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Genetics

A 5′-upstream region of a bovine keratin 6 gene confers tissue-specific expression and hyperproliferation-related induction in transgenic mice

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ABSTRACT **Keratins, the constituents of epithelial intermediate filaments, are precisely regulated in a tissue- and development-specific manner, although little is known about the molecular mechanisms underlying this regulation. The expression pattern of keratin 6 is particularly complex, since besides being constitutively expressed in hair follicles and in suprabasal cells of a variety of internal stratified epithelia, it is induced in epidermis in both natural and artificially caused hyperproliferative situations. Therefore, the regulatory sequences controlling keratin 6 gene activity are particularly suitable for target gene expression in a tissue-specific manner. More interestingly, they can be skin-induced in transgenic animals or in gene therapy protocols, particularly those addressing epidermal hyperproliferative disorders. To delimit the regions containing these regulatory elements, different parts of the bovine keratin 6 gene linked to a β-galactosidase reporter gene have been assayed in transgenic mice. A 9-kbp fragment from the 5′ upstream region was able to provide both suprabasal tissue-specific and inducible reporter expression.**

Keratins, a family of >30 polypeptides, constitute the cytoskeleton of intermediate filaments characteristic of epithelial cells. They are subdivided into two groups, type I or acidic and type II or basic, and heterodimers formed by polypeptides of each group are the subunits forming the keratin filaments (for reviews, see refs. 1–3). Specific combinations of type I–type II keratins are expressed *in vivo* in a tissue- and development-specific manner (1, 4) that is probably related to the cellular functions of this multigene family.

In humans, keratin 6 (K6) and its partner K16 are expressed in several stratified epithelia, such as those of the oral cavity (tongue, palate, and oral epithelium), esophagus, and the genital tract (vagina, exocervix, glans penis, etc.) (5). This keratin pair is not found in normal interfollicular epidermis, although it is present in the hair follicle outer root sheath (6, 7). These keratins are induced in a variety of epidermal hyperproliferative situations such as benign and malignant tumors, skin keratinocyte culture, psoriasis (refs. 8 and 9; for a review on this issue in mice, see ref. 10), or wound healing (11). Topical skin treatment with chemicals that modify keratinocyte proliferation, such as phorbol 12-myristate 13-acetate (PMA) or all-*trans*-retinoic acid (RA), also induces K6/K16 expression (12–14), although in cultured keratinocytes RA seems to repress these keratins (15, 16).

Little is known about the mechanisms controlling this complex tissue-specific and inducible K6 expression. There is evidence that in genes coding for keratins of stratified epithelia, including K6, transcriptional control plays a fundamental regulatory role (17–19). However, induction of K6 in human epidermis seems to be regulated, at least in part, posttranscriptionally (20). In addition, more than one gene coding for K6 has been described in humans (21), mice (ref. 22; J. Schweizer, R. Murillas, and J.L.J., unpublished data), and bovids (M. Navarro and J.L.J., unpublished data), and it is not known whether these genes are regulated differently. Chloramphenicol acetyltransferase assays and gel retardation experiments have shown an enhancer and several protein–DNA binding sites in the proximal 5′ upstream region of bovine and human K6 genes, respectively (23, 24). Considering that K6 has been localized in the postmitotic suprabasal cells of stratified epithelia (9, 25, 26), the relevance of these elements to the cell-type-specific expression of this gene is uncertain, as they were mapped by using cultured cells. And, as in cultured keratinocytes, K6 is synthesized permanently, this type of experiment does not allow the study of the mechanisms controlling K6 induction. An *in vivo* approach is thus necessary to identify the sequences controlling K6 expression and, in particular, its skin induction in response to hyperproliferative stimuli. We have therefore generated transgenic mice carrying a *lacZ* reporter gene coupled to different regions of the bovine K6 (BK6) locus and found that a 5′ upstream fragment of ≈9 kbp provides K6-like transgenic expression.

