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Genetics

Spatio-temporally controlled site-specific somatic mutagenesis in the mouse

(tamoxifen-inducible gene targeting/Cre recombinase/epidermis)

JACQUES BROCARD, XAVIER WAROT, OLIVIA WENDLING, NADIA MESSADDEQ, JEAN-LUC VONESCH, PIERRE CHAMBON*, AND DANIEL METZGER

Institut de Génétique et de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Université Louis Pasteur, Collège de France, BP 163, 67404 Illkirch-Cedex, Communauté Urbaine de Strasbourg, France

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ABSTRACT **The efficient introduction of somatic mutations in a given gene, at a given time, in a specific cell type will facilitate studies of gene function and the generation of animal models for human diseases. We have shown previously that conditional recombination–excision between two *loxP* sites can be achieved in mice by using the Cre recombinase fused to a mutated ligand binding domain of the human estrogen receptor (Cre-ER^T), which binds tamoxifen but not estrogens. DNA excision was induced in a number of tissues after administration of tamoxifen to transgenic mice expressing Cre-ER^T under the control of the cytomegalovirus promoter. However, the efficiency of excision varied between tissues, and the highest level (≈40%) was obtained in the skin. To determine the efficiency of excision mediated by Cre-ER^T in a given cell type, we have now crossed Cre-ER^T-expressing mice with reporter mice in which expression of *Escherichia coli* β-galactosidase can be induced through Cre-mediated recombination. The efficiency and kinetics of this recombination were analyzed at the cellular level in the epidermis of 6- to 8-week-old double transgenic mice. We show that site-specific excision occurred within a few days of tamoxifen treatment in essentially all epidermis cells expressing Cre-ER^T. These results indicate that cell-specific expression of Cre-ER^T in transgenic mice can be used for efficient tamoxifen-dependent, Cre-mediated recombination at loci containing *loxP* sites to generate site-specific somatic mutations in a spatio-temporally controlled manner.**

Homologous recombination in embryonic stem cells and transgenesis have made it possible to specifically target and mutate any genetic element in the germ line of mice (1, 2). By using these techniques, it is now possible to generate, more or less at will, mutations that result in gene inactivation (knock out), overexpression, or more discrete predetermined gene changes, which are all useful to assess physiological and pathological gene functions. For instance, mutations aimed at mimicking those observed in inherited or acquired diseases have been engineered in the germ line of mice to generate models of human diseases. There are, however, some serious limitations to this strategy, which are essentially due to the introduction of gene modifications into the germ line. For instance, a germ line mutation can be lethal or cause complex pleiotropic effects, or, on the contrary, its effects could be fully compensated during development. In many instances, this will prevent the determination of the role of a given gene product in a defined subset of cells at a given time of the animal life and also preclude the establishment of mouse model systems for human diseases generated by somatic mutations (3, 4).

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To overcome these limitations, strategies for conditional gene targeting in mice, based on cell type-specific or inducible expression of the bacteriophage P1 Cre recombinase, recently have been developed (refs. 5–10 and references therein). Indeed, this Cre recombinase efficiently can excise a DNA segment flanked by two *loxP* sites (floxed DNA) in animal cells (5). Placing the Cre gene under the control of either a cell-specific (6–8) or an inducible (9) promoter can lead, through the excision of the floxed DNA segment, to either spatially or temporally controlled somatic mutations, respectively. However, these conditional gene targeting systems for generating somatic mutations have also a number of limitations because they are only spatially or temporally controlled. Ideally, one would like to have a system that allows generation of somatic mutations in a defined gene at a given time of the life of the animal and in a specific cell type. To that end, we have generated a conditional tamoxifen-inducible Cre recombinase by fusing it with the ligand binding domain of the human estrogen receptor (Cre-ER^T) that binds tamoxifen but not estrogens (11, 12). We have shown that a floxed DNA segment can be excised in a number of tissues after administration of tamoxifen to transgenic mice expressing Cre-ER^T under the control of the human cytomegalovirus (CMV) promoter (12). The efficiency of excision was variable from one tissue to another, and the highest excision level was achieved in skin. This variability was ascribed to different levels of Cre-ER^T expression in different tissues (12, 13). This raised the question as to whether different cell types within a given tissue might differentially express Cre-ER^T, causing the excision of floxed DNA to be restricted to certain cell types in which its efficiency might reach 100% (12).

