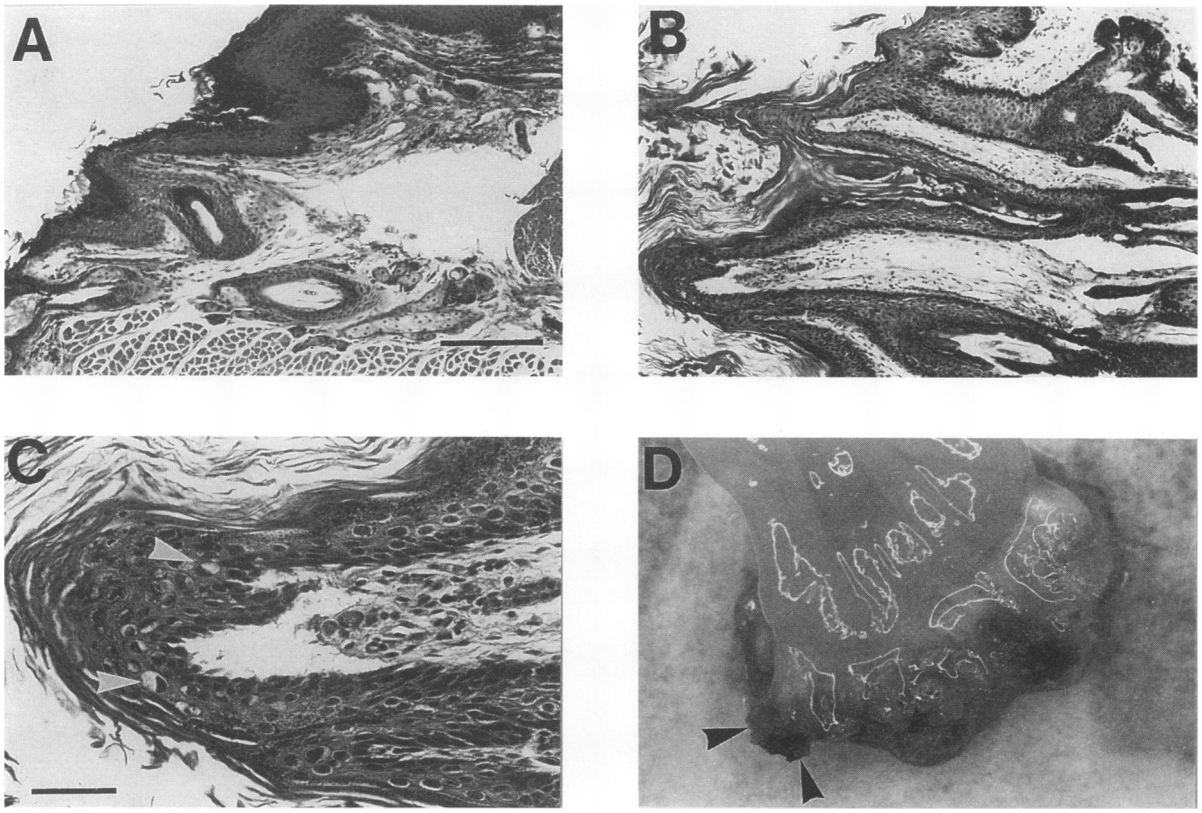

FIG. 1. (A) Longitudinal microscopic section of an anal canal from a nontransgenic mouse, showing the smooth transition from perineal skin to anal squamous mucosa. Bar = 150 μm. (B) Anal canal from a transgenic animal with visible anal papillomata (see panel D). There is a mass composed of frond-like epidermal projections topped by parakeratotic caps and containing a central vascular stalk, a classical papilloma. This mass occurred entirely within the anal canal, which was delineated by the superficial external sphincter muscle which marks the boundary between perineal skin and the mucosa of the anal canal. (C) Detailed view of one of the papillary fronds of this lesion, which demonstrates two cells which closely resemble kiolocytes (white arrowheads). Bar = 100 μm. (D) The actual bisected anus and lower rectum from the transgenic mouse, which shows a prominent papilloma and its stalk arising from the anal canal (arrowheads).

from other lines with similar marked ear involvement confirmed the histopathological analysis from this single line. The ear of a nontransgenic littermate was composed of four layers: columnar basal cells at the dermal-epidermal junction; cuboidal/transverse spinous cells; transverse granular cells; and overlying nonnucleated, eosinophilic cornified cells (Fig. 2A). The ear of a 1.5-month-old animal contained both mild and moderate epidermal hyperplasia (Fig. 2B). In areas of mild hyperplasia, the basal and granular layers were expanded to two cells in thickness, and the individual keratinocytes were slightly enlarged (Fig. 2B). While the overall maturation pattern of the epidermis was preserved, there were occasional parakeratotic cells in the stratum corneum, indicative of a focal failure of complete terminal differentiation. Adjacent microscopic fields demonstrating moderate hyperplastic changes were characterized by a further increase in the thickness of each of the epidermal cell layers and a greater degree of individual cell enlargement (data not shown). The ear epidermis from a 5.5-month-old animal contained both hyperplasia and dysplasia (Fig. 2C and D). In hyperplastic areas, there was marked increase in the size of individual cells, and each of the cell layers was increased three- to fourfold (Fig. 2C). The basal cells were spindle shaped and elongated with some nuclear irregularity, while the granular cells contained large, coarse granules. Despite these changes, the maturation pattern of the epidermis was preserved, parakeratosis was still focal, and the stratum corneum was covered with a hyperkeratotic layer several cells thick (Fig. 2C). An adjacent microscopic field from the same ear displayed atypical basaloid cells with enlarged, hyperchromatic, and irregularly bordered nuclei,