MATERIALS AND METHODS

DNA Constructs. The locus containing the BK6β gene (formerly named BKIV*) has been described (17). A 9-kbp *Eco*RI–*Nae* I fragment containing 5′ upstream and promoter sequences and 127 bp of the transcript of BK6β gene was obtained from phage λ6 (17). This fragment was introduced in a p-PolyIII-1 plasmid containing an *Escherichia coli lacZ* gene and simian virus 40 polyadenylylation sequences derived from pRSVLacZ (27), producing an in-frame translational fusion containing the first 18 K6β amino acids preceding the β-galactosidase (β-gal) sequence. This construct was called BK6β(−8.8).Z (Fig. 1*D*). BK6β.Z (Fig. 1*C*) was constructed by fusing, downstream of the *lacZ* gene in BK6β(−8.8).Z, a 9-kbp fragment of the BK6β locus spanning the rest of the coding region plus 2.5 kbp of 3′ flanking sequences. BK6β(EX)TK.Z (Fig. 1*B*) contained 5′ flanking sequences from −830 bp to −183 bp of BK6β (transcription initiation site is +1), a thymidine kinase promoter from pBL-CAT-2 (28), and the *lacZ* gene. All three constructs were able to direct β-gal synthesis in transient transfection experiments.

Transgenic Mice. Transgenes were excised from the plasmid vectors, purified by low-melting-point agarose gel electrophoresis and Elutip columns (Schleicher & Schuell), adjusted to a final concentration of ≈2 μg/ml, and microinjected into

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Abbreviations: β-gal, β-galactosidase; K*n*, keratin *n*; BK*n*, bovine keratin *n*; MK*n*, murine keratin *n*; RA, all-*trans*-retinoic acid; PMA, phorbol 12-myristate 13-acetate; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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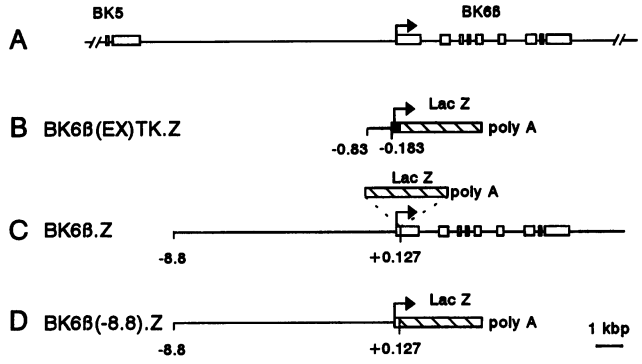

FIG. 1. Schematic representation of the BK5–BK6β locus (*A*) and the constructs used in this study (*B–D*). Open boxes, exons of BK5 and BK6β genes; stippled box, the *E. coli lacZ* gene; solid box, Herpes simplex virus thymidine kinase gene promoter; poly(A), the simian virus 40 polyadenylylation signal. Arrows indicate the transcription start site. Sizes in *B–D* are kbp with respect to the transcription initiation site.

(C57BL/10 × BALB/c)F_2 mouse embryos as described (29). Transgenic mice were identified by Southern blot analysis of tail DNA, by using the *lacZ* gene as probe.

5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) Histochemistry and Immunofluorescence. These techniques were performed as described (30). For X-Gal histochemistry, sections were stained overnight (except for tumors and reproductive epithelial, which were stained 2 h). Slides were counterstained with eosin, hematoxylin/eosin, or Ziehl's carbolfuchsin. Immunofluorescence was performed with a rabbit antiserum against a peptide specific for K6 that was provided by D. Roop (Baylor College of Medicine, Houston).

Hyperproliferative Treatments. RA (Sigma) was dissolved at 30 μg/100 μl of acetone; PMA (Sigma) was dissolved at 20 nmol/100 μl of acetone. Tail skins were treated topically with 100 μl of the RA solution daily for 14 days (unless otherwise specified) or with 100 μl of the PMA solution every second day (seven applications in 14 days). To study transgene expression in wound healing, the ears of transgenic animals were punched under light anesthesia and analyzed 7 days later; a spontaneous wound in the back skin of a transgenic mouse was also analyzed. Tumors were induced in the backs of 7-week-old female transgenic mice by a complete carcinogenesis protocol using 7,12-dimethylbenz[*a*]anthracene (31). Primary keratinocyte cultures were established from the dorsal skin of 10- to 12-day-old transgenic mice as described (32).

