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Both Conserved Region 1 (CR1) and CR2 of the Human Papillomavirus Type 16 E7 Oncogene Are Required for Induction of Epidermal Hyperplasia and Tumor Formation in Transgenic Mice

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High-risk human papillomavirus type 16 (HPV-16) and HPV-18 are associated with the majority of human cervical carcinomas, and two viral genes, HPV E6 and E7, are commonly found to be expressed in these cancers. The presence of HPV-16 E7 is sufficient to induce epidermal hyperplasia and epithelial tumors in transgenic mice. In this study, we have performed experiments in transgenic mice to determine which domains of E7 contribute to these in vivo properties. The human keratin 14 promoter was used to direct expression of mutant E7 genes to stratified squamous epithelia in mice. The E7 mutants chosen had either an in-frame deletion in the conserved region 2 (CR2) domain, which is required for binding of the retinoblastoma tumor suppressor protein (pRb) and pRb-like proteins, or an in-frame deletion in the E7 CR1 domain. The CR1 domain contributes to cellular transformation at a level other than pRb binding. Four lines of animals transgenic for an HPV-16 E7 harboring a CR1 deletion and five lines harboring a CR2 deletion were generated and were observed for overt and histological phenotypes. A detailed time course analysis was performed to monitor acute effects of wild-type versus mutant E7 on the epidermis, a site of high-level expression. In the transgenic mice with the wild-type E7 gene, age-dependent expression of HPV-16 E7 correlated with the severity of epidermal hyperplasia. Similar age-dependent patterns of expression of the mutant E7 genes failed to result in any phenotypes. In addition, the transgenic mice with a mutant E7 gene did not develop tumors. These experiments indicate that binding and inactivation of pRb and pRb-like proteins through the CR2 domain of E7 are necessary for induction of epidermal hyperplasia and carcinogenesis in mouse skin and also suggest a role for the CR1 domain in the induction of these phenotypes through as-yet-uncharacterized mechanisms.

Human papillomaviruses (HPVs) are small DNA viruses which infect epithelial cells of the epidermis as well as the upper respiratory and anogenital tracts. A subset of genital-specific papillomaviruses, including HPV type 16 (HPV-16) and HPV-18, are associated with more than 90% of cervical carcinomas and are referred to as high-risk HPVs (53). Integration of high-risk papillomavirus DNA genomes into the host chromosome has been demonstrated in a high percentage of cervical carcinomas (53) and has been correlated with an increase in the steady-state mRNA levels of two HPV oncogenes, E6 and E7 (19). The high-risk HPV E6 and E7 genes express oncoproteins which bind and functionally inactivate tumor suppressor proteins such as p53 and members of the retinoblastoma (Rb) tumor suppressor family of proteins (Rb, p107, and p130), respectively (10, 50). Formation of a complex between high-risk HPV E6 and the p53 tumor suppressor protein results in the rapid degradation of p53 through the ubiquitin-mediated proteolysis pathway (39). Binding of HPV E7 to Rb protein (pRb) family members abrogates the ability of these proteins to bind and inactivate host cellular proteins such as members of the E2F family of transcription factors (7, 33). The inactivation of cellular tumor suppressor proteins by high-risk HPV E6 and E7 is thought to contribute to the development of cervical cancer in HPV-infected women.

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HPV-16 E7 is an acidic 21-kDa nuclear phosphoprotein which has been shown in tissue culture to be necessary and sufficient to transform established rodent fibroblasts (6, 35, 45, 47, 49), to immortalize primary human keratinocytes (15), and to cooperate with an activated *ras* oncogene to transform primary rodent cells (27, 35, 44). E7 abrogates normal responses of cells to DNA damage (9, 41) and can cause genomic instability (51). E7 can induce aberrant proliferation of cells in vivo with or without concomitant cell death (16, 18, 31, 32). The capacity of E7 to transactivate various promoters has also been demonstrated (28, 35, 52), as has its ability to up-regulate c-*jun* transcriptional activation from a c-*jun*-responsive promoter (1). The amino terminus of E7 demonstrates substantial amino acid sequence similarity to two noncontiguous regions of the adenovirus (Ad) E1A protein, i.e., the conserved region 1 (CR1) and CR2 domains (35). Amino acid sequences in the CR1 domain of HPV-16 E7 are required for cellular transformation but are only marginally important for the transactivation activities of E7 (11, 34). The CR2 domain of HPV-16 E7 contains the amino acid sequences required for its association with pRb family members (5, 30). Deletion or mutation of amino acid sequences within the CR2 domain eliminates the cellular transforming and transactivating activities of E7 when analyzed independently of the entire HPV-16 genome, suggesting that binding to pRb family members is critical to both the transformation and transactivating functions of E7 (11, 34, 43, 48).

By using transgenic-mouse technology, the carcinogenic properties of high-risk HPV E6 and E7 have been demon-

strated in vivo (2–4, 12, 13, 18, 22, 25, 40), including the ability of HPV-16 E6 and E7 to function together to induce squamous cell carcinomas, the form of cancer most commonly associated with anogenital papillomavirus infection (24). Using the human keratin 14 (hK14) transcriptional promoter, we previously directed expression of HPV-16 E7 to mouse stratified squamous epithelia, the natural target tissue for papillomaviruses. HPV-16 E7 alone was sufficient to induce epidermal hyperplasia with high penetrance and to potentiate development of two types of skin tumors, squamous cell carcinomas and sebaceous gland epitheliomas, in adult animals (16). The importance of HPV-16 E7 in the development of cervical carcinomas is further supported by these studies.

To identify the domains of HPV-16 E7 required for induction of epidermal hyperplasia and carcinogenesis in vivo, we have compared the phenotypes of K14HPV16E7 transgenic mice that direct the expression of wild-type (16) or mutant (CR1 or CR2 deletions) HPV-16 E7 genes to stratified squamous epithelia. We demonstrate that in the epidermis of K14HPV16E7 transgenic mice carrying the wild-type E7 gene (henceforth referred to as K14HPV16E7^{WT}), levels of wild-type HPV-16 E7-specific mRNA expression from the K14 promoter vary with the age of the mouse and correlate with the severity of epidermal hyperplasia, as well as with altered growth properties pertaining to the proliferative and differentiation states of the epidermis. We demonstrate that deletions in the CR1 or CR2 domain abrogate the ability of HPV-16 E7 to induce epidermal hyperplasia and skin tumors. The significance of these findings is discussed in terms of the structure-function properties of HPV-16 E7 in vivo.

