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Targeted Expression of the E6 and E7 Oncogenes of Human Papillomavirus Type 16 in the Epidermis of Transgenic Mice Elicits Generalized Epidermal Hyperplasia Involving Autocrine Factors

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The E6 and E7 early genes of human papillomavirus type 16 have been shown in vitro to play a central role in the transforming capability of this virus. To explore their effects on differentiating epithelial cells in vivo, we used a bovine cytokeratin 10 (K10) promoter to target the expression of E6 and E7 to the suprabasal layers of the epidermis of transgenic mice. In two different lines of mice efficiently expressing the transgene, animals displayed generalized epidermal hyperplasia, hyperkeratosis and parakeratosis in the skin and the forestomach, both known to be sites of K10 expression. Northern (RNA) blot analysis revealed high levels of E6 and E7 transcripts, and in situ hybridizations localized these transcripts to the suprabasal strata of epidermis. In vivo labeling of proliferating cells showed two distinct effects of E6 and E7 expression in the epidermis: (i) an increase in the number of growing cells in the undifferentiated basal layer and (ii) abnormal proliferation of differentiated cells in the suprabasal strata. The expression of c-*myc* in the skin of transgenics was higher than that in control animals. The induction of c-*myc* transcription by topical application of tetradecanoyl phorbol acetate was prevented by simultaneous treatment with transforming growth factor β1 in nontransgenic skin but not in transgenic skin. In addition, transforming growth factor α was found to be overexpressed in the suprabasal layers of the transgenic epidermis. These findings suggest that autocrine mechanisms are involved in the development and maintenance of epidermal hyperplasia. Animals of both lines developed papillomas in skin sites exposed to mechanical irritation and wounding, suggesting that secondary events are necessary for progression to neoplasia. Collectively, these results provide new insights into the tumor promoter activities of human papillomavirus type 16 in epithelial cells in vivo.

Infection of epithelial cells with human papillomaviruses (HPVs) appears to be a key event in the initiation and development of carcinomas of the uterine cervix (22, 52). A large majority of cervical hyperproliferative lesions are infected by one or more HPV types. Of these, the so-called oncogenic HPVs (particularly HPV type 16 [HPV-16], -18, -31, -33, and -35) are found in nearly 90% of invasive cancers (40). HPV-16 is the most prevalent virus associated with squamous cell carcinomas of the cervix. It is present in primary tumors as well as metastases and cervical cancer cell lines, such as CaSki. The major transforming activities of HPV-16 have been localized to the E6 and E7 open reading frames (ORFs) (34, 50). Expression of these genes is consistently found in HPV-16-positive cancer cell lines. Further, they are expressed in suprabasal keratinocytes of mild intraepithelial lesions infected by HPV-16 and in abnormal undifferentiated cells of in situ carcinomas and invasive tumors (16, 43). Continuous E6 and E7 expression appears to be necessary to maintain the transformed and tumorigenic phenotype of HPV-containing cell lines (12, 47). In addition, keratinocytes and fibroblasts of rodent and human origins can be immortalized by introducing plasmids that stably express the E6 and E7 genes (4, 24, 26, 31, 48). The mechanisms by which the E6 and E7 proteins of oncogenic HPVs induce cellular transformation are not fully understood; however, this appears to be the consequence of E6 forming complexes with the p53 protein (50) and E7 binding to pRB (18), p107 (13, 17), cyclin A (17, 44), and cdk2 (32).

The onset of an invasive cancer in the stratified epithelium of the cervix is preceded by progressive intraepithelial abnormalities, which evolve from hyperplasia to severe dysplasia and carcinoma in situ. Since this evolution may span several decades, it has been suggested that secondary events are required for complete malignant transformation of HPV-16-infected epithelial cells (reviewed in reference 40). Thus, for instance, cigarette smoking and oral contraceptive use have been implicated by epidemiologic studies as independent risk factors in cervical cancer (46). The molecular basis of the mechanisms by which such factors cooperate with HPVs in the generation of cervical cancer remains obscure. Indeed, its study has been complicated by the absence of an animal model for HPV pathogenesis because of the strict species specificity of HPVs (38).

Previous work on transgenic mice showed fibropapillomas in the skin of animals carrying the entire bovine papillomavirus type 1 genome (26), germ cell tumors in mice expressing the HPV-16 E6 and E7 genes under the control of the mouse mammary tumor virus long terminal repeat (25), skin tumors in mice expressing ectopically the E6 and E7 genes of HPV-16 from an α-crystallin promoter (27), and very recently, squamous epithelial neoplasia in mice with targeted expression of the HPV-16 early region to the basal keratinocytes of the

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epidermis (1). However, in all these models, the mechanisms of neoplastic transformation by the respective papillomavirus transgenes remain unclear.

In order to assess the biological effects of HPV-16 E6 and E7 specifically on differentiating keratinocytes and to establish an in vivo system that may allow the dissection of the multistep carcinogenesis associated with this virus, we have produced transgenic mice expressing these viral oncogenes in the suprabasal compartment of the epidermis. To this end, the E6 and E7 ORFs of HPV-16 were placed under the control of the promoter-enhancer region of a bovine homolog of the human cytokeratin 10 (K10) gene (6). This cytokeratin is an early marker of terminal differentiation (29, 39) which has previously been reported to target the expression of a number of heterologous genes to the suprabasal differentiating cells of the epidermis in transgenic mice (2, 49). In this study, we describe the characterization of two lines of mice that express high levels of E6 and E7 transcripts in the suprabasal compartment of the epidermis. The results obtained reveal that this expression leads to epidermal hyperproliferative modifications, which suggests that epithelial growth factors, acting by autocrine and paracrine mechanisms, may play an important role in the initial stages of HPV carcinogenesis.