RESULTS AND DISCUSSION

Cell-Type-Specific Transgene Expression. The BK5 and BK6β genes are separated by ≈12 kbp (ref. 17 and Fig. 1*A*). An enhancer has been mapped between positions −830 and −183 upstream of the transcription start point, which when coupled to the thymidine kinase promoter, confers cell-type-specific expression to a chloramphenicol acetyltransferase reporter gene in transient transfection experiments (24). To study the *in vivo* activity of this enhancer, a similar construct replacing the chloramphenicol acetyltransferase reporter with a *lacZ* gene was used to generate transgenic mice [construct BK6β(EX)TK.Z, Fig. 1*B*]. Five founder mice were obtained, and lines were derived from three of them. In β-gal histochemical staining, the five founders and the animals of each line analyzed were negative for LacZ expression, although weak staining of scattered hair follicle cells was observed in some (results not shown). A region thus known to function as a cell-type-specific enhancer *in vitro* was inactive in transgenic mice. Therefore, another transgene was engineered that contained the BK6β locus from positions −8.8 to +9 kbp with respect to the transcription start point (construct BK6β.Z, Fig. 1*C*). Since the *lacZ* gene carries stop codons, translation of the generated mRNA does not proceed into the downstream K6β sequences and there is no fusion at the β-gal C-terminal end; therefore, the protein produced contains only 18 keratin amino acids at its N-terminal end. Three transgenic lines were obtained and analyzed. Transgene expression was reduced to a few scattered cells in hair follicles and oral epithelia, and the animals were considered as negative (results not shown).

In a third construct, named BK6β(−8.8).Z (Fig. 1*D*), the BK6β sequences after the *lacZ*/simian virus 40 poly(A) reporter gene in BK6β.Z were absent. Four founder mice and the corresponding lines were obtained. Three lines (containing 5–50 transgene copies) showed expression in several stratified epithelia and were used for subsequent analysis. The activity of BK6β(−8.8).Z, compared with the inactivity of BK6β.Z, suggests the presence of negative regulatory elements downstream of the transcription initiation region in the BK6β locus, although the existence of posttranscriptional mechanisms explaining this lack of expression cannot be ruled out.

Table 1 summarizes and Fig. 2 gives examples of the expression pattern observed in the three BK6β(−8.8).Z transgenic lines. β-gal activity was present in the innermost cells of the outer root sheath of hair and whisker follicles (Fig. 2*A*) and in the suprabasal cells of a variety of stratified epithelia of adult animals but not in interfollicular epidermis, following the established K6 expression pattern (1, 5, 6). Nonepithelial or simple epithelial tissues did not show β-gal activity.

In back skin, some mice showed scattered positive cells or small groups of cells (results not shown). Since the transgene can be induced in skin by hyperproliferative conditions (see below), this focal expression could be due to the healing of small wounds produced by scratching. The lack of transgene expression observed in plantar skin coincides with data demonstrating that, contrary to humans (5) and bovids (17), mice do not express K6 in this skin area (34). The bovine transgene thus appears to obey the endogenous murine K6 (MK6) regulatory mechanism. In tail skin, transgene expression was restricted to the area where the hairs emerge at the interscalar orthokeratotic regions and was not observed at the scalar parakeratotic regions (Fig. 3*A*). However, some orthokeratotic regions did not express the transgene, resulting in a mosaic expression pattern. In hair follicles, depending on the hair

Table 1. Tissue-specific expression of BK6β(−8.8).Z in transgenic mice

Tissue	Expression
Back skin	±
Tail skin (orthokeratotic)	+
Tail skin (parakeratotic)	−
Foot sole epidermis	−
Hair follicles	+
Oral mucosa	+
Palate	+
Esophagus	±
Stomach	+*
Tongue	+
Reproductive epithelia	
Vagina	+
Cervix	+
Uterus	−
Other tissues†	−

±, Few stained cells in some mice; −, no stained cells; +, more than 25% of stained cells in most mice.

*Epithelium of the transitional region between forestomach and glandular stomach.