MATERIALS AND METHODS

Construction of transgenes and generation of transgenic-mouse lineages. The wild-type E7-expressing plasmid, pK14HPV16E7, and derived transgenic mice have been previously described (16). Briefly, the plasmid contains the hK14 promoter linked to HPV-16 sequences from nucleotide (nt) 79 to nt 883, encompassing the tandemly positioned E6 and E7 genes, and carries a translational termination linker (TTL) early in the E6 open reading frame (ORF). Two additional plasmids were similarly constructed but harbored in-frame deletional mutations within the E7 ORF. The plasmid pK14HPV16E7$^{\Delta DLYC}$, containing a 4-amino-acid deletion in the CR2 domain of E7, was generated by inserting the 802-bp *Bam*HI fragment from plasmid pαAcryHPV16E6TTL/E7$^{\Delta DLYC}$ (31) into the *Bam*HI site in plasmid pG3Z-K14 (46). The plasmid pK14HPV16E6TTL/E7$^{\Delta PTLHE}$, containing a 5-amino-acid deletion in the CR1 domain of E7, was generated by ligation of a 404-bp *Bsf*RI-to-*Ban*II fragment from pmh1427 (29), containing a TTL in the E6 ORF, to a 386-bp *Bsr*FI-to-*Nde*I fragment from p1469 (30), containing a deletion of the residues encoding the amino acids PTLHE in the E7 ORF. The ligation product was amplified by PCR with oligonucleotides 1 (5′-GGCGGATCCTTTTATGCACCAAAAGAGAACTG-3′) and 4 (5′-CCCGGATCCTACCTGCAGGATCAGCCATG-3′), which add recognition sequences for the *Bam*HI restriction endonuclease to the 5′ and 3′ ends of the PCR product, respectively. Following *Bam*HI digestion, the 790-bp PCR product was cloned into the unique *Bam*HI site of pG3Z-K14 (46). pK14HPV16E6TTL/E7$^{\Delta DLYC}$ and pK14HPV16E6TTL/E7$^{\Delta PTLHE}$ were sequenced to verify integrity of the mutant E7 ORFs, the presence of the TTL in the E6 ORF, and the junction between the K14 and HPV sequences. For microinjection of the transgenes, the two plasmids were digested with the restriction enzymes *Hin*dIII and *Eco*RI to release 3.2-kb fragments containing the K14 promoter-enhancer region, the K14 3′ untranslated region (UTR) and polyadenylation signals, and the HPV sequences. The fragments were purified by gel electrophoresis as described by Griep et al. (14) and microinjected into the pronuclei of FVB/FVB one-cell embryos, as described previously (14, 17), at the University of Wisconsin Biotechnology Center's Transgenic Mouse Facility. Genomic DNA was prepared from tail biopsy specimens from 18-day-old founder mice, and the transgenes were detected by Southern analysis of the tail DNA after restriction with *Nsi*I, which cuts once within the injected transgene DNA fragment. The hybridization probe, an approximately 800-bp E6/E7 fragment generated by PCR amplification of a full-length HPV-16 clone with oligonucleotides 1 and 4, was ^{32}P radiolabeled by random primer labeling. To assess the copy number of the transgenes in each transgenic lineage, a series of copy number reconstruction standards containing 1, 5, 10, or 25 pg of digested pK14HPV16E6/E7 (25), equivalent to 1, 5, 10, and 25 copies of the transgene per diploid cell, respectively, was included in the Southern analysis. Southern blots were quantified with a Molecular Dynamics PhosphorImager. Lineages of transgenic mice were maintained on the inbred FVB genetic background in the American Association for the Accreditation of Laboratory Animal Care-approved McArdle Laboratory Cancer Center Animal Care Facility, and all offspring were screened for transgene status by Southern analysis.

Analysis of E7 expression by in situ hybridization. Skin samples were collected and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, transferred to PBS, embedded in paraffin, and cut into 5-μm-thick sections. Neighboring sections were examined by in situ hybridization, as described previously (26), to identify the pattern of E7 and K14 mRNA expression. Sense and antisense E7 cRNA probes were transcribed in vitro from plasmid pAB-E7, a pGem3Z vector containing the HPV-16 E7 ORF, and both [α-^{35}S]UTP and [α-^{35}S]CTP were incorporated. Sense and antisense K14 cRNA probes were transcribed from plasmid MK14-3′NC (23, 42), and [α-^{35}S]UTP was incorporated. Photographic-emulsion-coated slides were incubated for 4 weeks prior to development.

Immunohistological analysis of skin. To assess the proliferative indices of tissues, a solution consisting of 100 μg of 5-bromo-2′-deoxyuridine (BrdU; Sigma; catalog no. B-5002) and 6.7 μg of 5-fluoro-2′-deoxyuridine (Sigma; catalog no. F-0503) per g of body weight was administered by intraperitoneal (i.p.) injection in a volume of PBS equivalent to 10 μl/g of body weight. Animals were sacrificed 1 h postinjection. Skin samples were then collected and fixed as described above. Paraffin-embedded sections were analyzed for proliferative status by immunostaining with a mouse monoclonal antibody directed against BrdU. The slides were deparaffinized in xylenes and rehydrated by brief submersions in a series of aqueous alcohol solutions of decreasing alcohol content. A BrdU staining kit (BrdU Immunohistochemistry Kit; Oncogene Research Products; catalog no. HCS24) was used according to the instructions of the manufacturer except that the time of incubation with the biotinylated anti-mouse BrdU reagent was increased to 3 h. The slides were examined by light microscopy and photographed with Kodak Ektachrome T160 film. To determine the level of proliferation in the epidermis, the numbers of BrdU-positive cells in the stratum basale in 10 randomly selected microscopic fields (magnification of ×20) were counted. The percentage of cells in the stratum basale that were positive for BrdU was calculated for multiple independent skin samples for each line of mouse and is referred to as the proliferative index.

Paraffin sections were also subjected to K14-specific immunohistochemical staining. Sections were deparaffinized and rehydrated as stated above. Endogenous peroxidase activity was destroyed by treatment with 3% H_2O_2 in methanol for 30 min, and the sections were then washed in PBS. The samples were blocked for 30 min at ambient temperature with 5% normal goat serum–1% bovine serum albumin–PBS. The blocking agent was blotted off, and the samples were incubated 3 h at ambient temperature with K14 rabbit polyclonal antibody (36, 37) diluted 1:200 in 5% normal goat serum–1% bovine serum albumin–PBS. Following further washes in PBS, a rabbit immunoglobulin G Vectastain ABC system (Vector Laboratories, Inc.; catalog no. PK-4001) was employed in accordance with the manufacturer's directions. After being washed thoroughly in PBS, the specimens were subjected to immunohistochemical staining by incubating them in 3,3′-diaminobenzidine tetrahydrochloride for 5 min. The chromagen was quenched by submersion of the samples in H_2O, and then the sections were counterstained with hematoxylin, dehydrated through submersion in a series of aqueous alcohol solutions of increasing alcohol content and xylene, and coverslipped. The slides were examined by light microscopy and photographed with Kodak Ektachrome T160 film.

RESULTS

HPV-16 E7 structure-function. The HPV-16 E7 gene encodes an acidic nuclear phosphoprotein of approximately 100 amino acids that contains two domains designated CR1 and CR2 due to their amino acid sequence similarity to the CR1 and CR2 domains of the Ad5 E1A protein (35). In tissue culture, deletion of the amino acid sequence PTLHE (positions 6 to 10) (Fig. 1A) from the CR1 domain of HPV-16 E7 inactivates the transforming functions of E7 but does not affect the transactivating capacity of E7 (34). Deletion of the amino acid sequence DLYC (positions 21 to 24) (Fig. 1A) from the CR2 domain of HPV-16 E7 eliminates the capacity of E7 to bind pRb family members and abrogates biological functions of E7 involving transcriptional transactivation and transformation (34). To investigate the biological properties of the CR1 and CR2 domains of HPV-16 E7 in vivo, DNA fragments containing the HPV-16 E6 and E7 genes, from nt 79 to nt 883, with deletions in either the CR1 (ΔPTLHE) or CR2 (ΔDLYC) region of E7 were cloned into pG3Z-K14 to generate pK14 HPV16E6TTL/E7$^{\Delta PTLHE}$ and pK14HPV16E6TTL/E7$^{\Delta DLYC}$, respectively (Fig. 1B). We chose these mutant E7 genes because