TABLE 2. Classification of transgenic lineages into severity groups based on the number of different types of involved squamous epithelia and on the severity of hyperkeratosis, acanthosis, and papillomatosis of the ears and face, graded 1 to 4[a]

Severity group	Transgenic line(s)[b]	Type of involved anatomic site(s) (grade)
Severe	$E1_{ttl}$ 4 and 6; $E2_{ttl}$ 1	Ear (3–4), face (3–4), anus, diffuse epidermal hyperplasia cataracts
	wt 3; $E2_{ttl}$ 5 and 9	Ear (3–4), face (3–4), anus, diffuse epidermal hyperplasia
Moderate	wt 1	Ear (2), face (2), ulcer
Mild	$E1_{ttl}$ 1	Ear (1), ulcer, cataracts

[a] Grade 1 changes consisted of mild thickening of the ear skin, prominence of the conjunctival-epidermal junction, and slight thickness of the snout skin. Grades 2 and 3 included intermediate degrees of ear acanthosis and hyperkeratosis, moderate thickening of the conjunctivae junction, and mild to moderate papillomatosis of the snout. Grade 4 changes included marked thickening of the ear epidermis, with prominent hyperkeratosis, erythema, and papillomatosis, whereas conjunctival and snout skin changes included erythema and extensive papillomatosis.

[b] $E1_{ttl}$ has a TTL at nucleotide 1311, and $E2_{ttl}$ contains a TTL at nucleotide 2922. $E2_{ttl}$ 1 had grade 1 ear changes but otherwise was as affected by the remaining phenotypes as $E1_{ttl}$ 4 and 6.