†Muscle, brain, kidney, liver, colon, duodenum, spleen, pancreas, lung, and urinary bladder were tested. One line showed ectopic expression in some brain cells and in endothelial cells.

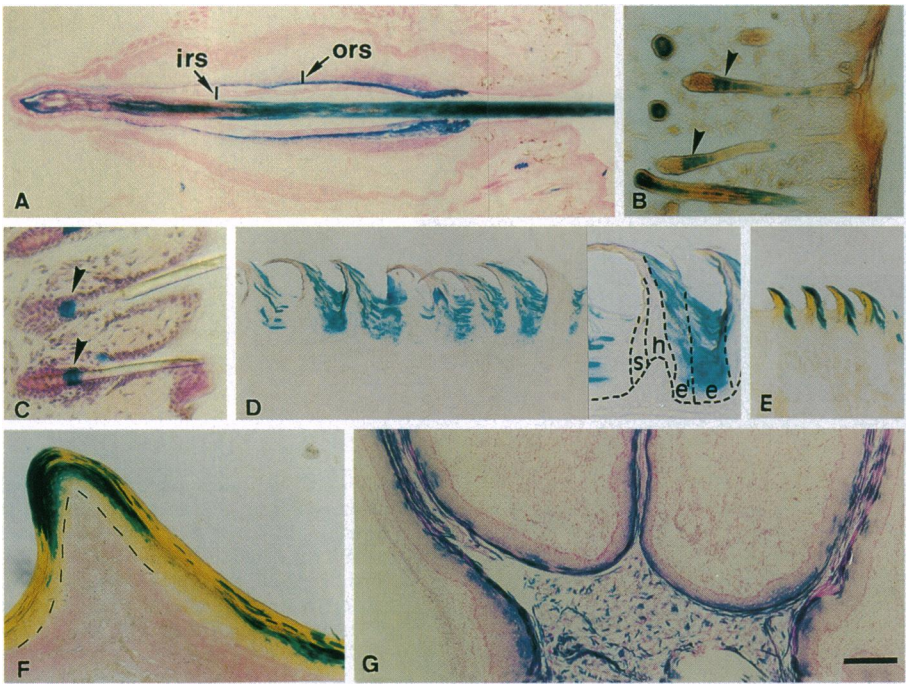

FIG. 2. Epithelium-specific expression of BK6β(−8.8).Z. β-gal was detected histochemically with X-Gal. (*A–C*) Expression pattern in hair follicles. (*A*) Anagen vibrissa from a 12-day-old animal. irs and ors indicate the inner and the outer root sheath, respectively. (*B*) Early-anagen hair follicles from the back of a 3-day-old animal. (*C*) Telogen follicles from the back of an adult animal. (*D* and *E*) Posterior and anterior tongue filiform papillae, respectively. (*D Inset*) Higher magnification of a papilla divided in compartments (33). (*F*) Palate. Dashed line represents the basement membrane. (*G*) Vaginal and cervical epithelia. (Bar: *A* and *D–F*, 120 μm; *D Inset*, 60 μm; *B* and *C*, 75 μm; *G*, 300 μm.)

growth phase, expression was found at locations other than the outer root sheath. In telogen, the neck hair (where the outer root sheath is being extruded) was positive (Fig. 2*C*, arrowheads), and in early anagen, staining was seen at the keratogenous zone of the shaft (Fig. 2*B*, arrowheads), which is a region of cellular proliferation during active hair growth. This is the cause of the β-gal activity present in some hairs (e.g., Fig. 2*A*).