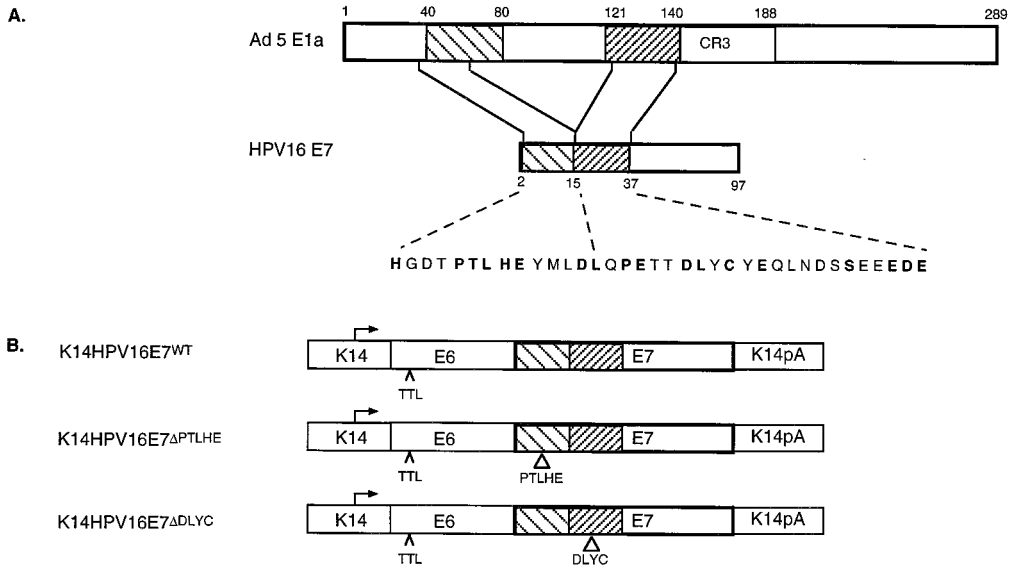

FIG. 1. Structure of transgenes. (A) Shown is the amino acid sequence similarity in the CR1 and CR2 domains of the Ad5 E1a and HPV-16 E7 oncoproteins (35). ▨, CR1 domain in E7; ▨, CR2 domain. The amino acid sequence from position 2 to 37 of HPV16E7 is shown, with boldfaced letters indicating amino acids that are identical for Ad5 E1a and E7. (B) Shown are schematic representations of each transgene. K14, hK14 promoter/enhancer; E6 and E7, HPV-16 E6 and E7 ORFs, respectively; K14pA, hK14 polyadenylation sequences. The relative positions of the TTL and of the PTLHE and DLYC deletions are indicated.

they are disrupted significantly within CR1 or CR2 yet give rise to stably expressed E7 protein species (34). While this region of HPV-16 contains both the E6 and E7 ORFs, a TTL inserted in the E6 ORF causes premature termination of translation early within the E6 ORF. The E6 ORF was included in the transgene construction because efficient expression of E7 may rely on the use of splice sites within this ORF. Fragments of 3.2 kb, consisting of HPV16E6TTL/E7$^{\Delta PTLHE}$ or HPV16E6TTL/E7$^{\Delta DLYC}$ positioned between the hK14 promoter and polyadenylation sequences, were subsequently microinjected into embryos to produce transgenic mice, hereafter referred to as K14HPV16E7$^{\Delta PTLHE}$ and K14HPV16E7$^{\Delta DLYC}$ mice, respectively. Because the HPV-16 E7 genes were under the control of the K14 promoter, their expression was directed to stratified squamous epithelia, which represent the cell type thought to be the precursor for HPV-associated tumor development. By Southern analysis of total genomic DNA extracted from tail biopsy specimens, founder candidates were screened for the presence of K14HPV16E7$^{\Delta PTLHE}$ or K14HPV16E7$^{\Delta DLYC}$ transgenes (data not shown). Copy number analyses indicated the presence of between 1 and 10 copies of the transgene in each of the transgenic lines (Table 1). Southern analysis also demonstrated that in each germ line all copies of the transgene were integrated at a single locus, as judged from the conservation of the Southern hybridization patterns among all offspring. Four K14HPV16E7$^{\Delta PTLHE}$ lines and five K14HPV16E7$^{\Delta DLYC}$ lines carrying germ line integrations were mated to produce offspring for experimental analysis.

TABLE 1. Summary of phenotypes of transgenic mice

Transgene	F_0 lineage[a]	Copy no.	Phenotype present							
			Overt[b]					Histological		
			Thickened ears	Wrinkled skin	Cataracts	Paucity of fur	Tumors[c]	Epidermal thickening	K14 staining	Proliferative index
K14HPV16E7^{WT}	2304	2	Yes	Yes	Yes	Yes	Yes (7/77)	Yes	Abnormal	High
K14HPV16E7$^{\Delta PTHLE}$	5205	10	No	No	No	No	No (0/36)	No	Normal	Normal
	5218	8–10	No	No	No	No	No (0/23)	No	Normal	Normal
	5220	10	No	No	No	No	No (0/65)	No	Normal	Normal
	5228	ND[d]	No	No	No	No	No (0/6)	ND	ND	ND
K14HPV16E7$^{\Delta DLYC}$	2901	1–2	No	No	No	No	Yes (1/43)	No	Normal	Normal
	2907	ND	No	No	No	No	No (0/42)	ND	ND	ND
	2913	ND	No	No	No	No	No (0/24)	ND	ND	ND
	2941	10	No	No	No	No	No (0/12)	No	ND	ND
	2948	8–10	No	No	No	No	No (0/48)	No	Normal	Normal

[a] See also reference 16 for a description of line 2304.
[b] The numbers of mice scored for overt phenotypes in each lineage were 77, line 2304; 36, line 5205; 23, line 5218; 65, line 5220; 43, line 2901; 42, line 2907; 24, line 2913; 12, line 2941; and 48, line 2948.
[c] The numbers in parentheses are ratios of the number of mice with tumors to the total number of mice scored.
[d] ND, not done.