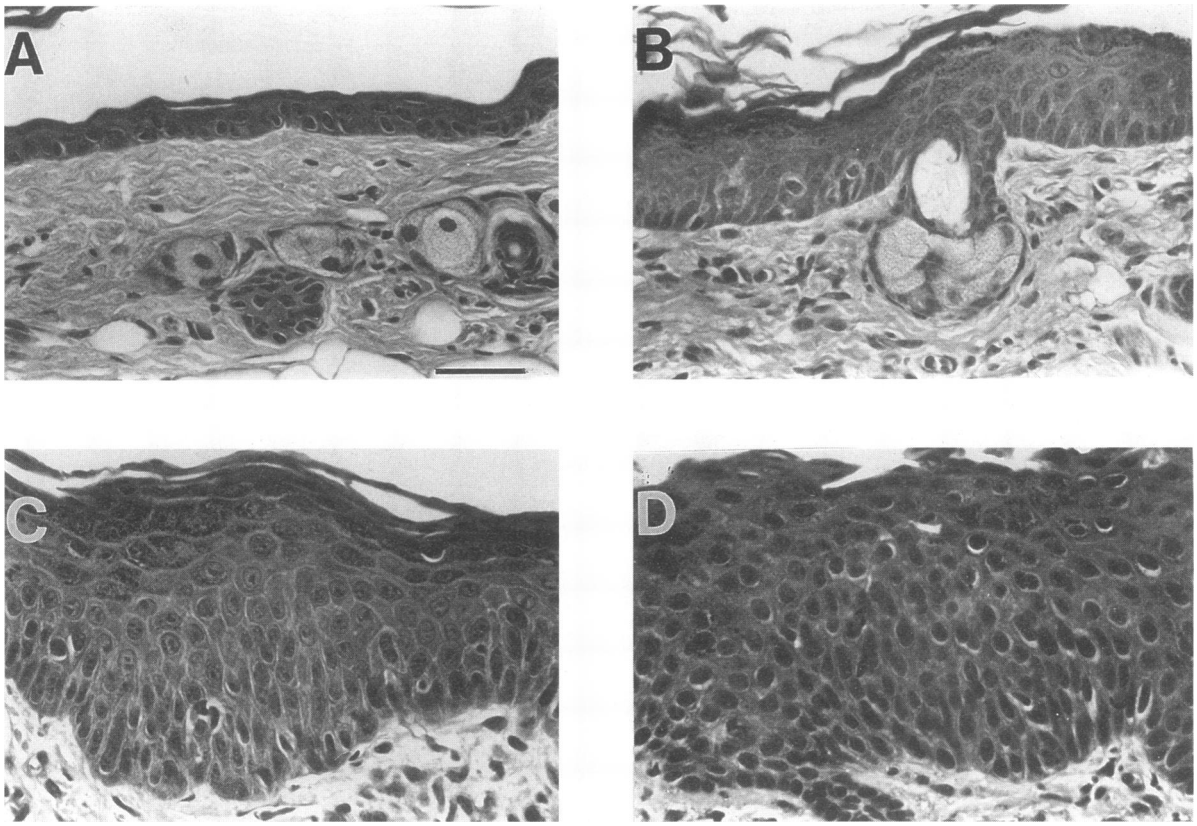

FIG. 2. The progressive histopathology of ear epidermis in K14-HPV16 $E1_{ttl}$ 4 transgenic animals biopsied at 1.5 and 5.5 months of age. (A) From a 1.5-month-old negative littermate. Note the discrete four-layer epidermis composed of basal spinous, granular, and cornified cells. Bar = 50 μm. (B) From a 1.5-month-old transgenic animal demonstrating mild epidermal hyperplasia with a twofold increase in the thickness of the basal layer and a widened hair follicle due to hyperplasia of the outer root sheath cells. (C) Microscopic field from a 5.5-month-old mouse, demonstrating epidermal hyperplasia. Each of the epidermal layers has increased three- to fourfold, with elongation of the basal cells and marked enlargement of the spinous and granular cells. The overall keratinocyte maturation is preserved in this sample. (D) Microscopic field adjacent to that in panel C, containing dysplastic changes consisting of basaloid cells with irregularly shaped and hyperchromatic nuclei extending from the dermal-epidermal junction to the stratum corneum. There is also a marked diminution in the granular cell layer, and in other fields there were marked increases in the extent of parakeratosis which overlay these areas (data not shown).

which extended into the granular layer, features consistent with a severe dysplasia or carcinoma in situ (Fig. 2D). These dysplastic areas were also characterized by a decrease in the number of granular cells and accompanied by a loss of overlying cornification (Fig. 2D). In addition, the animals developed papillomas which were similar in appearance to the anal lesions described above (data not shown). The focal nature of the histopathology, demonstrating variable degrees of hyperplasia or dysplasia in adjacent fields, was a characteristic feature of the K14-HPV16 transgenic mice and as such resembles the juxtaposition of normal cervical or anal mucosa to dysplasias observed in patients with HPV16 disease (10).

Increased and inappropriate proliferation in transgenic ear epidermis. A hallmark of neoplastic progression is inappropriate and increased frequency of proliferating cells, which can be identified by using S-phase markers or the histological identification of mitotic figures. Histological analysis of transgenic epidermis revealed an increased frequency of mitotic figures, some of which were bizarre (data not shown). These observations, combined with the progressive hyperplastic changes described above, suggested that DNA synthesis might be increased in affected transgenic epidermis. To examine DNA synthesis, in vivo pulse-labeling following a single intraperitoneal injection of BrdU was performed concomitant with histological analysis (see Materials and Methods). In normal epidermis, there were occasional basal and follicular outer root sheath cells in S phase, and the labeling index in these areas was 6% (Fig. 3A). In 1.5-month-old mildly hyperplastic ear epidermis, the BrdU incorporation was predominantly basal, with only an occasional spinous cell in S phase, and the labeling index was increased to 10% (Fig. 3B). In 5.5-month-old ear skin, there was a both an additional increase in the labeling index and a further extension of the abnormal distribution of S-phase keratinocytes. The labeling index in hyperplastic regions ranged from 20 to 25%, with an increased number of spinous cells and an occasional granular cell in S phase (Fig. 3C). An adjacent microscopic field with pronounced dysplasia demonstrated a further increase in the labeling index to 25 to 35%, with frequent S-phase cells throughout the granular layer (Fig. 3D). In normal epidermis, terminally differentiated keratinocytes are the sole occupants of the granular layer. Thus, the presence of numerous S-phase cells in the granular layer of dysplastic transgenic epidermis suggested that terminal keratinocyte differentiation was profoundly inhibited in these lesions.

Abrogation of terminal differentiation in transgenic epidermis. To further examine terminal differentiation of the keratinocytes in transgenic epidermis, we used immunohistochem-