Mouse tongue epithelium expresses different keratins in a compartmentalized manner (33, 35). In BK6β(−8.8).Z transgenic mice, although some variability was observed, filiform papillae showed β-gal activity at the orthokeratinized anterior unit (compartment e′ of ref. 33; Fig. 2*D*). This compartment-specific staining was more conspicuous in the papillae near the tip of the tongue, where staining of the parakeratinized posterior units was never observed (Fig. 2*E*). The interpapillary epithelium presented an anterior-to-posterior tongue staining gradient. In anterior tongue, these regions were negative (Fig. 2*E*); in central tongue, they were increasingly positive, reaching the same intensity as the papillae in posterior tongue (Fig. 2*D*). Therefore, the BK6β(−8.8).Z transgene expression pattern agrees with the division of tongue into different structural and functional compartments and also with the probable existence of different stem cells colonizing these compartments (33, 35–37). In tail and tongue epithelia, this transgene was expressed only at the orthokeratinized regions, contrary to a 65-kDa/48-kDa keratin pair described as being associated with parakeratotic differentiation in these tissues (35). The β-gal staining of other internal stratified epithelia was also suprabasal and mosaic. This was more apparent in palate, cervix, and vagina, where stacks of stained cells extending upward to the surface were frequently seen (Fig. 2 *F* and *G*). Whether these stacks represent the presume clonal organization of these tissues, as argued for other transgenes (38), remains to be determined.

Although transgene expression was clearly suprabasal in all positive stratified epithelia examined, it was frequently observed that the first suprabasal layers were negative and, in some instances, expression started closely below the stratum corneum. This appears to be at variance with the reported presence of K6 in all suprabasal layers in human and mouse hyperproliferative skin (9, 39). An explanation for this discrepancy could reside in the existence of more than one K6 gene in humans, bovids, and mice that could be subject to differential expression. Alternatively, the BK6β(−8.8).Z transgene may lack regulatory elements responsible for expression in all suprabasal layers.

Epidermal Transgene Induction. Prolonged treatment of mouse tail skin with RA leads to hyperplasia and changes the differentiation of the scalar parakeratotic regions to orthokeratotic; concomitantly, K6 is induced (12). When transgenic mice were treated with RA, the three BK6β(−8.8).Z-expressing lines clearly showed induced transgene activity in epidermis, whereas the line that did not express it constitutively also failed to induce the transgene. In hair follicles, expression was markedly induced above its constitutive expression level (compare Fig. 3 *A* and *B*). The induced expression level was variable between lines, as judged by X-Gal staining intensity, but was continuous along the epidermis. As occurred in other stratified epithelia (Fig. 2), only suprabasal cells and frequently only the most superficial of them were stained (Fig. 3*B*), whereas the endogenous K6 protein was induced in all cell layers (Fig. 3*C*).

Repetitive treatment of tail epidermis with the tumor promoter PMA also leads to skin hyperplasia and K6 induction, although it does not alter the characteristic alternating orthoparakeratotic differentiation pattern (12). Treatment of transgenic mice from the three expressing lines led to a clear induction of BK6β(−8.8)Z in suprabasal epidermis of the orthokeratinized interscale regions and in hair follicles (compare Fig. 3 *A* and *D*). At variance with this pattern, the endogenous K6 induction is continuous along the living epidermal layers (Fig. 3*E*). The results concerning hyperproliferative epidermis and other epithelia above mentioned suggest that transgene expression is restricted to orthokeratinized epithelial regions. The difference with the endogenous K6 protein, which is also present in parakeratotic regions, could be due, as already discussed, to (*i*) the lack of regulatory elements in the transgene or (*ii*) the existence of different K6 genes that could be differentially expressed in mammals.

Since K6 is also induced in wound healing, in epidermal tumors, and in *in vitro* skin keratinocyte cultures (8–11, 22), we studied the transgene induction in these situations. BK6β(−8.8)Z was induced in suprabasal cells of hyperproliferative epidermis involved in wound healing processes, as shown in Fig. 3 *F* and *G* for ear and back skin, respectively. As can be seen, the skin not affected by wounding was not stained. The transgene was also induced in suprabasal cells of back skin