We have analyzed the efficiency and kinetics of floxed DNA excision in the skin epidermis of 6- to 8-week-old double transgenic mice that carry both the CMV promoter Cre-ER^T transgene and the Cre recombinase reporter transgene (14) present in the chicken β-actin promoter *loxP*-CAT-*loxP*-lacZ (ACZL) transgenic line (10). We show that, upon tamoxifen administration to the double transgenic mice, excision of floxed DNA occurs in essentially all cells expressing the Cre-ER^T and therefore that cell-specific expression of Cre-ER^T in transgenic mice can be used to efficiently generate site-specific somatic mutations in a spatio-temporally controlled manner in mouse.

MATERIALS AND METHODS

Mice. The CMV–Cre-ER^T (12) transgenic mouse line was crossed with the ACZL reporter line (10) to generate double

Abbreviations: ACZL, chicken β-actin promoter *loxP*-CAT-*loxP*-lacZ; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; Cre-ER^T, fusion protein between the Cre recombinase and a mutated ligand-binding domain of the human estrogen receptor (G521R); DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

*To whom reprint requests should be addressed. e-mail: igbmc@igbmc.u-strasbg.fr.

heterozygous CMV–Cre-ER^T/ACZL transgenic mice. Genotyping was performed by PCR as described (12). For PCR detection of the ACZL transgene, the lacZ primers 5′-CGCCGACGGCACGCTGATTG-3′ and 5′-GTTTCAATATTGGCTTCATC-3′ were used. Administration (i.p.) of tamoxifen (1 mg in 100 μl of sunflower oil) was as indicated in the legends to the figures.

Immunohistochemistry and Histochemistry. Tail biopsies were embedded in OCT medium (Sakura Finetek Europe B.V., Zoeterwoude, Netherlands), immediately frozen on dry ice, and sectioned. Cre immunohistochemistry was performed on 10 μm-thick longitudinal sections mounted on gelatin-coated slides. Sections were incubated in PBT (0.1% Triton X-100 in PBS) containing 5% normal goat serum (Sigma) for 1 h at room temperature. A 1/10 dilution of purified rabbit polyclonal anti-Cre antibody (D.M., unpublished results) was applied to the slides for 2 h at 21°C. After 5 washes in PBT (10 min each), sections were incubated for 2 h at 21°C with a donkey anti-rabbit antibody coupled to the CY3 fluorochrome (Jackson ImmunoResearch) at a 1/500 dilution. Slides were washed 5 × 10 min in PBT, and medium for fluorescence (Vectashield, Vector Laboratories) containing 0.01% DAPI (4′, 6-diamidino-2-phenylindole dihydrochloride; Sigma) was applied. Pictures were taken on a Leica (Deerfield, IL) TSD 4 D confocal microscope.

For β-galactosidase histochemistry, 30 μm-thick longitudinal sections were stained with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) as described (ref. 15 and references therein), dehydrated through an increasing series of ethanol concentrations (70%, 90%, and 100%; 2 × 10 min each), embedded in epoxy (Epon 812), cut with an ultra-microtome (Reichert Ultracuts, Leica) in 2-μm semi-thin sections, and counterstained with safranine.

RESULTS

We have focused here on skin because our previous study had shown that it exhibited the highest excision level of a floxed DNA segment upon tamoxifen treatment of transgenic mice expressing Cre-ER^T under the control of the CMV promoter (CMV-Cre-ER^T; see ref. 12) and because it is an heterogeneous multilayered tissue that is comprised of the dermis and the epidermis. The epidermis itself is made up of several stratified layers of increasingly differentiated keratinocytes (Fig. 1). The proliferative basal keratinocytes (basal layer) are attached to the basement membrane that separates the dermis and epidermis. Differentiation of basal keratinocytes is coordinated with vertical migration into the next layer, known as the spinous layer; further differentiation accompanied by further vertical migration results in the granular layer that, with the spinous layer, constitutes the suprabasal layers. Continued differentiation is accompanied by further vertical migration yielding a terminally differentiated keratinocyte, the corneocyte or squame, which is present in the most superficial epidermal layer, the cornified layer or stratum corneum. Squames are lost daily from the surface of the skin, so the epidermis is a very dynamic tissue (reviewed in ref. 16).