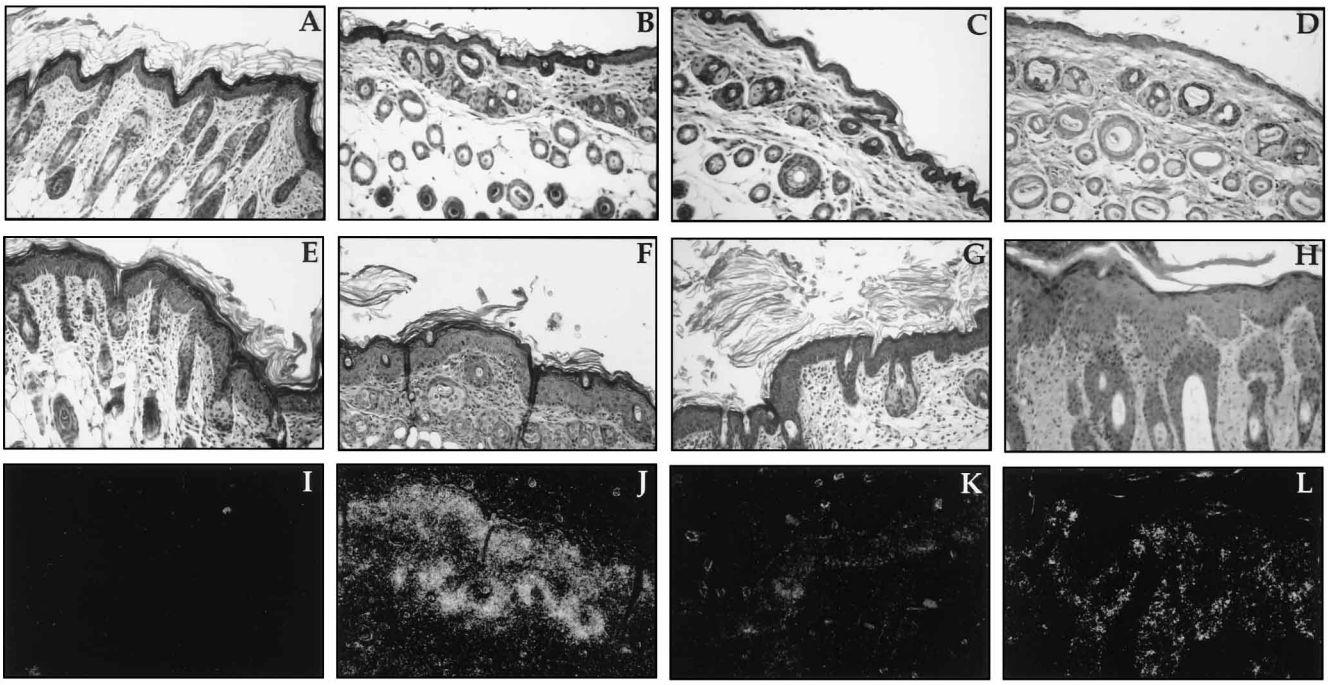

FIG. 2. In situ hybridization analysis of the skin of 6-, 12-, and 15-day-old K14HPV16E7^{WT} transgenic mice. All tissues were paraffin embedded and serially sectioned. Nontransgenic-mouse skin from postnatal days 6 (A), 12 (B), and 15 (C) and from adults (D) was stained with hematoxylin and eosin (H&E). Transgenic-mouse skin from postnatal days 6 (E and I), 12 (F and J), and 15 (G and K) was stained with H&E (E to H) or hybridized to E7 mRNA-specific probes (I to L), and dysplastic skin from an adult K14HPV16E7^{WT} mouse was treated similarly (H and L). Magnification, ×20.

Correlation of epidermal hyperplasia and E7-specific mRNA expression in the skin of K14HPV16E7^{WT} mice. K14HPV16E7^{WT} mice exhibited overt phenotypes, including wrinkled skin and thickened ears, which were apparent soon after birth (16). The skin and ears of all K14HPV16E7$^{\Delta PTLHE}$ and K14HPV16E7$^{\Delta DLYC}$ mice analyzed, however, were indistinguishable from age-matched nontransgenic control animals regardless of age (Table 1). To analyze further the consequence of HPV-16 E7 expression in the epidermis, skin samples collected from the torsos of K14HPV16E7^{WT} mice from a representative line, 2304, and of nontransgenic mice at postnatal days 6, 12, 15, and 18 were histologically examined. At postnatal day 6, the thickness of the epidermis of K14HPV16E7^{WT} mice (Fig. 2E) was slightly increased compared to that of nontransgenic age-matched controls (Fig. 2A). At postnatal day 12, the K14HPV16E7^{WT} mice exhibited a more pronounced epidermal thickening, characterized by an increased thickness of the nucleated-cell layer, and highly invaginated squamous epithelia, suggestive of a disproportionate lateral expansion of the epidermis relative to the underlying connective tissue (Fig. 2F). While the postnatal day 15 K14HPV16E7^{WT} mice also exhibited a thickened epidermis, the degree of thickening was decreased compared to that seen in postnatal day 12 K14HPV16E7^{WT} mice (Fig. 2F and G). In addition, while diminished, epidermal thickening persisted in the skin of the ears and torsos of these mice into adult life (Fig. 3).

To assess whether the temporal nature of the thickened epidermis correlated with levels of HPV-16 E7-specific mRNA expression in the epidermis, E7-specific in situ hybridization analyses were performed on skin sections of mice of different ages. Since HPV-16 E7-specific mRNA expression was placed under the control of the K14 promoter, E7 expression was predicted to be localized in the stratum basale, a single layer of undifferentiated epithelial cells that make direct contact with the basement membrane separating the dermis from the epidermis. The K14 promoter is also active in some suprabasal layers of the epidermis, its activity diminishing as the cells terminally differentiate, and in the epithelial structures of the dermis, including the hair follicles and sebaceous glands. Consequently, E7 expression was predicted to be detectable in these anatomical locations. HPV-16 E7-specific mRNA expression was not detectable in the epidermis of postnatal day 6 K14HPV16E7^{WT} mice, peaked at postnatal day 12, was noticeably diminished by postnatal day 15 (Fig. 2I to K), and was again not detectable at postnatal day 18 (data not shown). As predicted, expression of the transgene was noted in the hair follicles and sebaceous glands of the dermis and the inner suprabasal layers of the epidermis. These temporal and spatial patterns of expression were reproducibly seen in multiple in situ hybridization experiments. Hence, the kinetics of the severity of epidermal hyperplasia in the epidermis of K14HPV16E7^{WT} mice correlated with the age-dependent levels of HPV-16 E7-specific mRNA expression in the epidermis. These results indicate that at levels less than the lower limit of detection for in situ hybridization, E7 retains the capacity to induce aberrant epithelial cell growth, as demonstrated by analysis of skin from postnatal day 6 K14HPV16E7^{WT} mice (as well as postnatal day 18 mice [data not shown]), which exhibits a slightly thickened epidermis yet undetectable levels of HPV-16 E7 mRNA (Fig. 2E and I). In prior studies (16), we did observe expression of the E7 transgene in neonates by using the more sensitive reverse transcription-PCR technique. Thus, while expression is below the limit of detection by in situ hybridization at some ages, there is expression of the E7 transgene. Expression of HPV-16 E7-specific mRNA was also noted in the dysplastic skin of adult K14HPV16E7^{WT} mice (Fig. 2H and L), as well as in the nondysplastic skin of these mice, albeit