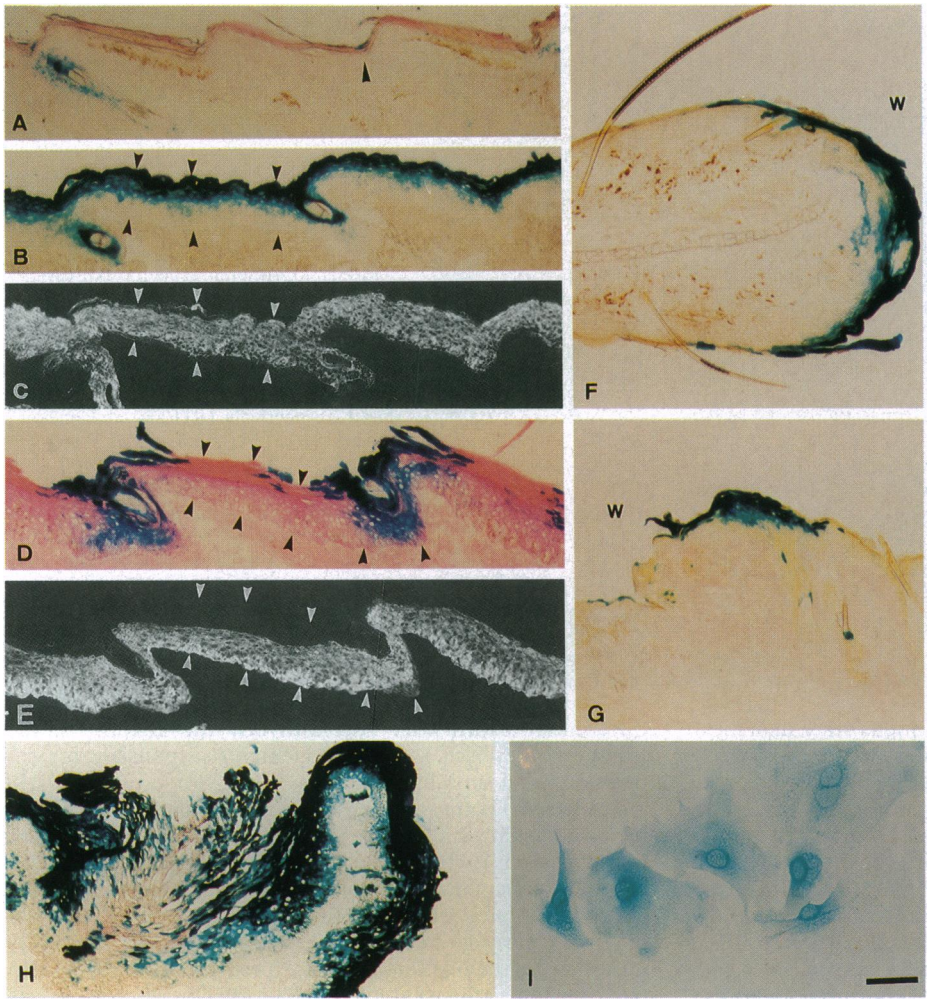

FIG. 3. Hyperproliferation-associated expression of BK6β(−8.8).Z transgene in skin. (*A*) BK6β(−8.8).Z expression in tail skin is restricted to hair follicles and some orthokeratinized regions (arrowhead). After treatment with RA (*B* and *C*) or PMA (*D* and *E*), transgene (*B* and *D*) and endogenous K6 (*C* and *E*) expression is induced. β-gal was detected histochemically with X-Gal and endogenous K6 by immunofluorescence with a specific antiserum. Note in *D* that transgene expression is restricted to orthokeratotic regions. Arrowheads in *B–E* delimit the epidermis. (*F* and *G*) Transgene expression in wound healing in ear and back skin, respectively. The wounded region is labeled w. (*H*) Transgene expression in a chemically produced skin papilloma. (*I*) Expression in a primary culture of transgenic skin keratinocytes. (Bar: *A–G*, 120 μm; *H*, 140 μm; *I*, 70 μm.)

papillomas (Fig. 3*H*) and in primary skin keratinocyte cultures (Fig. 3*I*). Therefore, the 9-kbp fragment of the BK6β locus included in this transgene contains regulatory elements that respond to physiologic, pathologic, and chemical hyperproliferative stimuli.

Kinetic Studies of K6 and BK6β(−8.8).Z Transgene Induction. To study the transgene induction process and to determine whether it parallels the behavior of the MK6 gene, both genes were compared in tail skin of animals treated with RA for various time periods (Fig. 4). In transgenic animals, increased transgene expression was already detected in hair follicles and interscale regions at treatment day 3 (compare Figs. 4*A* and 3*A*) and was conspicuous at day 5 (Fig. 4*B*). At day 7, some scale regions had lost their parakeratotic stratum corneum and showed transgene expression (Fig. 4*C*). At day 9, the skin has lost its interscale organization, and the epidermis is orthokeratinized and continuously blue-stained (Fig. 4*D*).

