Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 2809–2813, April 1992
Developmental Biology

Expression of the mouse tyrosinase gene during embryonic development: Recapitulation of the temporal regulation in transgenic mice

(melanocytes/retinal pigment epithelium/tyrosinase/transgenic mice/*in situ* hybridization)

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Communicated by Wolfgang Beermann, December 18, 1991

ABSTRACT **Pigment in mammals is produced in melanocytes of the skin derived from the neural crest and in the pigmented epithelial cells of the retina derived from the optic cup. Tyrosinase (monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1) is regarded as the key enzyme in pigment synthesis in both these cell types. In this study, we have investigated the temporal regulation of expression of the tyrosinase gene during early eye development and in the developing hair follicle of the mouse by *in situ* hybridization and have asked whether transgenes would precisely mimick this pattern. We show that the mouse tyrosinase gene is expressed in the pigment epithelium of the retina as early as day 10.5 of gestation. In the hair follicle, tyrosinase gene expression in melanocytes is detected from day 16.5 onwards. This cell type-specific and temporal expression is largely reproduced in transgenic mice carrying a tyrosinase minigene. Our results suggest that sequences in the immediate vicinity of the mouse tyrosinase gene are sufficient to provide cell-type specificity and developmental regulation in melanocytes and in the pigment epithelium.**

As illustrated by the analysis of the albino mutation, in particular by rescue of the albino phenotype in transgenic animals, the tyrosinase gene (monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1) is essential for pigmentation in skin and eyes (1–7). Pigment-producing cells stem from two different lineages: the pigment epithelium of the retina is derived from the outer wall of the optic cup, whereas melanocytes in the hair follicle and the choroid are of neural crest origin (1, 8). The mouse tyrosinase gene and its regulatory sequences have been isolated (5, 6). Tyrosinase minigenes under control of up to 5.5 kilobases (kb) of upstream sequences have been shown to direct expression to pigmented cell types and, furthermore, to restore pigmentation in albino mice (2–4, 9). In addition, the simian virus 40 early region under control of 2.5 kb of tyrosinase upstream sequence led to tumor formation of pigmented cell types (10, 11). By *in situ* analysis, we recently showed that as little as 270 base pairs (bp) of 5′-flanking sequence are sufficient for specific expression of the transgene in the pigmented cells of both lineages (9).

Development of the mouse eye begins with evagination of the optic vesicle from the forebrain at about day 8–9 of gestation. The lens placode is then formed, and at about 10–10.5 days, the lens rudiment and the optic vesicle together invaginate to become the lens cup and the optic cup. The inner and thicker wall of the optic cup will become the neuroretina, whereas the outer wall, which remains rather thin, generates the pigment epithelium. The first pigment in the retinal region appears around day 11–11.5 of gestation (12).

Pigmentation starts later in the skin. At the time of neural crest migration (day 8.5–12 of gestation), those cells giving rise to melanoblasts take a dorsolateral pathway around the periphery of the embryo after having left the neural tube. They migrate through the dermal mesoderm underlying the epidermis and invade the epidermis and colonize the skin and hair follicles, where they differentiate to melanocytes. At day 16–18, melanocytes enter the hair follicle as it develops from an invagination of the epidermis into the underlying dermal tissue and secrete melanin granules into the growing hair (8, 12).

We are interested in the regulation of expression from the mouse tyrosinase gene. We have analyzed its developmental profile of expression before birth by *in situ* hybridization and have asked whether expression is concomitant with melanin synthesis (1, 12). We have restricted our study to pigment cells of the eye and skin and show that expression is established in the retina on day 10.5 and is established in melanocytes within the hair follicle on day 16.5.

We then asked whether this temporal expression pattern was reproduced in transgenic mice that carried tyrosinase promoter sequences previously shown to be sufficient to establish cell type-specific expression in the newborn and the adult (2, 9). The developmental expression pattern from the tyrosinase minigenes largely followed that of the endogenous gene in melanocytes in skin and hair follicle and coincided with the onset of tyrosinase gene expression in the pigment epithelium of the eye.

MATERIALS AND METHODS

Animals. NMRI/Han mice carrying the albino mutation (*c*) were used for the experiments. This technique excludes interference of pigment granules with the hybridization signal. It has been shown recently that the albino mutation (*c*) represents a point mutation of the tyrosinase gene (4, 7, 13). Expression of the gene is not affected and does not differ from the wild-type allele (2, 9, 13, 14; unpublished observation).