MATERIALS AND METHODS

Plasmid construction and generation of transgenic mice. An *Xho*I-*Eco*RI fragment containing the E6 and E7 ORFs of HPV-16 (nucleotides 58 to 867) upstream of a simian virus 40 (SV40) poly(A) sequence (0.65 kb) was isolated and cloned in front of a 3.5-kb fragment of the bovine KVI (homolog of the human K10) promoter-enhancer region into plasmid pBKVI-CAT (6). Digestion of this plasmid with *Xho*I and *Eco*RI released the chloramphenicol acetyltransferase gene, which was replaced by the E6/E7-SV40 poly(A) fragment. The K10 promoter-enhancer fragment contained a TATA box but no cap site. A cap site was, however, provided by the E6/E7 sequence (p97 promoter).

The complete insert was isolated as a *Sal*I-*Eco*RI (~5-kb) fragment (see Fig. 1) and microinjected, at a concentration of 1 μg/ml, into the pronuclei of fertilized eggs to generate transgenic mice as described previously (10). At 3 weeks after birth, mouse tail DNAs were isolated and analyzed for the presence of transgenes by Southern blot, with a radiolabeled HPV-16 E6/E7 fragment as a probe.

RNA isolation and analysis. Total RNA was isolated from tissues of adult mice, electrophoresed (10 μg), transferred to nylon membranes, and hybridized to radiolabeled HPV-16 E6/E7 sequences as described elsewhere (10). Densitometric analysis of autoradiograms was performed with the NIH-Image program version 1.40. Slot blot hybridization of total RNA to a radiolabeled probe of rat transforming growth factor α (TGF-α) cDNA was performed on nitrocellulose with a Minifold apparatus (Schleicher & Schuell).

PCR analysis of cDNAs synthesized from total RNA samples by reverse transcription was performed essentially as previously described (5). E6/E7-specific single-stranded cDNAs were obtained from 0.5 μg of each RNA sample with an oligonucleotide primer hybridizing to the 3′ end of the E7 ORF (nucleotides 757 to 731 [5′ to 3′]). E6/E7 cDNAs were then amplified (30 cycles) by equal amounts of this primer and an oligonucleotide directed to the 5′ end of the E6 ORF (nucleotides 149 to 174 [5′ to 3′]). PCR products were electrophoresed through 1% agarose gels, transferred to nylon membranes, and analyzed by Southern blot hybridization with E6/E7 radiolabeled DNA.

In situ hybridization. In situ hybridization of frozen sections (6 μm) was performed as described elsewhere (20), with ^{32}P-labeled riboprobes complementary to the E6/E7 sequence of HPV-16.

Immunostaining of skin sections. Immunohistochemical detection of TGF-α was carried out with an anti-TGF-α (Ab-2) mouse monoclonal antibody (Oncogene Science, through Dianova, Hamburg, Germany). Frozen sections (6 μm) were fixed with methanol-acetone (1:1) for 5 min at 4°C, air dried, treated with 0.1% H_2O_2 in Tris-buffered saline (TBS) (125 mM NaCl, 10 mM Tris [pH 7.6]) for 5 to 10 min at room temperature to quench endogenous peroxidase activity, and treated for 1 h with 20% normal goat serum in TBS to block nonspecific binding of immunoglobulin G (IgG). Sections were incubated for 1 h at 37°C with monoclonal anti-TGF-α primary antibody, diluted to 6 μg/ml in TBS–5% normal goat serum, washed, and incubated for 1 h at room temperature with biotinylated goat anti-mouse IgG (Sigma) diluted to 5 μg/ml in TBS–5% normal goat serum. Subsequently, sections were incubated with peroxidase-conjugated streptavidin (Sigma) for 30 min, washed extensively in TBS, and rinsed in TBS–0.5% Triton X-100. Peroxidase activity was detected by incubation in DAB staining solution (0.025% diaminobenzidine–0.003% H_2O_2 in TBS) for several minutes (typically 5 min) at room temperature until color was visible. The reaction was stopped by adding sodium azide at a final concentration of 0.1%, and tissues were stained lightly with hematoxylin.

For immunofluorescence detection of cytokeratin 10, frozen sections (6 μm) were fixed as described above, rehydrated in phosphate-buffered saline (PBS) for 10 min, and incubated for 5 min at room temperature with a monoclonal anti-K10 antibody (1:100) described previously (23). Texas red-conjugated anti-mouse IgG (1:80) was used as the secondary antibody.

Labeling of proliferating keratinocytes with BrdU. Transgenic and control mice were injected intraperitoneally with bromodeoxyuridine (BrdU; 50 mg/kg of body weight) (Sigma). After 1 h, mice were sacrificed and skin and forestomach biopsy samples were frozen quickly in liquid nitrogen. Sections were fixed in methanol for 10 min at 4°C and stained with a mouse monoclonal antibody against BrdU conjugated to fluorescein (Boehringer GmbH, Mannheim, Germany), diluted to 50 μg/ml in PBS–0.1% bovine serum albumin, as described by the manufacturer.

Animal treatments. In all cases, animals were previously anesthetized with Ketavet (Parke-Davis). Tetradecanoyl phorbol acetate (TPA; Sigma) was administered topically 2 days before the mice were sacrified by slowly pipetting 200 μl of a 10^{-4} M TPA solution (in acetone) onto an approximately 1-cm^2 area of back skin. TGF-β1 (Serva, Heidelberg, Germany) was diluted to 20 ng/ml and injected intradermally (0.25 ml) into an area of the skin distant from that treated with TPA 2 h before the biopsies were taken. Double treatments were made by applying TPA and TGF-β1 to the same area of back skin by the protocols described above.