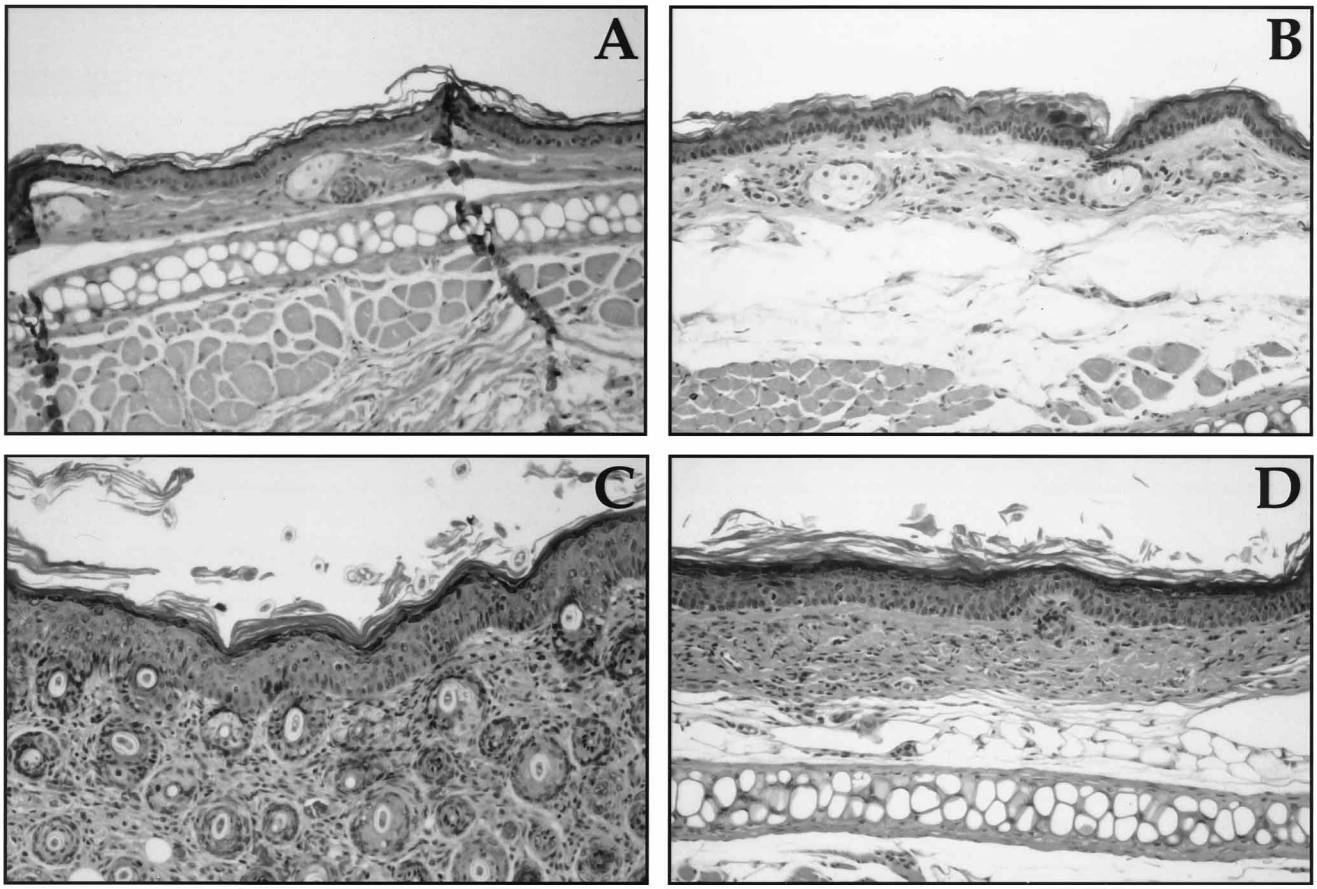

FIG. 3. Histological analysis of skin from the ears of K14HPV16E7^{WT} mice. All tissues were fixed in paraformaldehyde, embedded in paraffin, cut into sections, and stained with hematoxylin and eosin. Shown are neonatal ear skin from a nontransgenic mouse (A), adult ear skin from a nontransgenic mouse (B), neonatal ear skin from a K14HPV16E7^{WT} mouse (C), and adult ear skin from a K14HPV16E7^{WT} mouse (D). Magnification, ×20.

at lower levels (data not shown). Further evidence for persistent expression of the E7 transgene in adult skin was provided in our prior studies in which E7-specific mRNAs were detected in adult skin by reverse transcription-PCR (16). Therefore, E7 expression occurs throughout the life span of the mouse but is highest in postnatal day 12 K14HPV16E7^{WT} mice.

Proliferative index of the epidermis of K14HPV16E7^{WT} mice. In our prior study we had demonstrated that the epidermis of K14HPV16E7^{WT} mice displays an increased number of proliferating cell nuclear antigen (PCNA)-positive cells (16). To assess the proliferative state more directly and to determine whether there is an age-dependent variation in the proliferative index that correlates with the observed epidermal thickening in the K14HPV16E7^{WT} mice (Fig. 2), BrdU incorporation studies were carried out. This thymidine analog, which was administered to mice 1 h before sacrifice, is incorporated into newly synthesized DNA in cells in S phase. Sections of skin were subsequently subjected to BrdU-specific immunohistochemical staining. A proliferative index for the epidermis was determined by calculating the percentage of epithelial cells in the stratum basale that stained positive for BrdU. In nontransgenic mice, BrdU-positive cells were restricted to the stratum basale, the stratum in which cell proliferation normally occurs within the epidermis (Fig. 4A). The proliferative index in the nontransgenic-mouse skin was on average 15% at day 6 and steadily dropped to (on average) 10% by day 15 (Fig. 5). This drop corresponded to the reduced thickness of the epidermis at the later time points (from two to three nucleated cells thick at day 6 to one to two nucleated cells thick at day 15 [Fig. 2]). Conversely, while the proliferative index of the epidermis of postnatal day 6 K14HPV16E7^{WT} mice (Fig. 4G) was similar to that of nontransgenic age-matched controls (Fig. 4A), the spatial arrangement of nuclei staining positive for BrdU was slightly expanded, analogous to the observed expansion of PCNA-positive cells in the epidermis of postnatal day 6 K14HPV16E7^{WT} mice in earlier studies (16). Moreover, whereas there was a slight drop in the proliferative index at day 12 in nontransgenic mice, there was a 2.7-fold increase in this index in the epidermis of postnatal day 12 K14HPV16E7^{WT} mice (Fig. 5). In addition, a notable expansion of BrdU-positive cells into the suprabasal regions of the epidermis of these transgenic mice was observed (Fig. 4H). Between postnatal days 12 and 15 (Fig. 4H and I, respectively), the proliferative index of the epidermis of K14HPV16E7^{WT} mice decreased slightly but was still 2.5-fold higher than that of the age-matched nontransgenic mice (Fig. 5). The proliferative state of epidermis from adult nontransgenic and K14HPV16E7^{WT} mice (>6 months of age) was assessed qualitatively by immunohistochemical staining specific for PCNA (data not shown), a protein component of the cellular replicative apparatus that is detected in the nucleus during the late G_1 and S phases of the cell cycle (28a). In the epidermis of adult nontransgenic mice, PCNA-positive cells were restricted to the stratum basale, whereas an expansion of PCNA-positive cells into the