To analyze the efficiency of excision of a floxed DNA segment by Cre-ER^T at the cellular level, CMV-Cre-ER^T transgenic mice were crossed with the ACZL mouse reporter line (10). This line carries the chicken β-actin promoter *loxP*-chloramphenicol acetyltransferase (CAT) cassette-*loxP*-lacZ cassette transgene (14). β-galactosidase cannot be expressed from this transgene unless the CAT cassette is excised. Thus, β-galactosidase cannot be expressed in Cre-ER^T/ACZL double heterozygous animals unless Cre-mediated excision of the floxed CAT cassette has occurred.

To reveal the expression of Cre-ER^T in the skin of Cre-ER^T/ACZL mice, we performed immunohistochemistry on tail sections by using an anti-Cre rabbit polyclonal antibody and confocal microscopy. No Cre-ER^T expressing keratinocytes could be detected in the proliferative basal layer, and essentially all Cre-positive cells were located in the granular component of the suprabasal layers (Fig. 2 *b* and *b′*), whereas as expected no significant staining was observed on sections of wild-type mouse skin (Fig. 2 *a* and *a′*). Interestingly, labeling keratinocyte nuclei with DAPI clearly showed that the Cre-ER^T protein was essentially cytoplasmic in the absence of tamoxifen treatment (DAY 0, Fig. 2 *b* and *b′*). In marked contrast, it became progressively nuclear upon tamoxifen administration (see DAY 1, Fig. 2 *c* and *c′*) and was almost completely nuclear after 3 days of tamoxifen treatment (see DAY 3, Fig. 2 *d* and *d′*; the white color of the granular layer nuclei results from the superimposition of the red color of the anti-Cre signal and the cyan color of the DAPI staining). The translocation of Cre-ER^T from the cytoplasm to the nucleus of granular cells was clearly tamoxifen-dependent because Cre-ER^T was again mostly cytoplasmic when skin sections were examined 3 days (DAY 7, Fig. 2 *e* and *e′*) or 6 days (DAY 10, Fig. 2 *f* and *f′*) after the last tamoxifen administration (day 4).

Cre-ER^T/ACZL double heterozygote mice then were examined for induction of Cre-mediated excision upon tamoxifen treatment. To this end, skin sections were stained for β-galactosidase activity (X-Gal staining). Important to note, no X-Gal staining could be detected in the skin of untreated mice (Fig. 3*a*, DAY 0) whereas some staining, which was restricted to the granular layer, was visible after 1 day of treatment (Fig. 3*b*, DAY 1). As expected, this staining was cytoplasmic because β-galactosidase has no nuclear localization signal. All cells of the granular layer were stained clearly after 2–3 days of tamoxifen treatment (Fig. 3 *c* and *d*, DAYS 2 and 3), and, as expected, the intensity of the X-Gal staining