RESULTS

Transgenic mice carrying the K10-E6/E7 gene construct. The promoter region of a number of keratin genes has been used to target the expression of heterologous genes to stratified squamous epithelia of transgenic mice (2, 7, 45, 49). These studies have confirmed the overall tissue specificity of expression directed by keratin promoters. The K10 gene is expressed suprabasally in the epidermis and at very low concentrations in other stratified epithelia (29, 39). Here, we have used an

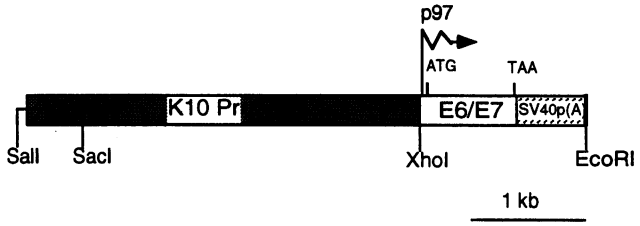

FIG. 1. Diagram of the K10-E6/E7 transgene construct. The HPV-16 E6 and E7 ORFs were cloned into the *Xho*I site of an expression vector containing the bovine K10 promoter-enhancer (6). SV40 poly(A) [SV40p(A)] was inserted immediately downstream of the E7 termination codon. K10Pr, cytokeratin 10 promoter; p97, transcription initiation site of the E6 and E7 ORFs; ATG, E6 translation start codon; TAA, E7 termination codon. The position of the unique restriction site for *Sac*I in the K10Pr sequence is indicated.

expression vector based on the bovine cytokeratin VI promoter which, in terms of tissue specificity, is equivalent to the human K10 promoter (2, 29). Figure 1 illustrates the K10-E6/E7 transgene construct as a 5-kb *Sal*I-*Eco*RI fragment devoid of plasmid sequences, which was used for microinjection into the pronuclei of single-cell mouse embryos. Southern blot analysis of tail DNAs from 26 mice showed that two of them (labeled 689 and 694) carried intact copies of the transgene integrated as head-to-tail arrays at a single locus. These founders were bred to generate two transgenic lines from which heterozygous animals were used in subsequent analyses. The copy number of the transgene, estimated by slot blot hybridizations, was 3 to 4 for line 689 and 12 to 15 for line 694.

Although there were no major phenotypic changes in transgenic animals compared with controls, the skin of the founders and their transgenic offspring was scalier than normal. This effect was particularly evident in regions of the skin that normally have little or no hair, such as the ears, which were markedly hyperkeratotic and somewhat thicker. This provided a rapid means by which to distinguish K10-E6/E7-positive mice from their negative littermates.

Transgene expression. To verify that the expression of the K10-E6/E7 transgene was targeted to the epidermis, total RNAs were isolated from different tissues of adult F_1 heterozygous mice. In both transgenic lines, Northern (RNA) blot analysis revealed high levels of E6/E7 expression in the different types of skin studied and in the forestomach (Fig. 2A). However, very low levels of expression were also seen in the esophagus, vagina, and thymus (data not shown). With a specific E6/E7 probe, three major transcripts of 4.5, 2.3, and 1.5 kb were detected in mouse tissues. These bands were also seen in a cervical carcinoma cell line, CaSki, carrying integrated copies of the HPV-16 genome. This pattern of E6/E7 transcription in CaSki cells and cervical carcinomas has previously been described (42). In agreement with the different copy numbers of the transgene carried by each line, the intensity of the signal for RNA samples from tissues of heterozygous animals of line 694 was higher than that for those of line 689, with three- to fourfold-fewer copies than the former (Fig. 2A). The splicing pattern of E6/E7 transcripts was analyzed by cDNA PCR (Fig. 2B). By this means, three different mRNA species had been identified previously in CaSki cells (42) and transgenic mice expressing the E6/E7 region from an α-crystallin promoter (27): full-length E6/E7, also called E6 because it is the transcript for translation of an intact E6 protein, and E6*I^E7 and E6*II^E7 spliced transcripts, which yield two truncated E6 proteins that differ in the carboxy terminus. As

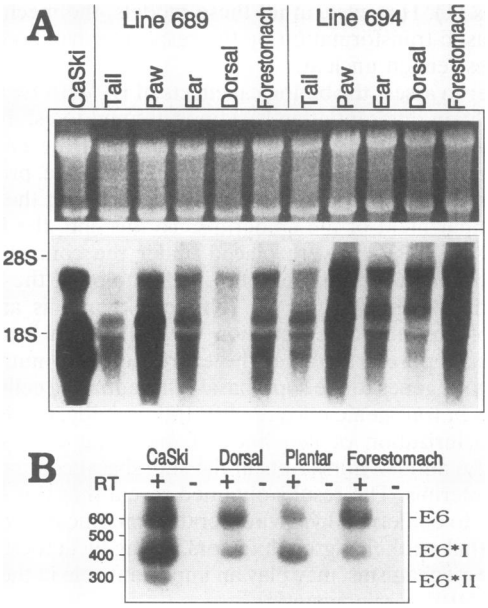

FIG. 2. Expression of E6/E7 mRNAs in the skin and forestomach. (A) Representative Northern blot analysis of HPV-16 E6/E7-specific RNAs produced in different regions of the skin and forestomach of heterozygous mice of low-copy-number (689) and high-copy-number (694) transgenic lines. Total RNA (10 μg) extracted from biopsy samples of the indicated body sites was separated on a 1% agarose gel, stained with ethidium bromide (upper panel), transferred to a nylon filter, and hybridized to radiolabeled HPV-16 E6/E7 DNA (lower panel). The positions of the 28S (4.7-kb) and 18S (1.9-kb) mouse RNAs are given. Total RNA (10 μg) of CaSki, an HPV-16-containing cell line, was used as a control. (B) PCR analysis of E6/E7 transcripts. cDNAs were generated from RNA samples, like those shown in panel A, amplified by specific primers, and analyzed by Southern blot hybridization. Radiolabeled E6/E7 DNA was used as the probe. Indicated on the right are the expected positions of the three amplification products on the basis of differential splicing. The positions of the 100-bp DNA ladder markers are indicated on the left. Shown are data for K10-E6/E7-694; data for K10-E6/E7-689 were identical with respect to the splicing pattern. RT, reverse transcription.