The transgenic mice (generated on a NMRI/Han background) carried tyrosinase minigenes that contained either 5.5 kb (ptrTyr4; ref. 2) or 270 bp (ptrTyr5; refs. 9 and 15) of 5′-flanking sequence and the coding sequence of mouse tyrosinase interrupted by the first intron. A simian virus 40 splice and polyadenylylation signal is included at the 3′ end of the constructs and enabled us to detect transgene-specific transcription. Cell lines 18 and 34 obtained with ptrTyr4 contain ≈10 and 5 copies of the transgene (2), and the cell lines harboring the shorter construct ptrTyr5 (9, 15) carry about 10 (cell line 334) or 20 copies of the transgene (cell lines 331 and 353).

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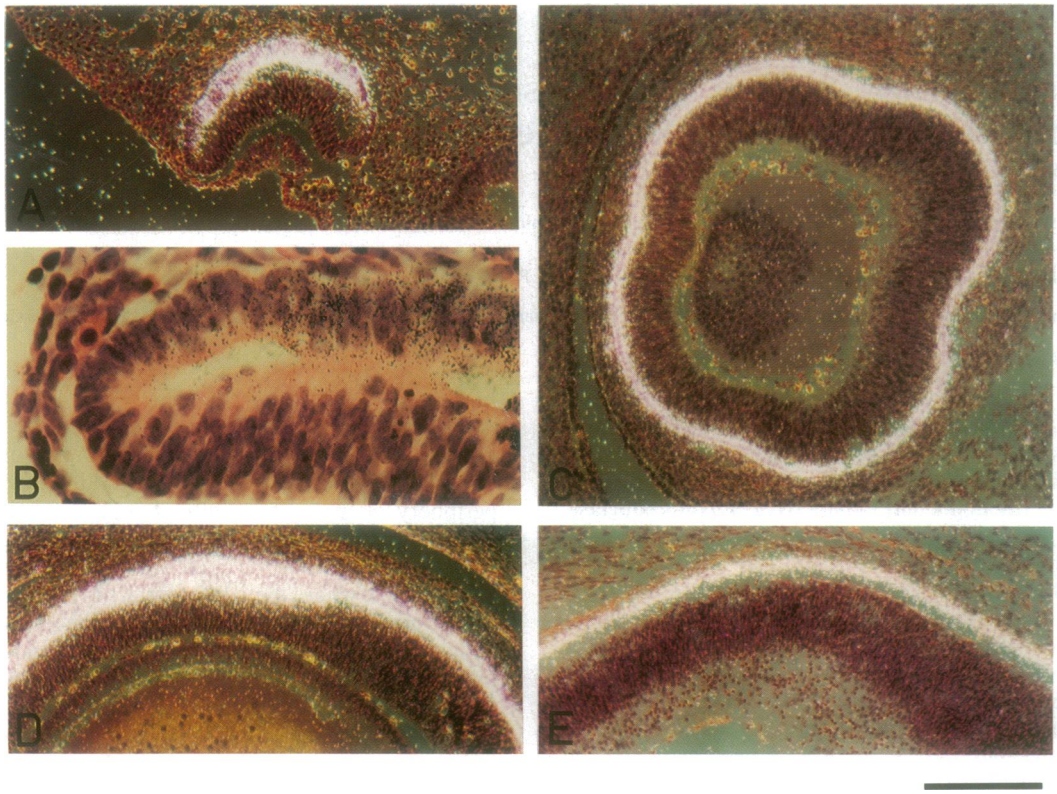

FIG. 1. Tyrosinase gene expression in early eye development. Sections are derived from embryos at day 10.5 (*A*), day 12.5 (*C*), day 14.5 (*D*), and day 16.5 (*E*) and are shown as dark-field photographs. Enlargement of hybridized section of optic cup of day 10.5 embryo (*A*) is shown as bright-field photograph (*B*). (Bar = 150 μm in *A*, *C–E*, and 40 μm in *B*.)