The kinetics of MK6 induction was analyzed by using a Northern blot of total tail skin RNA from treated animals. MK6 RNA levels increased continuously with treatment, reaching a maximum at day 11; as was observed with the transgene, this increase was detectable at treatment day 3 and conspicuous at day 5 (Fig. 4*E*). The same filter was hybridized with a MK5-specific probe to assess the RNA amount loaded in each lane (Fig. 4*F*). MK6 mRNA induction in skin thus

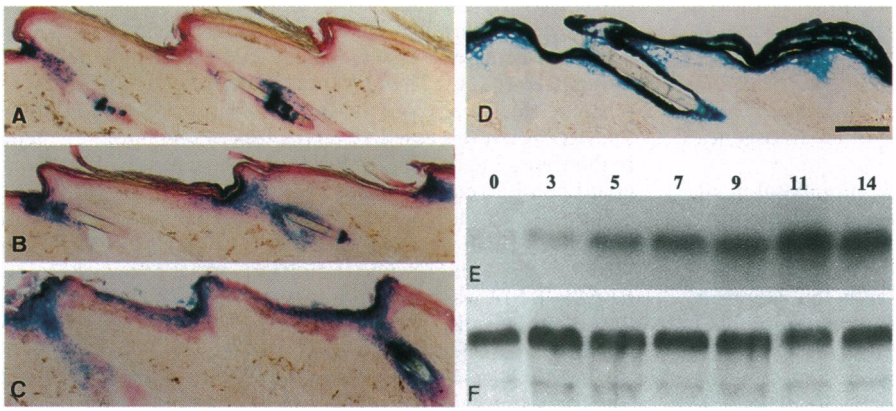

FIG. 4. Comparison of the kinetics of MK6 and BK6β(−8.8).Z induction in tail skin subjected to daily RA treatment for various times. (*A–D*) Histochemical detection of β-gal activity in tail skin of transgenic animals treated for 3, 5, 7, and 9 days, respectively. (Bar = 120 μm.) (*E* and *F*) Northern blot of total skin RNA of animals treated with RA as indicated (in days) and hybridized with the following probes: *E*, a K6-specific 377-bp *Eco*RI–*Hae* III fragment from the 3′ untranslated region of the MK6 region (22); *F*, a K5-specific 800-bp *Pst* I fragment spanning the eighth exon of MK5.

begins shortly after RA treatment and the timing of transgene induction parallels that of the endogenous MK6 gene at the mRNA level. However, Schweizer *et al.* (12) have shown, by one-dimensional protein gel electrophoresis in similar experiments, that MK6 protein is not induced in interfollicular epidermis until day 8 of RA treatment. Whether this discrepancy is due to the different experimental approaches used or to posttranscriptional control of MK6 synthesis remains to be elucidated.

In summary, the 9-kbp fragment of the BK6β locus included in the BK6β(−8.8).Z transgene contains sufficient information to provide both tissue-specific and inducible transgene expression similar to that of endogenous MK6.

Expression in oral mucosa and whisker follicles of a BMP-4 gene cloned under the control of BK6β in a 21-kbp vector containing a minilocus with the linked BK5 and BK6β genes has been reported (40). Our results, however, indicate that the 9 kbp used here are sufficient to mimic K6 expression. We expect that the characteristics of the regulatory region described will be of utility in targeting the expression of genes of biomedical or biotechnological interest to stratified epithelia. In particular, this region's expression in response to hyperproliferative stimuli could be of interest in designing gene therapy protocols for diverse epidermal hyperproliferative situations such as tumors, wound healing, or psoriasis, where the corresponding genetic construct not only will be activated but also will be automatically switched off when the epidermis returns to a normal state of proliferation/differentiation. Its easy skin induction in response to topical treatment with RA or PMA should be of utility in targeting to skin gene products in a spatially and temporally controlled manner.

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