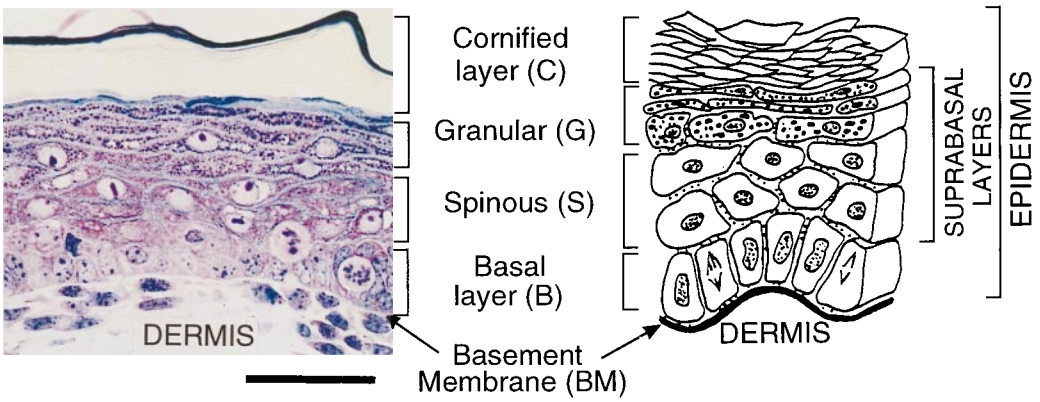

FIG. 1. Epidermal stratification of mouse skin. Toluidine blue-stained, 2-μm, semi-thin section of newborn dorsal skin. A diagrammatic representation is given on the right side. Note that mitosis is restricted to the proliferative basal cells. (Bar = 25 μm.)