Mouse embryos were derived from matings of nontransgenic NMRI/Han females to either NMRI/Han males ortransgenic males (both hemizygous and homozygous). The day of vaginal plug was counted as day 0.5 of gestation. After mating

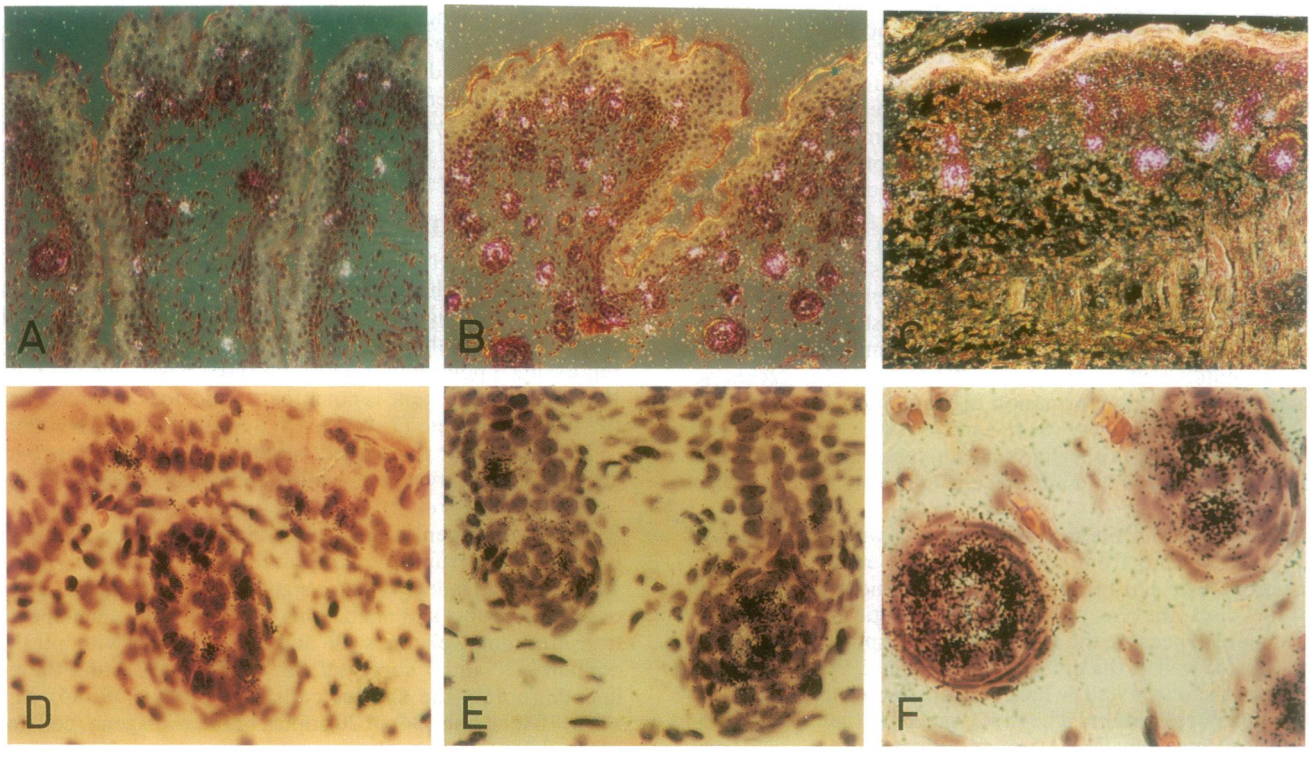

FIG. 2. Tyrosinase gene expression in melanocytes of developing hair follicle. Hybridization of tyrosinase probe to melanocytes in skin and in hair follicle is demonstrated for day 16.5 (*A* and *D*), day 17.5 (*B* and *E*), and day 18.5 (*C* and *F*) of gestation. *A–C* are dark-field and *D–F* are bright-field photographs. (Bar = 150 μm in *A*; 200 μm in *B* and *C*, and 40 μm in *D–F*.)