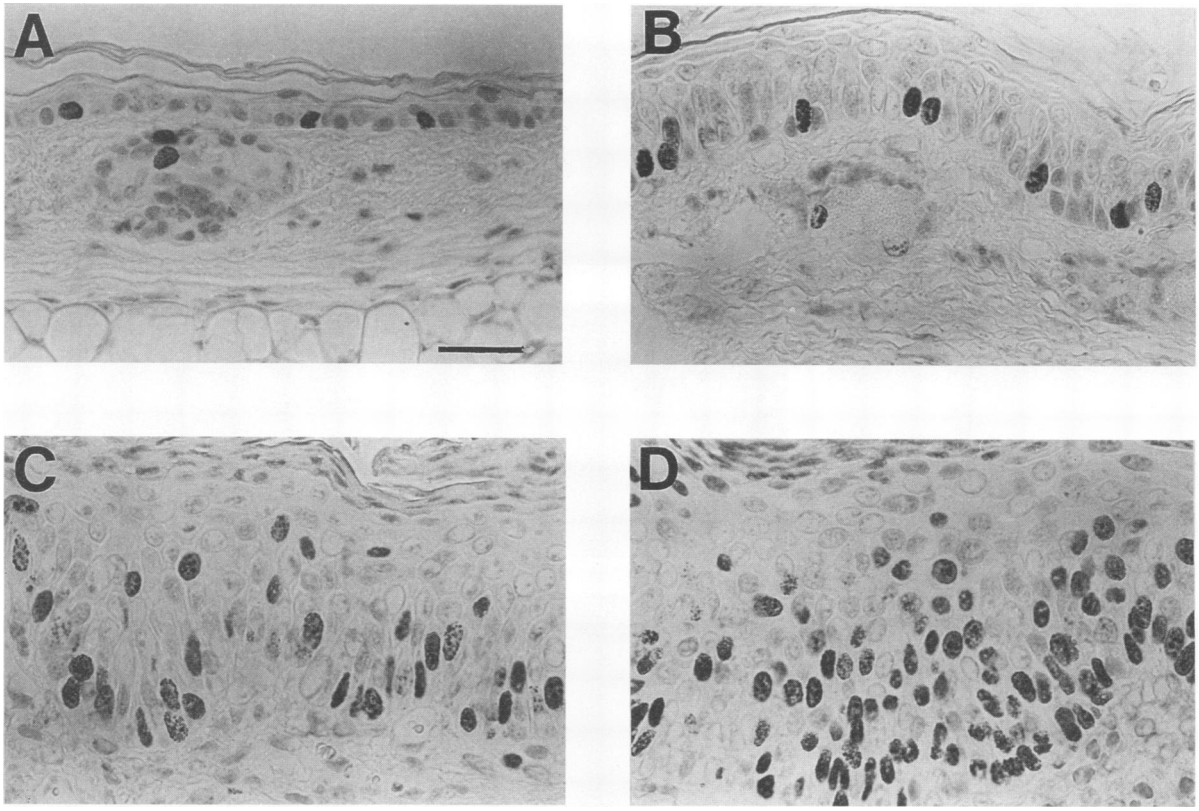

FIG. 3. BrdU labeling in sections adjacent to those of Fig. 2. (A) A section from nontransgenic ear reveals infrequent S-phase keratinocytes confined to the basal layer and the hair follicle and a labeling index of 6%. Bar = 50 μm. In panel B, from a 1.5-month-old transgenic animal, there is an increase in the labeling index to 10%, but the distribution of S-phase keratinocytes is still predominantly basal. Panel C is developed hyperplastic epidermis from a 5.5-month-old transgenic mouse. The labeling index is 20%, with an increased frequency of spinous and lower granular cells in S phase. Panel D is an adjacent field, with marked dysplasia, from the same 5.5-month-old transgenic mouse. The labeling index has increased to 35%, and there numerous spinous, upper granular, and subcorneal transition S-phase cells.

istry to analyze the expression of specific keratins or envelope-associated proteins in adjacent sections of ears. Basal keratinocytes exclusively express K14 and K5 (18, 32). Committed terminal differentiation occurs at the transition between the basal and spinous cells. In spinous keratinocytes, K1 and K10 mRNAs and proteins are expressed at high levels, whereas K5 and K14 expression is markedly downregulated (46). The expression of K1 and K10 also extends into the granular layer, and in addition, these cells express proteins associated with envelope formation such as filaggrin, which macroaggregates the K1/K10 intermediate filaments in preparation for envelope formation and thus is a marker for the complete differentiation of the keratinocyte (18). We used specific antibodies directed against several of these molecules to precisely characterize the extent and completeness of differentiation in adjacent tissue sections of progressive, $E1_{ttl}$ 4 transgenic epidermis described in the previous experiments.

Expression of basal cell-specific keratins was investigated by using an antiserum specific for either K5 or K14. The results using either reagent were identical, but since the K5 antibody had less background, the data for this reagent are presented in Fig. 4A to C. In nontransgenic epidermis, K5 expression was sharply restricted both to the basal layer and to the outer root sheath cells of the hair follicle (Fig. 4A). In mildly hyperplastic, 1.5-month-old transgenic epidermis, this basal distribution of keratin expression was maintained, but an occasional K5-staining cell was present in the spinous layer (Fig. 4B). In dysplastic, 5.5-month-old transgenic epidermis, there was an overall diminution of K5 staining in the basal layer, along with the striking presence of intensely stained K5-expressing cells in the upper granular layer (Fig. 4C). Examination of K10 immunostaining revealed minor changes in mildly hyperplastic epidermis compared with nontransgenic skin (Fig. 4D and E). In dysplastic epidermis, there was a marked diminution in K10 staining throughout the entire suprabasal layers, which was focally undetectable (Fig. 4F). The completeness of terminal differentiation was assessed by examining filaggrin expression by the granular keratinocytes. In nontransgenic skin, filaggrin immunostaining was confined to the compressed single layer of subcorneal granular cells (Fig. 4G). In hyperplastic 1.5-month-old transgenic epidermis, filaggrin expression was spread over several layers of suprabasal cells but was similar to nontransgenic epidermis in its level of expression per keratinocyte (Fig. 4H). In dysplastic 5.5-month-old transgenic skin, there was a striking failure to detect filaggrin over wide areas of the suprabasal layer, with only scattered positive cells detectable (Fig. 4I). In summary, the persistence of K5-expressing cells in the upper granular layers of dysplastic epidermis suggests that basaloid keratinocytes are inappropriately present in these layers. The diminution of K10 expression and the near absence of filaggrin indicate that terminal differentiation is abrogated, especially in dysplastic epidermis.