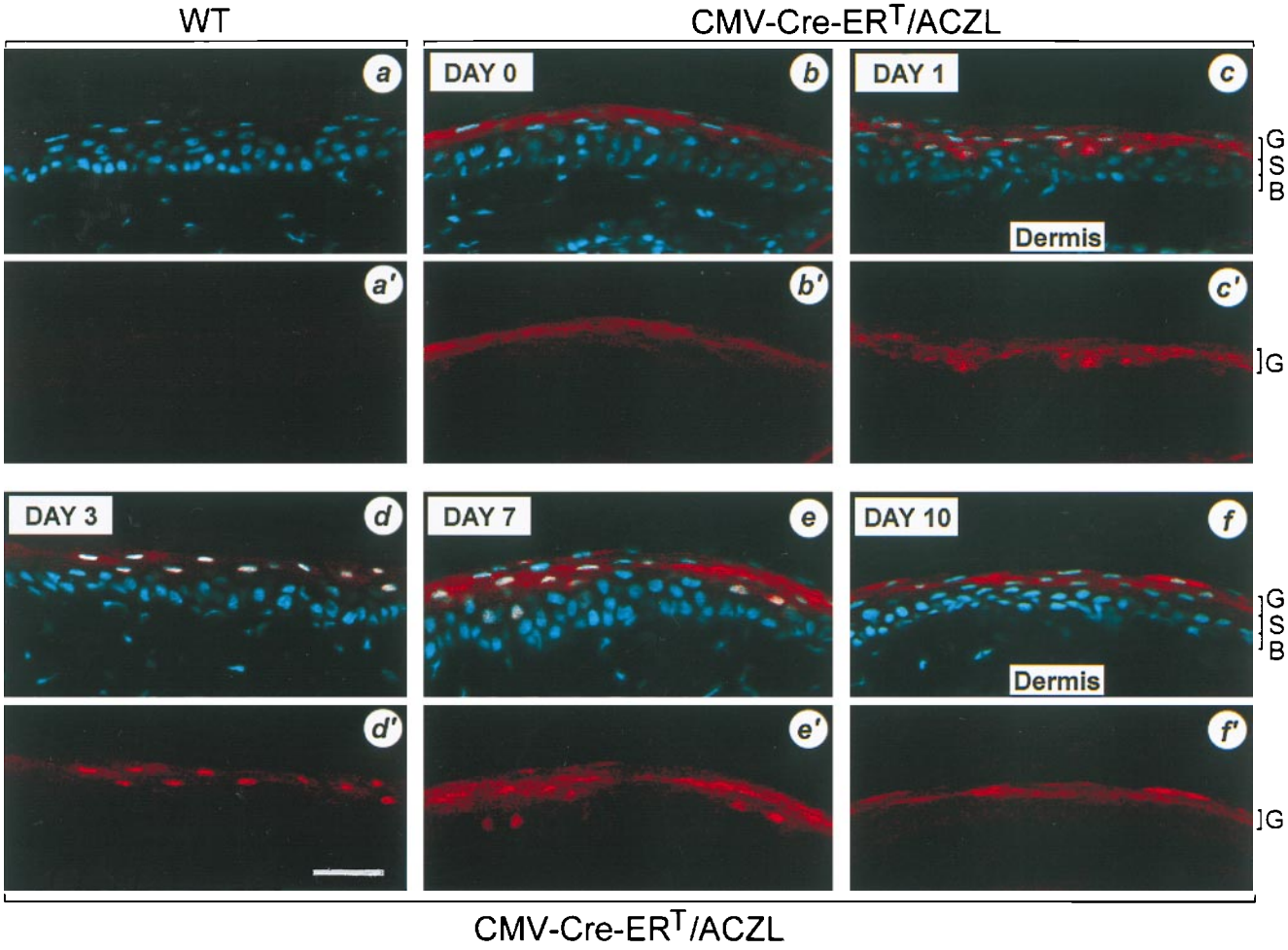

FIG. 2. Pattern of Cre-ER^T expression in the tail epidermis of double transgenic mice. Immunohistochemistry with anti-Cre antibody was performed on sections (10 μm-thick) of tail biopsies of 6- to 8-week-old wild-type (WT; *a*, *a'*) and CMV-Cre-ER^T/ACZL double heterozygous transgenic mice (*b–f*, *b'–f'*). Double transgenic mice were injected for 5 consecutive days (days 0–4) with tamoxifen (1 mg/day). Sections were stained with DAPI and anti-Cre antibody. DAY 0 (*b*, *b'*), before the first tamoxifen injection; DAY 1 (*c*, *c'*) and DAY 3 (*d*, *d'*), after 1 and 3 days of tamoxifen treatment, respectively; DAY 7 (*e*, *e'*) and DAY 10 (*f*, *f'*), 3 and 6 days after the last tamoxifen injection, respectively. The cyan color corresponds to the DAPI staining (*a–f*), and the red color corresponds to the staining of Cre-ER^T (*a–f*, *a'–f'*). B, S, and G, basal, spinous, and granular layers, respectively (see Fig. 1). (Bar = 25 μm.)

increased with time (Fig. 3 *b–f* and data not shown). It is important to note that, under various conditions of tamoxifen treatment, the expression of β-galactosidase always was restricted to the granular layer where Cre-ER^T expression was specifically detected.

If, as indicated by the above results, excision of the floxed CAT cassette does not occur in the proliferative basal keratinocytes, one can expect that the X-Gal staining will progressively disappear from the granular layer and move into the cornified layer. This prediction was supported by further examining, at later times, the CMV-Cre-ER^T/ACZL mice that were initially treated for 5 days (days 0–4) with tamoxifen (Fig. 4). Three days later (day 7), the X-Gal staining indeed had migrated vertically into the upper part of the granular layer, as well as into the lower part of the cornified layer, whereas very little X-Gal staining was left at day 10 in the lower part, and almost none was left at day 15 in the upper part of the cornified layer.

The observation that the newly differentiated granular cells were not X-Gal-stained indicates that no Cre-mediated recombination had occurred in the proliferative basal keratinocytes or, alternatively, that, for unknown reasons, the tamoxifen treatment could have silenced the expression of the lacZ gene. This latter possibility is clearly ruled out by the reappearance of X-Gal staining in the granular layer of tail biopsies of CMV-Cre-ER^T/ACZL mice to which tamoxifen was readministered between days 25 and 29 (Fig. 4*f*).

DISCUSSION

We have shown previously that the tamoxifen-inducible Cre-ER^T recombinase expressed under the control of the CMV promoter can be used in transgenic mice to excise a floxed DNA segment in various tissues (12). However, the efficiency of the tamoxifen-induced excision was very variable from one tissue to another, being efficient ($\approx$40%) in the skin and very low in other tissues. This variability tentatively was ascribed to variations in the level of expression of the CMV-Cre-ER^T. We speculated that, in the case of skin that contains multiple cell types, the activity of the CMV promoter could be cell-specific (13) and therefore the Cre-ER^T expression restricted to certain cell types in which recombination would be very efficient (12). The present study fully supports this assumption and demonstrates that cell-specific expression of Cre-ER^T can be used in adult mice to generate spatio-temporally controlled, site-specific excision mutagenesis upon tamoxifen treatment.