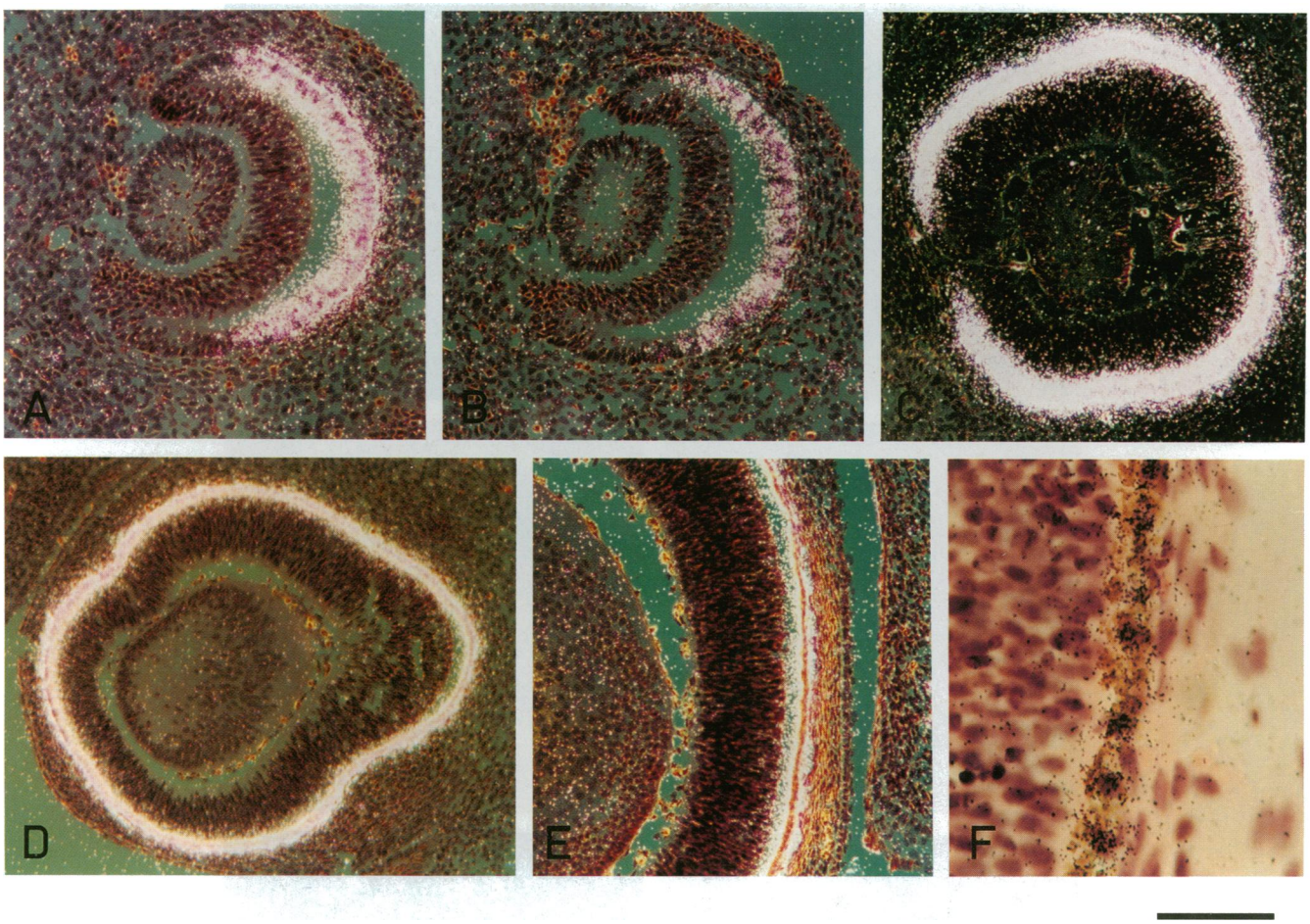

FIG. 3. Expression from ptrTyr4 coincides with that of endogenous tyrosinase gene in eye. Sections are derived from embryos at 10.5 (*A* and *B*), 11.5 (*C*), 12.5 (*D*), 14.5 (*E*), and 16.5 (*F*) days of gestation and hybridized to transgene-specific probe pSV-H (*B–F*). In *A*, a section adjacent to that of *B* was hybridized with tyrosinase probe pmcTyr54. (Bar = 120 μm in *A–C* and *E*, 150 μm in *D*, and 30 μm in *F*.)

of females with hemizygous transgenic males, transgenic embryos were detected by Southern blots of DNA prepared from placentas (16) or by eye pigmentation. Embryos were collected each day from day 9.5 or 10.5 to date of birth.

***In Situ* Hybridization.** Either whole embryos (up to 15.5 days of gestation) or skin and eyes (16.5 days of gestation and older) were isolated, washed in phosphate-buffered saline, and fixed in 4% paraformaldehyde. *In situ* hybridizations were carried out on paraffin-embedded tissue sections essentially as described (17, 18) by using ^{35}S-labeled RNA probes generated from pmcTyr54 and pSV-H (2, 9). After 1–3 weeks of exposure, the sections were developed with Kodak D19 solution and stained with hematoxylin/eosin. Microscopical analysis and photography were done by using both bright-field and dark-field optics from Zeiss (Oberkochen, F.R.G.).