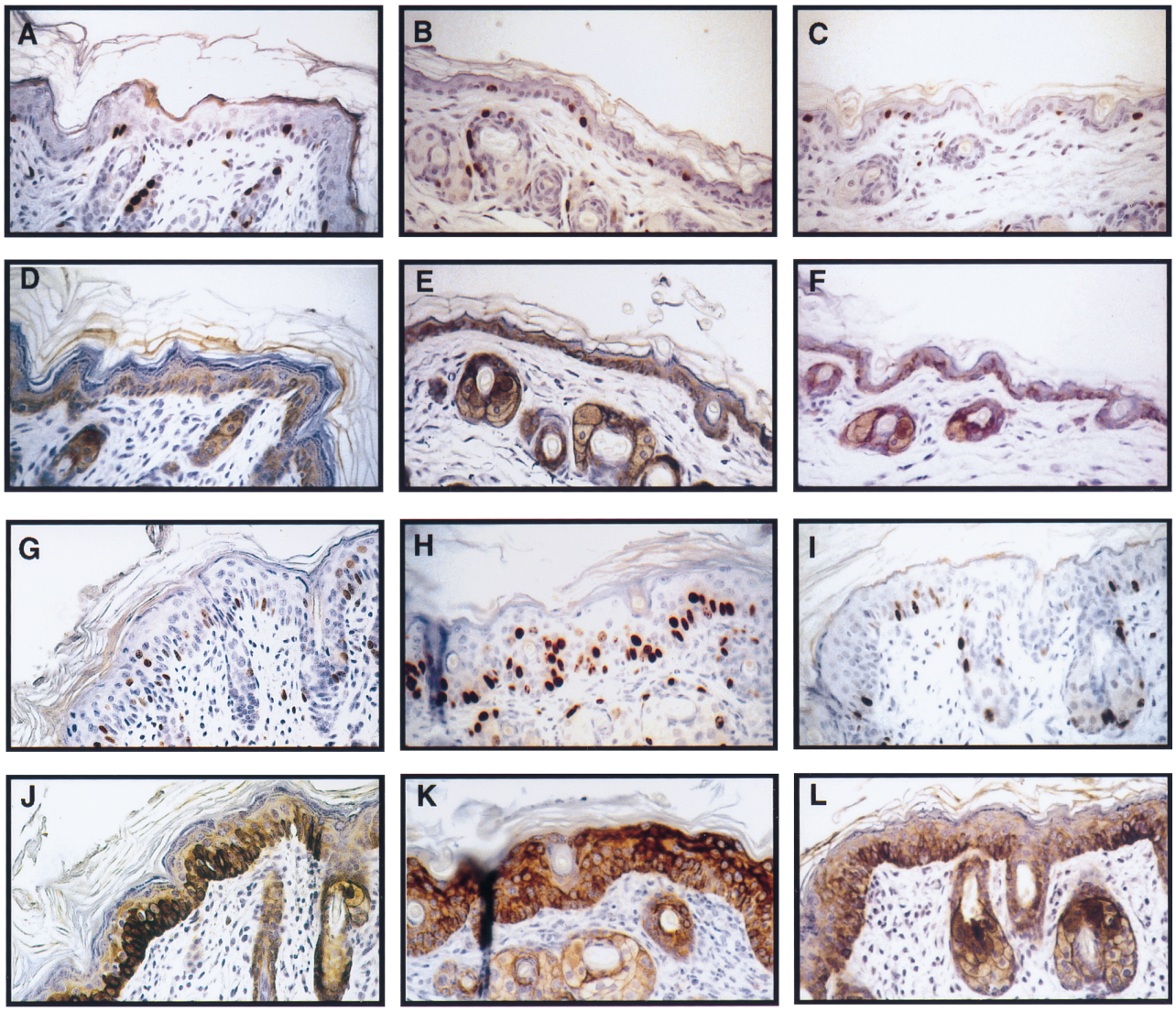

FIG. 4. BrdU and K14 analyses of skin from nontransgenic and K14HPV16E7^{WT} transgenic mice. All tissues were embedded in paraffin and sectioned. Nontransgenic-mouse skin was stained with BrdU or K14 at day 6 (A and D), day 12, (B and E) and day 15 (C and F). K14HPV16E7^{WT} transgenic-mouse skin was stained with BrdU or K14 at day 6 (G and J), day 12 (H and K), and day 15 (I and L). Magnification, ×40.

suprabasal layers of the epidermis was observed in adult K14HPV16E7^{WT} mice, albeit a less severe expansion than that observed at postnatal day 12 (data not shown). Thus, the kinetics of the intensity of the proliferative state in the epidermis of the K14HPV16E7^{WT} mice correlates with age-dependent levels of HPV-16 E7-specific mRNA expression in the epidermis.

Differentiation state of the epidermis of K14HPV16E7^{WT} mice. Histological sections of epidermis from K14HPV16E7^{WT} mice were subjected to K14-specific immunohistochemical staining to determine whether the differentiation state of these mice was affected by age-dependent levels of HPV-16 E7-specific mRNA expression in the epidermis. The expression of K14 is predominant in the cells of the stratum basale of the epidermis and diminishes as the cells terminally differentiate (38). K14 staining in the epidermis of the K14HPV16E7^{WT} mice was indistinguishable from that in the epidermis of nontransgenic mice at postnatal day 6 (Fig. 4J and D, respectively), with K14-positive cells being present in the suprabasal as well as the basal layers. K14 staining in the epidermis of postnatal day 12 and 15 nontransgenic mice is most pronounced in the basal layer (Fig. 4E and F). However, a significant vertical expansion in K14 staining within the epidermis of the postnatal day 12 K14HPV16E7^{WT} transgenic mice was observed (Fig. 4K). This vertical expansion in K14 staining within the epidermis of K14HPV16E7^{WT} transgenic mice was diminished by postnatal day 15 (Fig. 4L), consistent with the decrease in the level of HPV-16 E7-specific mRNA expression, epidermal thickening, and proliferative index. K14 staining was also evident in the hair follicles and sebaceous glands, which correlated with the expression pattern of the K14-directed E7 transgene (Fig. 2).

Expression of E7-specific mRNA in the skin of K14HPV16 E7$^{\Delta PTLHE}$ and K14HPV16E7$^{\Delta DLYC}$ mice. To assess whether K14HPV16E7$^{\Delta PTLHE}$ and K14HPV16E7$^{\Delta DLYC}$ transgenes are expressed appropriately in the epidermis, we performed in situ hybridization analyses. The expression pattern seen for these transgenes was similar to that seen for the K14HPV16E7^{WT}

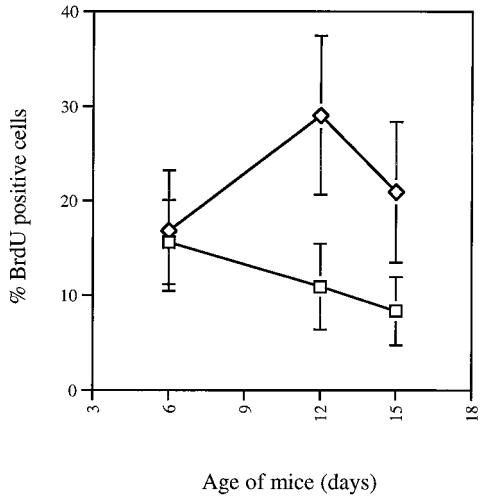

FIG. 5. Proliferative indices of the epidermis of nontransgenic and K14HPV 16E7^{WT} transgenic mice. The percentages of BrdU-positive cells shown are the numbers of positively stained basal cells versus the total numbers of cells in the basal layer of the epidermis. A minimum of 10 fields of postnatal day 6, 12, and 15 skin samples from two independent experiments were examined at a magnification of 40×. Squares, nontransgenic mice; diamonds, K14HPV16E7^{WT} transgenic mice.

transgene (Fig. 2). Strong E7-specific in situ hybridization signals were observed in the epidermis and epithelial structures (hair follicles and sebaceous glands) of the dermis. Maximal expression of the K14HPV16E7$^{\Delta PTLHE}$ and K14HPV16 E7$^{\Delta DLYC}$ transgenes appeared to occur slightly earlier (between 5 and 7 days after birth [Fig. 6E and F]) than with the K14HPV16E7^{WT} transgene (between 10 and 15 days after birth [Fig. 2]).