The Cre-ER^T system appears particularly attractive because, in the presence of tamoxifen, recombination occurs in all cells expressing the fusion protein whereas none could be detected in the absence of tamoxifen. The present observation that

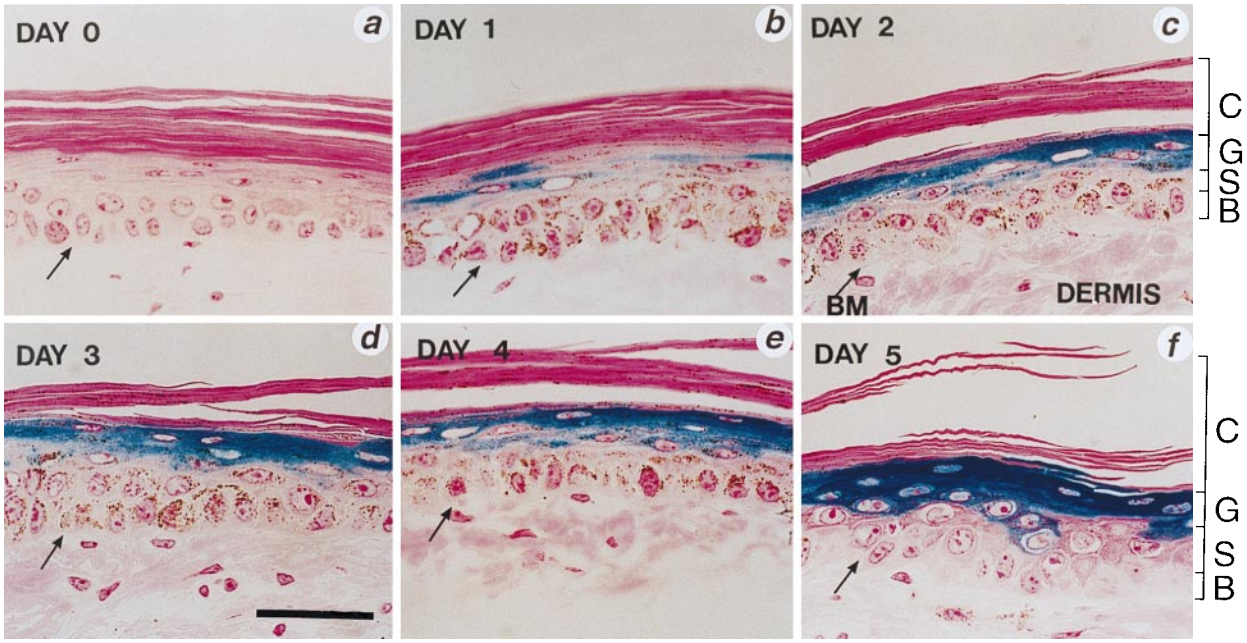

FIG. 3. Kinetics of β-galactosidase expression in tail epidermis granular layer of CMV-Cre-ER^T/ACZL double heterozygous mice. CMV-Cre-ER^T/ACZL double heterozygous mice (6–8 weeks old) were injected daily with tamoxifen from day 0 to 4 (see legend to Fig. 2). Tail biopsies were collected just before the first tamoxifen injection (DAY 0, *a*) and at DAYS 1–5 (*b–f*) and treated as described in *Materials and Methods* (2-μm, semi-thin sections). Arrows point to the basement membrane (BM). B, S, G, and C, basal, spinous, granular, and cornified layers, respectively (see Fig. 1). (Bar = 25 μm.)