Expression of HPV16 E7 RNA and protein in transgenic neonatal skin. HPV transgene expression was first analyzed by

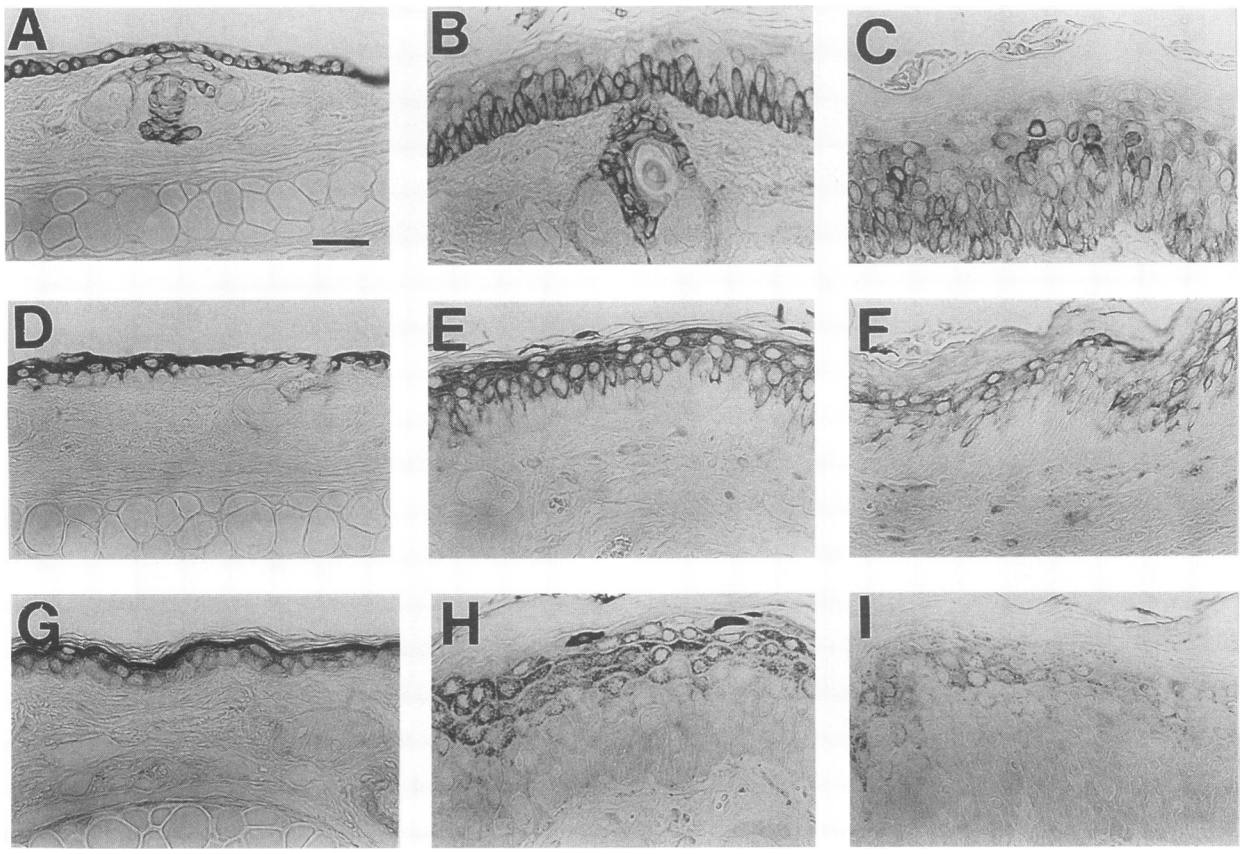

FIG. 4. Keratin and filaggrin immunohistochemistry in K14-HPV $E1_{ttl}$ 4 animals with progressive ear changes. (A to C) Sections stained with a K5 antibody. Bar = 50 μm. In nontransgenic epidermis, K5 staining is tightly restricted to the basal cells. Panel B is a section of mild epidermal hyperplasia from the 1.5-month-old transgenic mouse. There is an increase in the number of K5-staining basal cells, consistent with the twofold increase in the histological thickness of this layer, and an infrequent K5-staining spinous cell. In panel C, dysplastic epidermis from the 5.5-month-old transgenic mouse, there are K5-staining keratinocytes throughout all cell layers, including intensely positive cells in the granular layer. (D to F) Stained with a K10 antibody. In nontransgenic epidermis (D), K10 staining is evenly distributed suprabasally. (D). In mild hyperplasia (E), K10 staining is similar to that in nontransgenic skin, whereas in dysplastic epidermis, K10 staining is markedly decreased throughout the suprabasal area and focally absent (F). (G to I) Sections stained with a filaggrin antibody. In nontransgenic epidermis (G), filaggrin is evenly distributed in the thin subcorneal granular cells. In hyperplastic epidermis (H), there is a uniform expansion of granular cells expressing filaggrin. In dysplastic epidermis (I), there is nearly a complete absence of filaggrin staining, with only an occasional positive cell.