shown in Fig. 2B, E6/E7 and the E6*I were detected in RNAs from the skin and forestomach of transgenic animals. However, under the conditions used for the amplification of transgene cDNAs, E6*II was not visible. Nevertheless, it has been suggested previously (41) that E6*I may provide for more efficient translation of E7 protein than E6*II does because it has a much larger space between the ORFs. Accordingly, E7 protein was present in E6/E7-expressing tissues, as it could be immunoprecipitated in protein extracts of skin from these animals (data not shown).

To examine the location of transgene mRNAs, serial sections of tissues of adult heterozygous mice were hybridized with radiolabeled E6/E7 antisense RNAs. E6/E7 mRNAs were localized to the suprabasal strata (spinous and granular) of the transgenic epidermis, as shown in Fig. 3 for back skin. Basal cells (Fig. 3) showed only background levels of hybridization. The same distribution of E6/E7 transcripts was observed in the skin of the footpad, ear, and tail and in the forestomach. In contrast, no hybridization was obtained in the same tissues with the sense probe or in control (nontransgenic) tissues with the antisense probe (data not shown).

The phenotype of K10-E6/E7 mice correlates with transgene expression. Histopathologic analysis of biopsies from 30 ani-

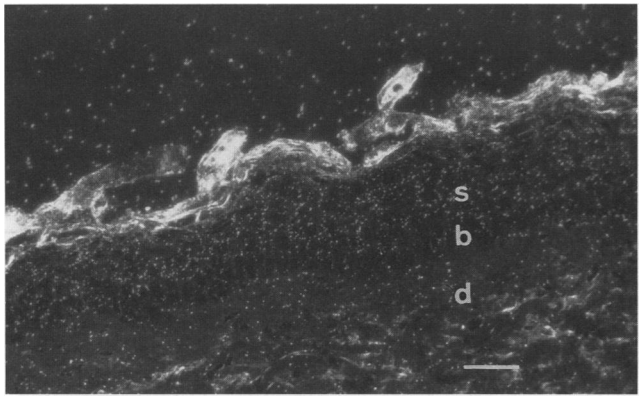

FIG. 3. In situ localization of transgene E6 and E7 mRNAs. Tissue was snap frozen in liquid nitrogen, sectioned (5 μm), and processed for in situ hybridization with ^{32}P-labeled RNA probes complementary to the E6 and E7 sequences of HPV-16. After hybridizations, sections were dipped in LM1 emulsion (Amersham) and exposed for 2 weeks at 4°C. After being developed, tissue sections were counterstained with hematoxylin and eosin. A representative example of E6/E7-specific hybridization in the back skin epidermis of an adult heterozygous mouse of line 694 is shown. Exposed silver grains, seen by dark-field microscopy, are mostly localized over suprabasal cells of the epidermis, whereas basal cells show only background levels of hybridization. In situ hybridizations with samples from line 689 mice were similar with respect to localization, but with reduced E6/E7 signals. Bar, 30 μm. Abbreviations: d, dermis; b, basal layer; s, suprabasal layer of the epidermis.

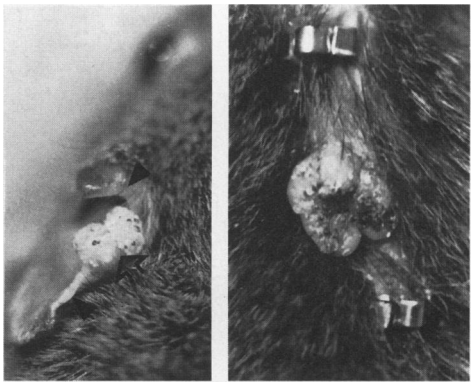

FIG. 5. Papillomas developed at wound sites in adult transgenic mice. (Left panel) Small papilloma (arrow) on the ear of a 14-month-old mouse of line 689. The tumor (approximately 0.5 by 0.3 by 0.3 cm) is located along the cut edge (arrowheads) of the ear tag punch hole. The ear tag had detached previously. Note the increased thickness of ear edges. (Right panel) Back of a mouse of line 694 (17 months old), showing an ulcerative papilloma (1 by 0.8 by 0.4 cm) growing on the scar of a previous incision performed to remove a sample of skin for histological analysis. Both animals were heterozygous for the K10-E6/E7 transgene. Histological examination indicated that these tumors were well differentiated.

mals of both transgenic lines and 20 controls revealed that the epidermis of the skin and forestomach was markedly thicker in transgenic animals than in controls (see sections of transgenic and control tissues in Fig. 4 and 6). Hyperthickening was observed in all types of epidermis in newborns as well as in adult and old animals without regional variations. However, in some areas, the founders exhibited alternating patches of thickened and normal epidermis, and this was interpreted as the result of mosaicism. The density of cells in the basal layer was increased, and in some stretches, two layers were present (Fig. 4A). The spinous and granular strata were increased proportionally relative to control epidermis, and the size of the cells in these layers was greater than normal. In addition, the transgenic epidermis exhibited signs of abnormal keratinization; the stratum corneum was markedly thickened (hyperkeratosis), with cell nuclei present throughout (parakeratosis). The dermis was normal in thickness and cell density, and hair follicles also appeared to be normal. No significant histologic changes in the stratified epithelia of the esophagus and vagina were observed.