tamoxifen is required for the translocation of the unliganded cytoplasmic Cre-ER^T to the nucleus may account at least in part for this very low background of recombination in the absence of tamoxifen. The excision kinetics only can be estimated indirectly from the β-galactosidase activity. At least some recombination must take place within the first 24 h after tamoxifen injection (Fig. 3). This is also the time at which some Cre-ER^T has been translocated into the nucleus although complete translocation may take 2–3 days of tamoxifen treatment (Fig. 2). It appears therefore that the intracellular level of tamoxifen could be a more limiting factor than the amount of expressed Cre-ER^T. In any event, our data indicate that a tamoxifen treatment for 3 days is sufficient to excise the floxed CAT cassette in all epidermis granular cells. It is worth noting that we could not detect (data not shown) the expression of Cre-ER^T in the granular layer of our present transgenic mice by using the Cre antibody that enabled Tsien *et al.* (7) to detect the Cre protein expressed under the α-calcium–calmodulin-dependent kinase II promoter in the brain of their transgenic mice. It appears therefore that it should be possible to use the

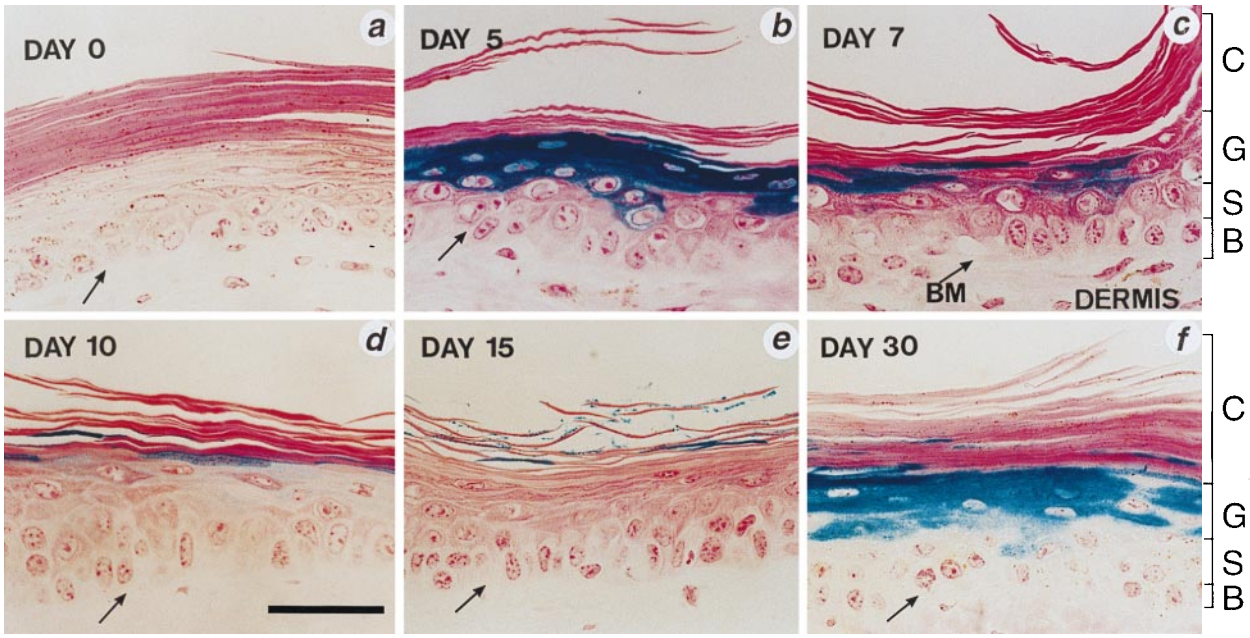

FIG. 4. Vertical migration of granular layer-restricted β-galactosidase expression in tail epidermis of CMV-Cre-ER^T/ACZL double heterozygous mice. Two series of daily tamoxifen injection, from day 0 to 4 and from day 25 to 29, were administered to 6- to 8-week-old CMV-Cre-ER^T/ACZL double heterozygous mice. Tail biopsies were collected just before the first tamoxifen injection (DAY 0, *a*) and at different days after the first injection, as indicated (*b–f*). BM arrows, B, S, G, and C are as defined in legend to Fig. 3. (Bar = 25 μm.)

conditional Cre-ER^T expressed under cell-specific promoters to excise floxed DNA segment in a spatio-temporally controlled manner. Among other possibilities, the tamoxifen-inducible Cre-ER^T system will be useful in combination with homologous recombination to generate cell type-restricted null mutations in adult mice in a temporally controlled manner.

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