RESULTS AND DISCUSSION

Expression of Tyrosinase Gene During Early Eye Development. At about day 8–9, eye development starts with the

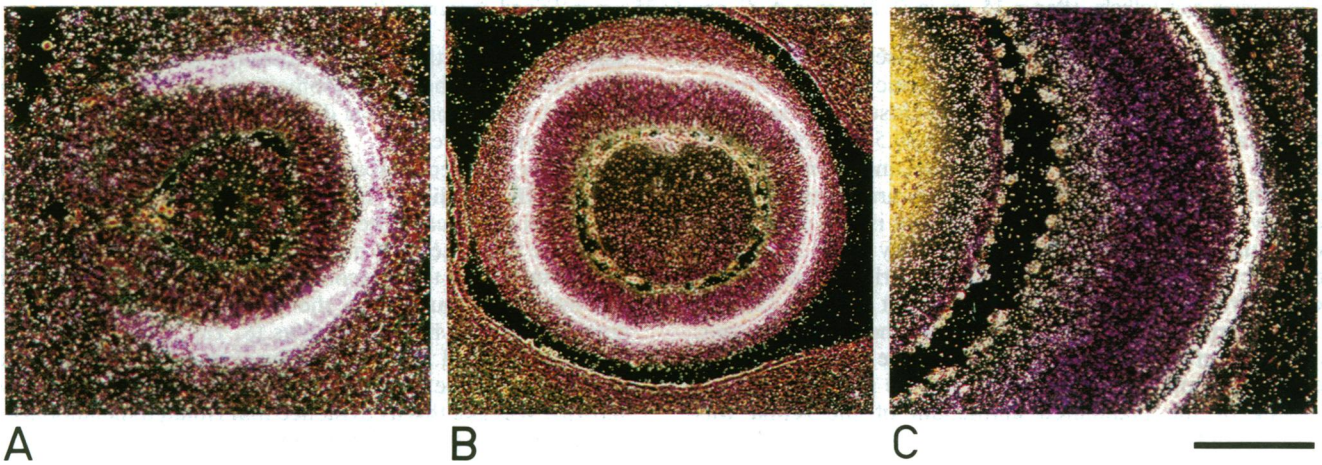

FIG. 4. The 270 bp of 5′ sequence are sufficient for temporal regulation in pigment epithelium. Dark-field photographs show expression from ptrTyr5 at day 10.5 (*A*), day 13.5 (*B*), and day 17.5 (*C*) of gestation. (Bar = 120 μm in *A*, 200 μm in *B*, and 150 μm in *C*.)