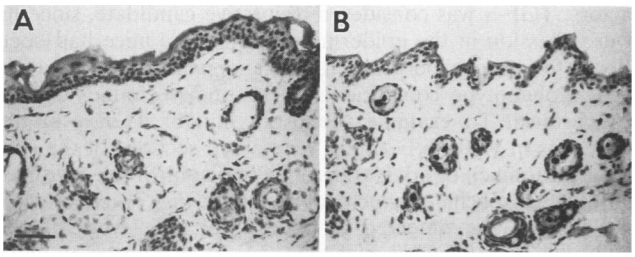

FIG. 4. Histopathology of transgenic mouse skin and control mouse skin. Representative skin samples taken from the backs of a K10-E6/E7 adult heterozygous mouse of line 689 (A) and a normal nontransgenic mouse (B). Tissues were fixed with formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin. Note the differences in epidermal thickening and, most importantly, the overall increase in the keratinocyte population of the basal and suprabasal layers in the transgenic epidermis. Bar, 70 μm.

Adult transgenic mice, maintained at least until the age of 20 months, developed a limited number of benign skin papillomas in areas of mechanical abrasion, like the ear tag attachment site or scars of previous scalpel incisions from skin biopsies (Fig. 5). Papillomas were observed more frequently in line 694 (5 of 16 animals) than in line 689 (3 of 13) and were never seen in control mice under the same conditions. Histological analysis of these papillomas showed a very thickened and hyperkeratotic epidermis, with extensive areas of keratinization underneath, supported by a thin underlying dermis (data not shown).

Increased basal cell proliferation and suprabasal mitotic activity. In order to analyze whether the hyperthickening of the epidermis was truly due to a hyperproliferative status of keratinocytes, we compared the numbers of epidermal cells synthesizing DNA in the skin and forestomach of transgenic and control animals. Proliferating cells were labeled with BrdU in vivo, and cryosections of the skin and forestomach were subsequently stained with an antibody against BrdU. The number of labeled basal cells was markedly increased (two- to threefold) in the epidermis of skin and forestomach of heterozygous transgenic mice relative to nontransgenic controls, as determined by counting labeled and unlabeled cells in photographs from five random fields, like those illustrated in Fig. 6. There was little difference in the relative increase of proliferating cells among the three types of epidermis analyzed (plantar skin, back skin, and forestomach), despite the different levels of transgene RNA detected in such tissues (Fig. 2A). Consistent with this, no significant differences between the two (high-copy-number and low-copy-number) transgenic lines, which also displayed differences in transgene expression, were observed (Fig. 2A).

An observation from these studies was that the suprabasal layers of the hyperplastic epidermis contained labeled cells