using RNA-PCR in the skin of K14-HPV16 mice with primers flanking the entire E6 ORF (see Materials and Methods). A specific band consistent with spliced HPV E6* mRNA was present in samples of skin from each of the eight transgenic lines (data not shown). This fragment is amplified from a combined E6*/E7 transcript, which is the predominant mRNA produced from the HPV16 E6/E7 ORF and from which the E7 protein is translated (44, 45).

Previously we demonstrated that the HPV16 E7 protein could be detected in tissues of transgenic mice by a combined immunoprecipitation-Western blotting procedure (2). This technique was used to determine whether HPV16 E7 protein expression was detectable in skin of the K14-HPV16 mice. Full-thickness skin pelts, encompassing all of the dorsal and ventral skin between the fore and hind limbs, were obtained from 2-day-old neonates. At this age, the thickness of the epidermis, determined by microscopy, is equivalent in all of the lines despite the severity of their adult phenotype (data not shown). E7 protein expression was analyzed from representatives of six lines which varied in adult phenotype severity from mild ($E1_{ttl}$ 1) to moderate (wt 1) to severely affected (wt 3, $E1_{ttl}$ 4, and $E1_{ttl}$ 6) (Table 2). Immunoprecipitation performed on equivalent amounts of protein extracts from these samples revealed specific 18-kDa bands, consistent with the migration of HPV16 E7 protein, present in all of the lines examined (Fig. 5). There was no correlation between the amount of detectable neonatal skin E7 protein and the eventual severity of the adult phenotype. Note the intensity of the E7 band in the mildly affected $E1_{ttl}$ 1 line compared with the markedly affected wt 3 and $E1_{ttl}$ 4 lines. A similar lack of correlation existed between the transgene copy number, determined by Southern blotting, and ultimate phenotype severity (data not shown). Preliminary in situ hybridization analysis of transgenic ear epidermis suggests that focal upregulation of K14-HPV16 expression occurs during progression and correlates with the extent of pathology (data not shown). Thus, while the distribution and ultimate severity of the adult phenotype cannot be predicted from the level of neonatal transgene expression, focal upregulation of the HPV early region in individual keratinocytes may be the key determinant of phenotype severity. The mechanisms underlying the focal transgene expression and its progressive increase will be addressed in future studies.

K14-HPV16 transgenic mice: a model of multistep epidermal neoplastic progression. The changes in histopathology,

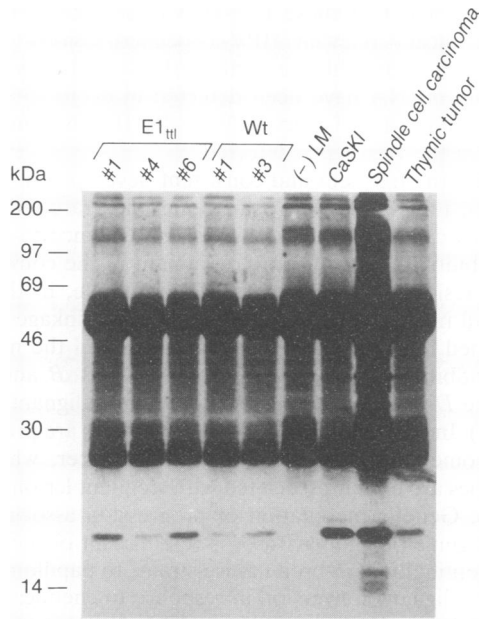

FIG. 5. Expression of HPV16 E7 oncoprotein in transgenic 2-day-old neonatal mice. An aliquot of tissue extract equivalent to 500 μg of total protein was immunoprecipitated with a polyclonal E7 antibody, electrophoresed, transferred to nylon, and detected with a monoclonal E7 antibody and the enhanced chemiluminescence system. All of the transgenic lines exhibited a specific 18-kDa band similar to that seen in CaSKI cells. The spindle cell carcinoma was a subcutaneous tumor that arose in a sterile K14 wild-type (Wt) founder. Thymic tumors develop in mice transgenic with a major histocompatibility complex class I-regulated HPV16 E6/E7 transgene (1a). The levels of E7 expression in the neonatal skin samples from these representative K14-HPV16 lines were similar to those seen in the thymuses of H2K E6/E7 animals, suggesting that the K14 enhancer/promoter used in these experiments produces levels of expression which are similar to those produced by a strong constitutive promoter in lymphatic tissue. Comparison of the level of neonatal transgene expression between the different lines demonstrated that the E7 band was more intense in the epidermis from the mildly affected $E1_{ttl}$ 1 line (lane 1) than in the moderate and markedly affected wt 1 and 3 lines (lanes 4 and 5). These data indicated the expression of E7 protein in 2-day neonatal skin was not predictive of the severity of the phenotype that eventually developed in adult mice.