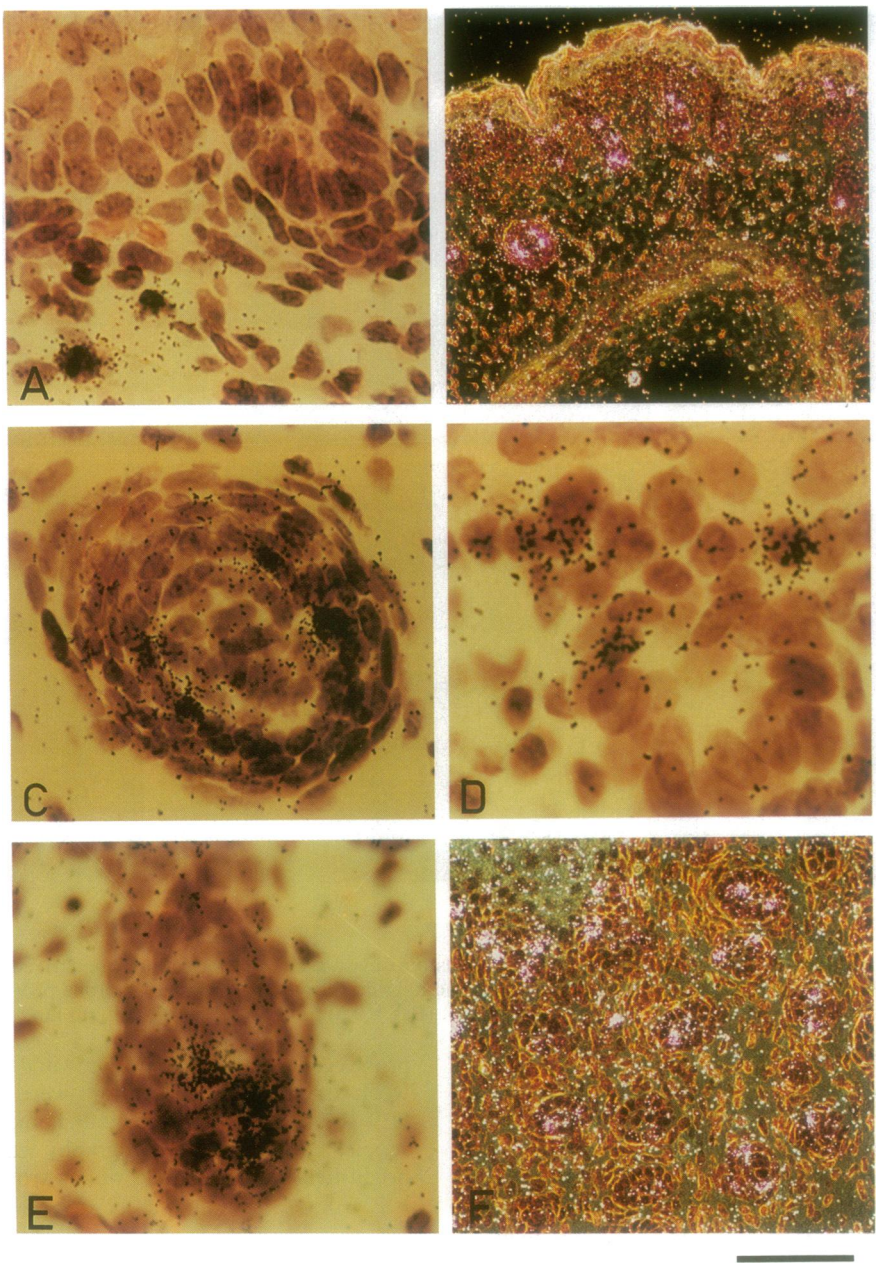

FIG. 5. Transgene-specific expression in melanocytes of developing hair follicle (ptrTyr4, 5.5 kb). Sections derived from day 16.5 (*A*), day 17.5 (*B–D*), and day 18.5 (*E* and *F*) embryos were hybridized to pSV-H and show specific labeling of melanocytes located in dermis, epidermis, or developing hair follicle. (Bar = 15 μm in *D*; 25 μm in *A*, *C*, and *E*; 75 μm in *F*; and 150 μm in *B*.)

appearance of the optic vesicle, which arises from the neural ectodermal wall of the forebrain. The optic vesicle converts to the optic cup, which then begins to close and first comes into contact with the ectoderm at day 10.5 (12, 19). At this stage the prospective pigment epithelium and the neuroretina are prominent (Fig. 1). These two layers are continuous and are derived from neuroectoderm. Pigment is first detected in the presumptive pigment epithelium at day 11–11.5 (12, 19). No transcripts were detected by *in situ* hybridization on serial sections from a day 9.5 embryo (data not shown). On day 12.5, the lens becomes separated from the epidermis and, during further development of the eye, the sensory layer of the retina becomes much thicker than the pigmented layer. Labeling of the pigmented epithelium of the retina was detected continuously from day 10.5 onwards (Fig. 1). No qualitative changes in expression were apparent, although the intensity of the hybridization signal seemed weaker in older stages (e.g., at day 16.5 of gestation, see Fig. 1*E*). The data suggest that the mouse tyrosinase gene is transcriptionally active throughout development of the pigment epithelium and is already expressed at day 10.5, before the first pigment appears in the eye.

Melanocyte-Specific Expression of Tyrosinase Gene in Developing Hair Follicle. The first melanoblasts migrating from the neural crest reach the skin by 12 days of gestation. They penetrate the basement membrane and enter the epidermal ectoderm, where they become incorporated into developing hair follicles. Around birth, the melanocytes secrete melanin granules into the hair as it grows (8, 12).