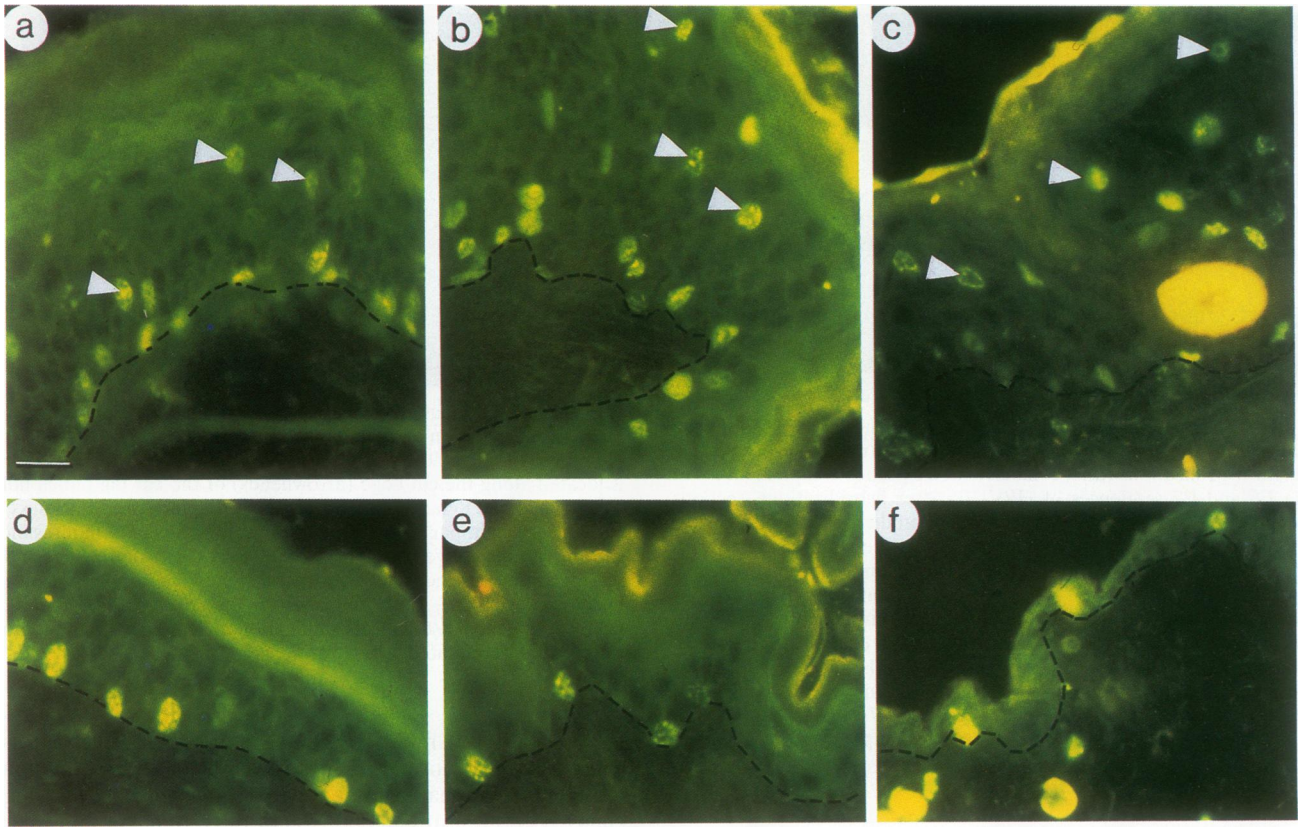

FIG. 6. Detection of proliferating keratinocytes in the epidermis of adult mice by BrdU labeling. BrdU was injected intraperitoneally 1 h before biopsies were taken. Sections were stained with a fluorescein isothiocyanate-conjugated antibody against BrdU. Shown are representative sections of plantar skin (a and d), forestomach (b and e), and skin from the backs (c and f) of F_1 heterozygous transgenic mice (a, b, and c) and nontransgenic controls (d, e, and f). Note the increased number of stained nuclei in the basal layer of the transgenic epidermis and the presence of labeled cells in the suprabasal strata (arrowheads indicate some examples). Note also the difference in the thickness of the epidermis of transgenic and nontransgenic mice. The positions of the basal lamina, which separates the epidermis from the dermis, are denoted by dashed lines. Bar, 24 μm (a to e) and 48 μm (f).

(arrowheads in Fig. 6), whose number was estimated to be 15 to 20% of that of proliferating cells. The brevity of exposure to BrdU (1 h) before biopsies were performed and the fact that labeled cells are distant from the basal layer (in some cases, more than 100 μm; Fig. 6b) indicated that these were truly suprabasal cells and not undifferentiated labeled cells migrating from the basal layer. Moreover, in time course experiments of BrdU labeling, we observed that the progression of labeled cells from basal to cornified layers in the transgenic epidermis lasted about 1 week, an interval similar to that in nontransgenic skin (data not shown). Further, double immunofluorescence analyses allowed us to determine that suprabasal cells synthesizing DNA expressed K10 (data not shown), which is an early marker of terminal differentiation (39), thus suggesting that these were indeed differentiated cells with proliferating capacity. In contrast, BrdU-incorporating cells in the basal layer did not contain K10.

In addition, the proliferating cell nuclear antigen, an auxiliary peptide of DNA polymerase, whose expression is associated with the S phase of the cell cycle and is normally restricted to undifferentiated keratinocytes of the basal layer (8, 9), was detected in suprabasal cells by immunostaining (data not shown). In contrast, neither the synthesis of DNA nor proliferating cell nuclear antigen expression was detected in suprabasal cells of the epidermis in control mice or mice treated with TPA, a tumor promoter that, administered topically, causes stimulation of mitotic activity in the epidermis (reviewed in reference 15).

Overexpression of TGF-α in the suprabasal layers of the hyperplastic epidermis. Although expression of the K10-E6/E7 transgene took place primarily in suprabasal cells of the epidermis (Fig. 3), an increased number of proliferating cells was observed in the basal layer. This led us to investigate whether basal cells could be stimulated by autocrine factors secreted by E6/E7-expressing suprabasal cells. Among these factors, TGF-α was considered a putative candidate, since its overexpression in the epidermis of transgenic mice had been shown to cause hyperproliferation and papilloma formation (45), a phenotype comparable to the one we found in K10-E6/E7 mice. To examine the expression of TGF-α in the epidermis of K10-E6/E7 mice, we stained sections of the skin and forestomach of adult transgenic and control mice with an anti-TGF-α-specific monoclonal antibody (Fig. 7). High levels of TGF-α were detected in the suprabasal layers of the epidermis in K10-E6/E7 mice (Fig. 7A, b), in contrast to more discrete staining, also in the suprabasal compartment, in nontransgenic controls (Fig. 7A, a). This increase in the expression of TGF-α was observed in the epidermis of the types of skin studied (e.g., back skin and footpad) and the forestomach of heterozygous animals of both transgenic lines, regardless of age (data not shown).

The level of increase in TGF-α expression was determined

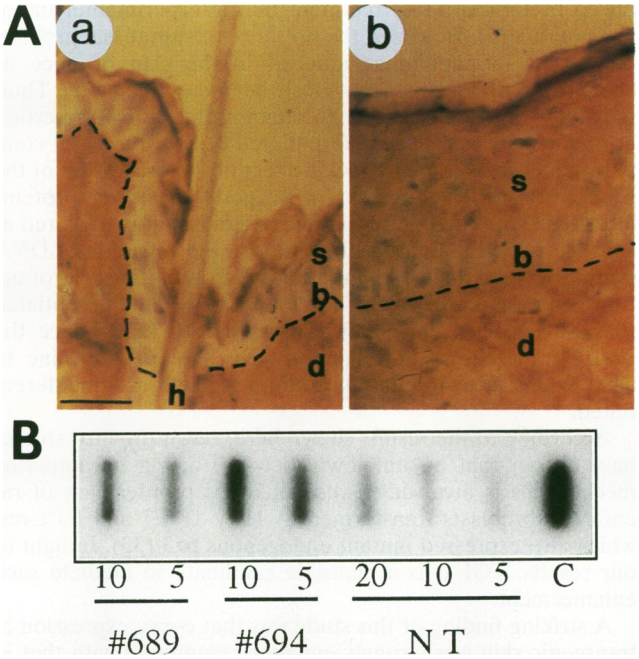

FIG. 7. Expression of TGF-α in the epidermis of transgenic and control mice. (A) Frozen sections of skin from the backs of an adult heterozygous mouse of line 694 (b) and a nontransgenic mouse (a) were stained with a monoclonal anti-TGF-α antibody by the biotin-avidin-peroxidase system (see Materials and Methods). The background of peroxidase activity seen in the underlying dermis is due to nonspecific binding of the secondary antibody (goat anti-mouse IgG), since it was also observed when the primary antibody was omitted. In contrast, no peroxidase activity was detected in the epidermis when the anti-TGF-α antibody was omitted. Dashed lines show the positions of the basement membrane separating the epidermal strata from the dermis. Bar, 24 μm. b and s, basal and suprabasal layers of the epidermis, respectively; d, dermis; h, hair shaft. (B) Slot blot analysis of TGF-α RNA levels in the skin of heterozygous adult mice of both lines and a nontransgenic control (NT). Numbers indicate the different amounts (in micrograms) of total RNA loaded. A positive control (C) consisting of approximately 10 pg of TGF-α cDNA used for hybridization was included.