BrdU incorporation, and keratin/filaggrin immunohistochemistry during ear epidermal progression in markedly affected transgenic mice are summarized in Table 3. Similar to HPV16 disease in humans, there is focal variation in these changes, with adjacent microscopic fields displaying different appearances and biochemical abnormalities. This focal variance prevents a strict correlation of one type of lesion (i.e., mild hyperplasia versus hyperplasia versus dysplasia) with increasing age. Rather, what is correlated with increasing age is the frequency and extent of a particular lesion. In 3- to 6-week-old mice, mild and established hyperplasia are present in approximately equal frequency. Mild epidermal hyperplasia has a BrdU labeling index ranging from 10 to 15% and a keratin/filaggrin immunohistochemistry profile similar to that of a nontransgenic ear, but with changes consistent with an increase of the basal cell layer to twice that of normal skin (Table 3). By 2 months of age, there is an almost uniform distribution of hyperplasia, with rare foci of dysplasia. The labeling index of hyperplasia ranges from 20 to 25%, and the keratin/filaggrin immunohistochemistry profile is similar to that of mild hyperplasia, with more pronounced changes consistent with a two- to threefold expansion of all of the epidermal cell layers (Table 3). By 3 to 5 months of age, there is a marked increase in the frequency of dysplasia. The BrdU labeling index of dysplastic lesions ranges from 25 to 35%, with numerous S-phase keratinocytes in the upper granular layer. The keratin/filaggrin immunohistochemistry profile is markedly abnormal, with K5/K14-expressing keratinocytes present in the upper granular layer and marked diminution in suprabasal K10 and filaggrin expression (Table 3). These changes in keratin immunohistochemistry are consistent with a profound abrogation of epidermal keratinocyte differentiation in dysplastic ear lesions.

DISCUSSION

This study demonstrates that expression of the early region of HPV16 can be targeted to the basal keratinocytes of transgenic mice and of stable transgenic lineages established from founder animals. K14-HPV16 transgenic mice develop various degrees of persistent epidermal and squamous mucosal hyperplasia. Benign papillomas also occur at multiple sites, including the ear, the conjunctival-epidermal junction of the eyelid, and the squamous mucosa of the anal canal. In more affected lineages, the ear epidermis regularly progresses from mild hyperplasia to extensive hyperplasia, followed by dysplasia. The multistep progression of these K14-HPV16 transgenic mice may be a unique result of expressing the HPV16 oncogenes in the basal keratinocyte.

The cloning and analysis of the regulatory regions of several human and bovine keratin genes (3, 22, 51) have enabled expression of growth factors, cytokines, and oncogenes to be targeted to specific keratinocyte subtypes in transgenic mice (6, 26, 47, 48, 49). Basal keratinocyte expression of transforming growth factor α, a growth factor known to be overexpressed in many squamous cancers (14), under control of the K14 pro-

TABLE 3. Characteristics of the stages of epidermal neoplastic progression in K14-HPV transgenic mice

Stage	Histology	Labeling index (%)[a]	Expression[b] of:		
			K5/K14	K10	Filaggrin
Normal	4-layer epidermis	6	Restricted to basal layer	Suprabasal layer	Granular layer
Mild hyperplasia	Basal layer increased 2×	10–15	Present in basal/spinous layer	Suprabasal layer	Granular layer
Hyperplasia	All layers increased in thickness, differentiation normal	20–25	Extends to lower granular layer	Decreased in suprabasal layer	Present in multiple granular layers
Dysplasia	Basaloid cells in granular layer	25–35	Detectable in upper granular layer	Further decreased in suprabasal layer	Barely detectable in granular layer

[a] Determined by the incorporation of BrdU following a 2-h pulse administered intraperitoneally.
[b] Assessed by immunohistochemistry.

moter produced diffuse neonatal epidermal hyperplasia and runting and focal hyperplasia of the scrotal epidermis in adults (49). Truncal papillomas developed in response to surgical incisions or topical application of the tumor promoter tetradecanoyl phorbol acetate (50). Suprabasal expression of v-Ha-*ras* regulated by a bovine K-VI promoter elicited hyperplasia of both the epidermis and the forestomach squamous mucosa, whereas a K1–v-*fos* transgene induced sporadic focal papillomas at 6 to 8 months of age (3, 22, 23). The combined expression of these two transgenes in K1-*ras/fos* double transgenic animals resulted in accelerated papilloma development, which persisted and developed foci of dysplasia (21), consistent with previous cell culture assays demonstrating complementary activities of *ras* and *fos* (24).