Absence of epidermal hyperplasia in the skin of K14HPV16 E7$^{\Delta PTLHE}$ and K14HPV16E7$^{\Delta DLYC}$ mice. K14HPV16 E7$^{\Delta PTLHE}$ and K14HPV16E7$^{\Delta DLYC}$ lineages shown to express E7-specific mRNA were observed for overt phenotypes. Unlike the K14HPV16E7^{WT} lineages, these lines, which carry deletions in the E7 gene, do not exhibit any overt phenotypes (Table 1). Skin samples were collected from transgenic mice carrying mutant E7 genes at the ages at which apparent maximal transgene expression was observed, postnatal days 7 and 5 for K14HPV16E7$^{\Delta PTLHE}$ and K14HPV16E7$^{\Delta DLYC}$ mice, respectively. Histological sections were subsequently examined after immunohistochemical staining. Neither a thickening of the nucleated-cell layer nor a lateral expansion of the epidermis of K14HPV16E7$^{\Delta PTLHE}$ and K14HPV16E7$^{\Delta DLYC}$ mice was observed upon comparison to the nontransgenic age-matched controls (Fig. 7A to D). The proliferative indices of the epidermis in postnatal day 7 K14HPV16E7$^{\Delta PTLHE}$ (12.5%) and postnatal day 5 K14HPV16E7$^{\Delta DLYC}$ (9.8%) mice (Fig. 7F and H) were comparable to those of the nontransgenic age-matched mice (11.5% and 10.4%, respectively) (Fig. 7E and G). In addition, while BrdU-positive cells were present in the suprabasal region of the K14HPV16E7^{WT} mouse epidermis, they were not observed in the epidermis of K14HPV16 E7$^{\Delta PTLHE}$ or K14HPV16E7$^{\Delta DLYC}$ mice (Fig. 7F and H). An additional immunohistochemical analysis to assess the differentiation state was performed by staining histological sections with K14-specific antibody. The vertical expansion of K14-positive cells detected in the epidermis of K14HPV16E7^{WT} mice (Fig. 4) was not observed in the epidermis of K14HPV16 E7$^{\Delta PTLHE}$ or K14HPV16E7$^{\Delta DLYC}$ mice (Fig. 7J and L). We conclude that functions associated with the CR1 and CR2 domains of HPV-16 E7 are required to alter the proliferative and differentiation states of mouse epithelial cells in vivo.

Development of skin tumors in K14HPV16E7^{WT} mice compared to that in K14HPV16E7$^{\Delta PTLHE}$ and K14HPV16E7$^{\Delta DLYC}$ mice. Ten percent of line 2304 K14HPV16E7^{WT} mice develop skin tumors late in life, with a median age of onset of 10.6 months (16). In comparison, less than 1% of nontransgenic FVB inbred mice develop skin tumors by the age of 12 months. The skin tumors in the K14HPV16E7^{WT} mice originated from squamous cells (papillomas and carcinomas) or sebaceous

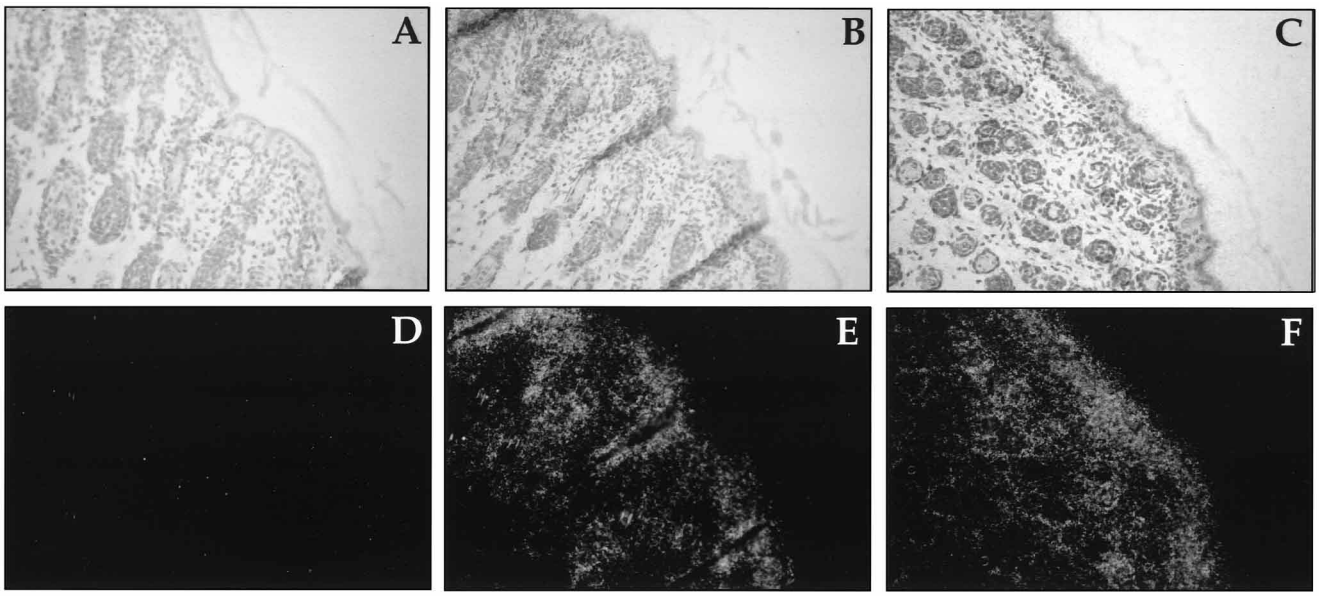

FIG. 6. In situ hybridization analysis of skin from nontransgenic mice and from K14HPV16E7$^{\Delta PTLHE}$ and K14HPV16E7$^{\Delta DLYC}$ transgenic mice. All tissues were embedded in paraffin and sectioned. Skin from postnatal day 5 nontransgenic mice (A and D) and from postnatal day 7 K14HPV16E7$^{\Delta PTLHE}$ (B and E) and postnatal day 5 K14HPV16E7$^{\Delta DLYC}$ (C and F) transgenic mice was stained with hematoxylin (A to C) or hybridized to E7 mRNA-specific probes (D to F).

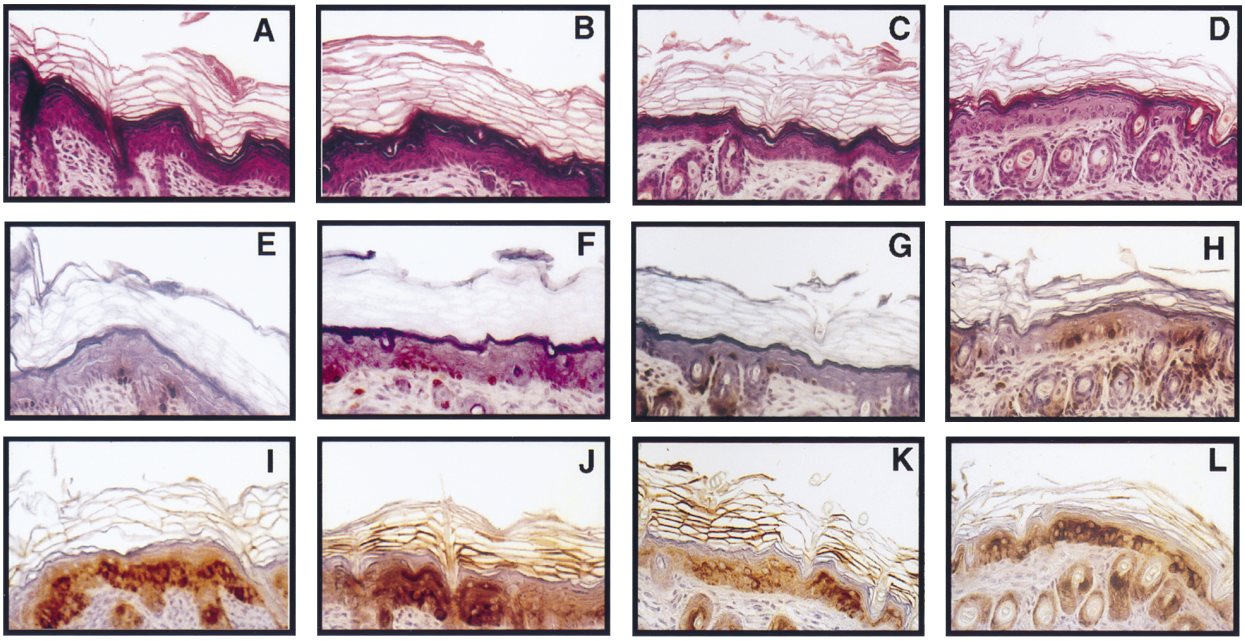

FIG. 7. BrdU and K14 analyses of skin from nontransgenic mice and from K14HPV16E7$^{\Delta PTLHE}$ and K14HPV16E7$^{\Delta DLYC}$ transgenic mice. All tissues were paraffin embedded and sectioned. Skin from postnatal day 7 (A, E, and I) and day 5 (C, G, and K) nontransgenic mice and from postnatal day 7 K14HPV16E7$^{\Delta PTLHE}$ (B, F, and J) and day 5 K14HPV16E7$^{\Delta DLYC}$ (D, H, and L) transgenic mice was stained with hematoxylin and eosin (A to D), BrdU (E to H), or K14 (I to L).