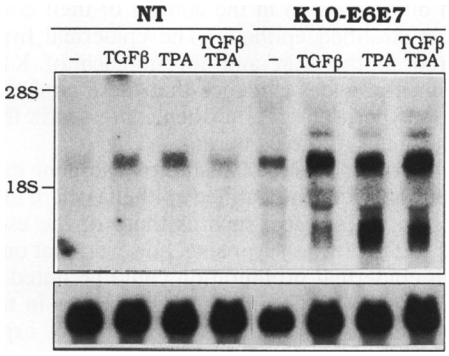

FIG. 8. Northern blot comparison of c-*myc* expression in the skin of an adult F_1 transgenic mouse (K10-E6/E7) of line 689 and a control nontransgenic littermate (NT). After as much dermis as possible was removed by scraping with a scalpel, total cellular RNAs were isolated from four distinct ~1-cm^2 areas of back skin; one was untreated (−), and the other three were treated with TGF-β1 or TPA or with both compounds administered sequentially (see Materials and Methods). RNAs were electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and hybridized to a ^{32}P-labeled probe containing the first exon of mouse c-*myc*. The positions of mouse 18S (1.9-kb) and 28S (4.7-kb) rRNAs, used as markers, are given on the left. Rehybridization of the stripped filter with a glyceraldehyde-3-phosphate dehydrogenase radioactive probe (lower panel) was used as a control for the quantity and integrity of RNAs.

by slot blot hybridization of total RNA from the skin of mice of both lines and a nontransgenic control to a TGF-α cDNA probe (Fig. 7B). Densitometric analysis of the autoradiographic bands revealed increases of nearly threefold for line 689 and fivefold for line 694. The higher level in the latter correlates with the higher level of E6/E7 transcripts found in these animals (Fig. 2).

Expression of c-*myc* in the transgenic epidermis. Cultured skin keratinocytes expressing HPV-16 E7, like those expressing SV40 large T antigen or adenovirus E1A, which also bind the retinoblastoma gene product (pRB), have been demonstrated to be resistant to the growth-inhibitory effect of TGF-β1 (30, 35). Moreover, in these cells, TGF-β1 was unable to suppress c-*myc* transcription as it does in keratinocyte cell lines in which pRB is not affected. These data point to the possible involvement of pRB in the mechanism of suppression of c-*myc* transcription by TGF-β1 (30).

In this context, we wondered whether inactivation of a cellular protein (most likely pRB) by E7 might be a primary event in the epidermis of our transgenic mice, which could interfere with suppression of c-*myc* transcription by TGF-β1, thus inducing proliferation in the suprabasal compartment. The first implication of this hypothesis is that c-*myc* should be overexpressed in the skin of CK10-E6/E7 mice. We tested this by comparing Northern blot hybridizations of total RNA extracted from the skin of transgenic and control mice with a radioactive mouse c-*myc* DNA probe. In control nontransgenic skin, the c-*myc* probe detected a single band of 2.4 kb; in contrast, this band and three additional bands of 3.2, 1.6, and 1.2 kb were found in samples from transgenic skin (Fig. 8). In addition, these experiments showed that the level of the c-*myc* 2.4-kb mRNA was 5- to 10-fold higher in transgenic skin than in control skin, as determined by densitometric tracings of the autoradiographic bands of blots like that shown in Fig. 8. Treatment with TPA, which is known to induce transcription of c-*myc* in vitro (11) and in vivo (37), caused an increase in the 2.4-kb transcript levels of both transgenic skin and control skin. Moreover, the 1.2-kb band underwent a strong increase in transgenic skin. Treatment with TGF-β1 increased c-*myc* transcription in transgenics as well as in controls. In contrast to TPA, TGF-β1 did not cause a significant increase of the 1.6-kb transcript in transgenic skin. Finally, when TGF-β1 was administered to a skin area previously stimulated with TPA, the amount of the 2.4-kb c-*myc* transcript decreased almost to the basal level in the control, whereas it was found to be increased in transgenics. On the contrary, the relative amount of the 1.2-kb band was lower after the double treatment (TPA and TGF-β1) than after treatment with TPA only. Altogether, these results suggest that c-*myc* is involved in the epidermal hyperproliferation induced by the E6/E7 transgene and are in agreement with a block of TGF-β1 suppression of c-*myc* transcription in the transgenic epidermis.

DISCUSSION

Although in vitro studies with cultured cells have helped to determine the oncogenic capability of HPV-16 and provided useful models for examining the transforming activities of its E6 and E7 ORFs, studies with transgenic mice have provided an assessment of the effects of these genes on keratinocyte

growth and differentiation in the context of their constitutive expression in stratified epithelia. The epidermal hyperplasia that occurred in the skin and forestomach of K10-E6/E7 transgenic mice provides evidence that these viral oncogenes cause intense hyperproliferation when expressed in the suprabasal compartment of the epidermis.