Two groups have reported squamous cancers resulting from epidermal expression of oncogenes regulated by nonkeratinocyte promoters. A single line of transgenic mice carrying a ξ-globin-regulated v-Ha-*ras* oncogene developed papillomas, of which 30% progressed to two types of malignancies, squamous cell carcinomas and dermal fibrosarcomas (35). An α-crystallin-promoted HPV16 E6/E7 transgene produced invasive squamous carcinomas that developed from antecedent hyperplastic skin lesions in 20% of the transgenic mice (34). Both phenotypes may reflect integration site biases conferred on these gene regulatory regions, which are not known to be expressed in the epidermis. In contrast, the use of the K14 regulatory region in this study resulted in the production of multiple lines of transgenic mice that evidence various degrees of epidermal pathology.

The most prominent feature of the K14-HPV16 transgenic mice was progression of the epidermal lesions through stages with distinctive characteristics and the consistent development of severe dysplastic lesions. Both this study and previous studies suggest that dysplasia may be the most direct consequence of HPV16 oncogene expression in squamous epithelia. Keratinocytes transfected with HPV16 DNA frequently formed dysplastic epithelial sheets when transplanted under nude mouse skin (52) or when placed in organotypic raft cultures (36, 39). Proliferation was increased in all cell layers of these raft cultures (36), which is similar to the extensive granular cell BrdU labeling of the dysplastic lesions of K14-HPV16 mice. Expression of involucrin and filaggrin in the transplants and raft cultures was significantly reduced at both the mRNA and protein levels. Immunohistochemical staining for these markers of terminal differentiation was restricted to mosaic patches of the upper layers of the raft cultures (36), in a pattern which resembled the low-level scattered granular expression of filaggrin in the dysplastic lesions of our K14-HPV16 animals. Similar levels and patterns of filaggrin and involucrin expression were also demonstrated in biopsies of dysplastic cervical lesions from HPV16-infected patients (8). The data from these previous studies and from the progressive lesions in the K14-HPV16 mice suggest that expression of HPV16 in the basal keratinocyte may maintain this cell in a relatively undifferentiated state, preventing the normal suprabasal downregulation of K5/K14 expression and the upregulation of K1/K10 and filaggrin expression (32). Since the primary locus and target of clinical HPV16 disease is the basal keratinocyte of cervical epithelium (10, 11), clinical dysplasia may also be produced by an enhanced expression of viral oncogenes in this cell layer in the cervical and anal mucosa.

Another interesting feature of the K14-HPV16 transgenic mice was the development of hyperplasia and papillomas in squamous mucosa. The appearance of eyelid, snout, and anal papillomas demonstrated that murine squamous mucosa was susceptible to neoplastic conversion in response to HPV16 expression and that these K14-HPV16 transgenic mice may model clinically relevant HPV-associated lesions. The anal lesions of these animals are particularly interesting, since HPV DNA and mRNA have been detected in up to 85% of anal condylomata and anal cancers (20), with HPV16 comprising up to 60% of the viral types detected.

Despite the frequent and consistent development of severe dysplasia, the K14-HPV16 transgenic mice rarely developed squamous cancers. There is increasing evidence that genetic susceptibility is a significant determinant in the conversion of HPV16 lesions to malignancies. For example, in the Shope cottontail rabbit papillomavirus model, tight linkage has been established between papilloma regression and the major histocompatibility complex class II markers *DRαB* and *DRαE*, while the *DRαG* allele is associated with malignant progression (27). In patients, specific HLA haplotypes are prevalent in those women who progress to cervical cancer, while other haplotypes are closely associated with frequent lesional regression (1). Genetic modulation of progression associated with the oncogenic papillomaviruses is reminiscent of the differential susceptibility of in-bred mouse strains to papilloma formation or malignant conversion in response to chemical carcinogens. In-bred strains such as CD-1, and its derivative SENCAR, are exquisitely sensitive to initiation and promotion, while others such as DBA and C57BL/6 are resistant (19, 43). Recently the in-bred strain FVB/n has been shown to be permissive for malignant conversion of carcinogen-induced papillomas (28), and the susceptibility of this strain may also contribute to malignant conversion in transgenic skin carcinogenesis (34, 35). Thus, the lack of malignancies in our K14-HPV16 animals may have been due to genetic background effects, since both of the in-bred strains used in this study, C57BL/6 and BALB/c, have been shown to be resistant to the development of either papillomas or cancers in response to chemical carcinogens. Therefore, we have initiated backcrosses of the K14-HPV16 transgene into two strains, SENCAR and FVB/n. Notably, cancers are developing in the early backcross K14-HPV16 × FBV/n animals, suggesting that as in other studies (28, 34, 35), this strain may be permissive for malignant progression of genetically induced epidermal lesions. It will be of interest to determine the incidence and temporal progression of these squamous cancers. If the rate is significant, further genetic analysis (15) will be used to identify loci that may contribute to malignant conversion.

In conclusion, the K14-HPV16 animals develop proliferative lesions at multiple epidermal and squamous mucosal sites, which progress from mild hyperplasia to hyperplasia and result in papillomatosis and dysplasia. Given the resemblance to patients with HPV disease, further characterization of these transgenic mice could shed light on the molecular and cellular changes necessary for the development of carcinomas under the influence of the HPV16 oncogenes.

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