At day 12.5–13.5, we found the first melanocytes, which express tyrosinase mRNA in the region of the eyelid (data not shown). We take this as evidence that isolated melanoblasts/melanocytes express the tyrosinase gene and can be identified before they produce detectable amounts of pigment. We focused our further interest on the developing hair follicle and took sections from the back skin of embryos of day 16.5 of

gestation and older. At day 16.5 of gestation, melanocytes expressing tyrosinase mRNA were detected in both dermis and epidermis but were rarely detected in the developing hair follicles (Fig. 2 *A* and *D* shows its presence). The number of cells containing tyrosinase mRNA increased from day 16.5 to 17.5, at the same time as which increased numbers of melanocytes appear to enter the hair follicle. The hair follicles then differentiate rapidly and at day 17.5 (Fig. 2 *B* and *E*) and day 18.5 (Fig. 2 *C* and *F*) of gestation almost all hair follicles contain expressing melanocytes. Thus, melanocytes seem to express the gene either before or as soon as they enter the hair follicle, long before melanin is produced and secreted into the growing hair that emerges only after birth (8, 12).

Spatial and Temporal Developmental Patterns of Expression Are Reproduced in Transgenic Mice. Transgenic mice carrying tyrosinase minigenes with either 5.5 kb (ptrTyr4) or 270 bp (ptrTyr5) of 5′-flanking sequence have been produced and shown to express the transgene in a cell type-specific manner after birth (in 4-day-old mice)—i.e., in the pigment epithelium of the retina and in melanocytes of the hair follicle and the choroid (2, 9). We now asked whether these transgenes display the temporal pattern of expression of the endogenous gene before birth.

At day 10.5 of gestation, transgene-specific transcripts are detected in the developing pigmented epithelium of embryos transgenic for ptrTyr4 (Fig. 3*B*) and ptrTyr5 (Fig. 4*A*). These transcripts were detected in both ptrTyr4 lines analyzed but were detected in only one of two lines obtained with ptrTyr5. Expression in the other ptrTyr5 line was detected at day 11.5 in the pigmented epithelium. Transcription from both constructs was detected continuously throughout subsequent embryonic development (Figs. 3 and 4). No transgene-specific transcripts were detected in the eye region in serial sections of a day 9.5 embryo (line 18, ptrTyr4), a stage at which expression of the endogenous gene was also not seen (data not shown).

Transgene-specific expression in single melanocytes is already detected at day 13.5–14.5 in sections of the region around the eye from animals carrying ptrTyr4 but is detected first at day 16.5–17.5 in animals generated with the short construct (data not shown). At day 16.5, melanocytes expressing ptrTyr4 are only rarely seen within developing hair follicles or the epidermis and are restricted mainly to the dermis (Fig. 5*A*). Expression of the ptrTyr4 transgene is much more pronounced in melanocytes of developing hair follicles at day 17.5 and 18.5 (Fig. 5). In contrast to both the endogenous gene and ptrTyr4, expression of the shorter construct is first detected in melanocytes of the hair follicle at day 18.5 but becomes prominent only after birth (data not shown) (9).

In conclusion, cell type-specific and temporal expression of the endogenous gene seems to be reproduced when the transgene contains 5.5 kb of the 5′-flanking sequence. In mice carrying the construct with less flanking DNA, the transgene is expressed in a cell type-specific manner but with an apparent delay, especially in skin melanocytes. The fact that expression of this transgene did not exactly correspond to that of the endogenous gene may reflect a quantitative difference, which may be further exacerbated by the different lengths of the transgene-specific and the tyrosinase-specific RNA probes rather than a real temporal difference. Transient transfections of tyrosinase chloramphenicol acetyltransferase (CAT) fusion genes into melanoma cells have shown a higher level of expression for longer constructs (9). In these experiments, 6.1 kb of tyrosinase 5′ sequence resulted in a 3- to 4-fold higher chloramphenicol acetyltransferase activity in comparison to a construct containing only 270 bp (see figure 1 in ref. 9).

Our results have demonstrated that as little as 270 bp of upstream sequence gives cell type-specific expression and faithful temporal regulation. This finding restricts the search for the responsible cis elements to a very defined region of the tyrosinase promoter.

We thank Drs. E. Hummler and G. Kelsey for critical reading of the manuscript, Drs. P. Dollé and D. Duboule for technical advice with the *in situ* hybridization, Drs. E. Hummler and S. Ruppert for continuous support of the project, and C. Schneider for secretarial assistance. The work was supported by the Deutsche Forschungsgemeinschaft through SFB 229 and the Leibniz Programm and the Fonds der Chemischen Industrie.

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