gland cells (sebaceous epitheliomas) (16). No skin tumors were observed in 130 adult K14HPV16E7$^{\Delta PTLHE}$ mice that cumulatively arose from the four lines analyzed in this study (Table 1). Of the 169 mice observed among the five K14HPV16 E7$^{\Delta DLYC}$ lines, only one mouse developed tumors, and these tumors were benign papillomas that arose after 12 months of age (Table 1). Thus, the incidence of tumors in the K14HPV16 E7$^{\Delta DLYC}$ and K14HPV16E7$^{\Delta PTLHE}$ mice was no greater than the background frequency observed in nontransgenic FVB mice.

DISCUSSION

The expression of wild-type HPV-16 E7 in squamous epithelia of transgenic mice leads to epidermal thickening, an increased proliferative index, and an expanded population of K14-positive cells (16). The epidermal thickening observed in the skin of K14HPV16E7^{WT} mice peaked at postnatal day 12, which correlated with maximum age-dependent levels of HPV-16 E7-specific mRNA expression. However, while diminished compared to that observed at postnatal day 12, the observed epidermal thickening persisted in the epidermis of the ears and torsos of these mice into adult life when compared to nontransgenic age-matched controls. Since HPV-16 has recently been shown to induce aberrant apoptosis (31, 32), increased apoptosis in the skin of K14HPV16E7^{WT} mice could potentially explain the regression of epidermal thickening by postnatal day 15. This is not likely to be the cause since E7 does not induce aberrant apoptosis in the epidermis of K14HPV16E7^{WT} mice at either postnatal day 6 (16) or day 10 (41a). Nevertheless, experiments measuring the number of apoptotic cells in mouse epidermis between postnatal day 10 and postnatal day 18 will be designed to address this hypothesis.

The HPV-16 E7 amino terminus encodes two domains, CR1 and CR2, which exhibit amino acid sequence similarity to the CR1 and CR2 regions of the Ad E1A protein. In this study, we created amino acid deletions in each of these domains to determine their contributions to the ability of wild-type E7 to alter epidermal growth properties. Since it has previously been argued that the CR1 domain of HPV-16 E7 possesses transformation activity (20, 34), a deletion in the CR1 domain (ΔPTLHE) of E7 was used to generate K14HPV16E7$^{\Delta PTLHE}$ transgenic mice. The proliferation and differentiation statuses of the epidermis of K14HPV16E7$^{\Delta PTLHE}$ mice were equivalent to those observed in nontransgenic mice. These data suggest that a biological activity associated with the CR1 domain is necessary for E7 to alter cell growth in the epidermis. However, a definitive biological activity has not been attributed to the CR1 domain of HPV-16 E7. Conversely, biochemical properties associated with the CR2 domain of HPV-16 E7 have been well described, including its capacity to bind pRb family members and the correlation of this binding with the transformation activity of HPV-16 E7. To investigate the consequences of inactivating the CR2 domain in vivo, a 4-amino-acid deletion (ΔDLYC) in the CR2 domain of E7 was used to generate K14HPV16E7$^{\Delta DLYC}$. Histological analyses again revealed that the proliferation and differentiation statuses of the epidermis of K14HPV16E7$^{\Delta DLYC}$ mice were indistinguishable from those observed in nontransgenic mice. These results suggest that the interaction between E7 and any, or all, of the pRb family members is involved in the alteration of growth properties of epithelial cells in the epidermis.

In this study, maximal expression of E7^{WT} was found to occur at postnatal day 12. Several K14HPV16E7^{WT} transgenic founder mice, and a high frequency of transgene-hemizygous offspring from one line (2350) and transgene-homozygous offspring from another line (2304) of K14HPV16E7^{WT} mice, were found to die at this age (16). These mice exhibited stunted growth and showed a paucity of mother's milk in their stomachs. We ascribe the lethal phenotype to the hyperproliferation of the stratified squamous epithelium lining the upper digestive tracts of these mice (data not shown), which we believe was responsible for the apparent feeding difficulty of these young animals. While most of these mice died before 2

weeks of age, those that survived grew to normal adult weight and bred successfully. The age restriction of this morbid phenotype correlated with the age-dependent expression of E7.

The capacity of HPV-16 E7 to potentiate tumorigenesis in the skin of K14HPV16E7^{WT} mice was previously demonstrated to be independent of either HPV-16 E6 or mutational inactivation of p53 or Rb (16). The tumors observed in the skin of these mice belonged to two classes, low-grade squamous cell carcinomas and sebaceous gland epitheliomas. The frequency of tumors observed in the skin of K14HPV16E7$^{\Delta PTLHE}$ and K14HPV16E7$^{\Delta DLYC}$ mice was not above levels noted in nontransgenic mice. These data indicate that in addition to the importance of the HPV-16 E7 CR1 and CR2 domains in the alteration of cell growth, activities associated with these domains are also necessary for the induction of squamous epithelial cell-derived tumors in the skin of K14HPV16E7^{WT} mice. Due to the lack of increased squamous epithelial cell proliferation and tumorigenesis in the skin of K14HPV16E7$^{\Delta PTLHE}$ and K14HPV16E7$^{\Delta DLYC}$ mice, we conclude that there exists a link or synergy between activities associated with the CR1 and CR2 domains of E7 and HPV-associated carcinogenesis.

Previous studies have suggested that the activity associated with the CR1 domain of E7 may be functionally equivalent to the activity associated with the N-terminal p300-binding region of E1A (8a). Recent evidence also suggests that the CR1 domain of E7 is involved in the degradation of Rb (21). These hypothesized CR1-associated activities have been inactivated in the K14HPV16E7$^{\Delta PTLHE}$ mice but could potentially be complemented by the intact CR1 region present in the K14HPV16E7$^{\Delta DLYC}$ mice, resulting in the aberrant growth and cancers observed in the skin of K14HPV16E7^{WT} mice. Hence, complementation analyses that involve mating K14HPV16E7$^{\Delta PTLHE}$ mice with K14HPV16E7$^{\Delta DLYC}$ mice have been initiated to generate K14HPV16E7$^{\Delta PTLHE/\Delta DLYC}$ hybrid mice. In a tissue culture system, E7 proteins with mutations within the Rb-binding region were unable to complement either non-p300-binding E1A or N-terminal E7 mutants (8a). However, data demonstrating that the E7 oncoprotein can form oligomeric complexes in vivo (8, 54) lend support to the possibility that complementation between HPV-16 E7$^{\Delta PTLHE}$ and HPV-16 E7$^{\Delta DLYC}$ in a transgenic-mouse background may occur. Experiments will also be carried out to investigate whether these E7 CR1 and CR2 transgenes display tumorigenic phenotypes when coexpressed with an HPV-16 E6 transgene. These experiments will investigate the in vivo relevance of the observation made in tissue culture that CR2 domain mutants of E7 display transforming properties when expressed in cells in the context of the full-length viral genome (20).

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