Hyperproliferation was seen at sites of transgene expression and was never observed in stratified epithelia where little or no K10 expression takes place, such as those of the esophagus, tongue, and vagina. It was surprising, however, that only minor variations in epidermal proliferation could be noted between the two lines analyzed, despite their differences in transgene copy number and expression (Fig. 2). A possible explanation for this could be a posttranscriptional negative regulation of E6/E7 expression. Another possibility is that the differentiated state of E6/E7-expressing suprabasal cells prevents perturbation of the homeostatic control of epidermal growth beyond a certain limit, regardless of the amount of viral proteins. This would also explain the fact that the turnover rates of keratinocytes in the skin of transgenic and control mice were similar (data not shown).

Labeling DNA-synthesizing cells with BrdU revealed that hyperplasia resulted not only from enhanced proliferation in the basal layer but also from ectopic proliferation in the suprabasal compartment. The mechanisms for these two effects should be distinct since expression of the E6/E7 transgene was targeted to the suprabasal layers of the epidermis. Thus, basal hyperproliferation cannot be directly linked to E6/E7 expression; instead, it seems to be a secondary event. The regulation of epidermal growth is complex and involves factors produced locally as well as circulating factors. It is unlikely, however, that viral proteins could act systemically because the effects of the K10-E6/E7 transgene were limited to the epidermis. Moreover, we found that TGF-α, a major autocrine regulator of epidermal growth which acts as a potent mitogen (14), was overexpressed in the suprabasal layers of the epidermis in both transgenic lines. The increase in TGF-α expression correlated well with the level of E6/E7 expression for each line, suggesting that this factor may be involved in the development and maintenance of the hyperproliferative state of this tissue. TGF-α overexpression has been implicated in human hyperproliferative disorders of skin and mucosae, like psoriasis (19) and vulvar condylomas (51), respectively. In addition, overexpression of TGF-α from a basal cytokeratin promoter in the skin of transgenic mice elicited epidermal hyperplasia and papillomas (45), a phenotype very close to the one we found in our mice.

In the normal epidermis, expression of the epidermal growth factor (EGF) receptor, which is thought to mediate TGF-α effects (28), is restricted to the basal layer. Moreover, expression of the EGF receptor was not modified in basal cells overexpressing TGF-α in the epidermis of transgenic mice (45) nor induced in suprabasal cells of the same epidermis. Indeed, suprabasal proliferation in such mice was not observed. Considering these data, the proliferating activity in the suprabasal layers of the epidermis of our transgenic mice does not seem to be an effect of the accompanying TGF-α overexpression. However, we cannot rule out the possibility that the transgene induced ectopic expression of EGF receptor in suprabasal cells, thereby making the effect of TGF-α on these cells possible. Studies of the distribution of EGF receptors in the epidermis and the papillomas of our transgenic mice will help to clarify this point.

Suprabasal proliferation in the epidermis is an abnormal phenomenon. It is never found in naturally occurring epidermal hyperplasia (e.g., by mechanical irritation) nor is it induced by topical TPA treatment during experimental tumor promotion (15). However, the presence of suprabasal proliferating cells in papillomas induced in the skin of mice by chemical treatments has previously been described (21). Thus, the ability of suprabasal cells to retain proliferating properties, normally restricted to undifferentiated cells of the basal compartment, seems to be a specific effect of the expression of the E6/E7 transgene. Moreover, it suggests that viral proteins interfered with the differentiation program that is triggered as cells leave the basal layer and results in the cessation of DNA synthesis. Nevertheless, the suprabasal proliferating cells of the transgenic epidermis were not found to be undifferentiated (basal-like cells), since they expressed K10 (and hence the E6/E7 transgene). Studies in progress should determine to what extent such suprabasal proliferating cells are undifferentiated.

According to the results shown here, recent in vitro studies have shown that an unknown factor(s) acting by autocrine mechanisms is involved in the enhanced proliferation of rat embryo fibroblasts transformed by HPV-16 E7 and EJ c-*ras*, which also expressed mutant endogenous p53 (33). In light of our results, TGF-α is a plausible candidate to mediate such enhancement.

A striking finding of this study was that c-*myc* expression in transgenic skin was strongly enhanced compared with that in normal or TPA-treated (hyperthickened) skin of controls. The origin and meaning of the 1.2-kb c-*myc* transcript that appeared in transgenics still need to be elucidated. The expression of c-*myc* has been shown to be necessary for keratinocyte proliferation (3, 35) and has been found to be increased in at least one-third of stage I and II squamous cell carcinomas of the uterine cervix (36). In addition, topical treatment with TGF-β1 failed to block induction of c-*myc* expression by TPA in the skin of transgenics but not in controls. These findings are in agreement with the results of previous in vitro studies showing that skin keratinocytes transformed by HPV-16 are resistant to the growth-inhibitory effect of TGF-β1, possibly as a result of pRB inactivation (30). More-extensive studies are required to determine whether c-*myc* mediates the effects of viral proteins in the epidermis of these mice and whether it is involved in the promotion of proliferating activity by the transgene in suprabasal cells.

The fact that under certain circumstances, such as wounding, our mice developed papillomas in the skin suggests that the E6/E7 transgene provides a genetic predisposition to neoplasia and that secondary events are necessary for progression from hyperplasia to papillomas. Therefore, these mice constitute a useful system for studying the mechanisms by which such factors cooperate with E6 and E7 in inducing neoplastic progression and thereby a valuable in vivo model for the multistep tumorigenesis associated with HPV-16 infection. Finally, our animals may be used to test the effectiveness of vaccines based on E6/E7 immunization as well as of therapeutic agents based on antisense strategies; for instance, antisense oligonucleotides directed against the E6 and/or E7 sequences could be administered topically to determine their effects on the hyperproliferating epidermis of K10-E6/E7 transgenic mice.

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