Clonal coat color variation due to a transforming gene expressed in melanocytes of transgenic mice
(simian virus 40 large tumor antigen/tyrosinase promoter/pigmentary patterns/melanocyte development/phenoclonies)

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ABSTRACT
Transgenic mice of an inbred black strain were previously produced with the Tyr-SV40E transgene, comprising simian virus 40 transforming sequences driven by the tyrosinase promoter, in order to obtain melanomas; the animals were found to be lighter than normal in coat color, to various degrees. As described here, hypopigmentation resulted from diminished differentiation of melanized pigment granules in the melanocytes of the hair bulbs in vivo and occurred autonomously in cultured melanocytes. Whereas some of the mice had single-color coats, most (7/13) had coats of two or three colors; in addition, one single-color founder produced a two-color descendant. These eight mice had patterns seen in natural genotypes; the most striking were transversely striped to various extents, with regions of left–right asymmetry on either side of the dorsal midline. The patterns visualized the same clonal developmental territories of coat melanocytes displayed in allogenic mice that are formed from conjoined early embryo cells of different color genotypes. Some of the Tyr-SV40E transgenics were also cellular genotypic mosaics, probably arising by late integration of the transgene. However, one transgenic founder with a completely striped coat proved to be true-breeding, with autosomal inheritance of the pattern. The inherited striped pattern thus exemplifies the formation of phenotypically different but genetically identical developmental clones, or phenoclonies, among cells of the same type. This line of transgenic mice provides exceptional material for experimental analysis of the molecular basis for clonal variation in gene expression and of the fate of oncogenic phenoclonies of melanocytes occurring in the same individual.

Transgenic mice were recently described that are prone to melanoma in the eyes and skin (1) and to other tumors associated with melanosis (2). The melanomas arose through expression of the transgene in pigment cells, as expected, due to its tyrosinase gene promoter controlling expression of the simian virus 40 (SV40) early region transforming sequences, including the large tumor antigen (T antigen). Reduced coat pigmentation occurred, to varying degrees, reflecting the relative numbers of transgene copies in many but not all cases. As reported here, the light coat colors were found to arise from subnormal and sporadic melanization of pigment granules in the melanocytes of the hair bulbs. This result is consistent with incomplete differentiation of the cells and may have arisen either by impairment of their maturation or by reinforcement of their proliferative potential (3), rather than by a direct effect of the transgene on melanin synthesis.

Given the fact that many single-gene mutations in mice lead to multicolored coats (4), one would expect that any transgene capable of affecting color would also produce some multicolored individuals. This could occur by differential reduction of pigment in various areas of the coat of animals from genetically black eggs, as proved to be the case in the majority of mice in the present study. It could also occur by differential induction of pigment in parts of the coat of mice from genetically albino eggs, as was the case in an unrelated series injected with another transgene (B.M. and M.B., unpublished data).

In natural genotypes of mice with a coat color pattern due to gene expression in melanocytes, the patterns are ideal or modified versions of the clonal developmental history of melanoblasts, in which separate clones may be of different colors or color intensities. This conclusion was drawn from models in allogenic mice experimentally produced from conjoined early embryo cells of dissimilar color genotypes (5–8). Melanoblasts originating in the neural crest (9), in predetermined numbers, migrate dorsoventrally on each side and form transverse mitotic clones (5). In the allogenic animals, clonal color differences are due to genotypic differences (10). This is not the case in nonexperimental mice of natural genotypes. Among the latter, single-allele activation per cell, and per clone, explains the patterns in X chromosome-linked heterozygotes (11). Among other genotypes, the autosomal homozygotes are the most baffling. Their patterns are apparently due to phenoclonies—phenotypically different but genetically identical clones in which the same gene must be yielding more than one kind or amount of product in mitotic lineages of the same cell type (6–8). The phenomenon is presumably not limited to pigment cells.

We report here that the multicolored patterns in our Tyr-SV40E transgenic mice resemble those previously seen in allogenic mice and further confirm the developmental lineages of melanocytes. Some of the transgenic cases are clearly due to genetic mosaicism, possibly resulting from late integration of the transgene. At least one autosomal transgenic case is clearly an example of phenoclonies and thus provides unique material for analyses of gene expression and of susceptibility to melanomas.

MATERIALS AND METHODS

The group of transgenic mice previously described (1) provided the material for this study. The animals were produced by injecting Tyr-SV40E recombinant DNA into fertilized eggs of the C57BL/6 (black nonagouti) inbred strain. The fusion construct contains the SV40 early region genes, including the coding sequences of the transforming large tumor (T) and small tumor (t) antigens, under the control of 2.35 kilobases (kb) of mouse tyrosinase gene promoter sequence.

Positive animals were identified, and the copy number of the transgene determined, by Southern blot analysis of DNA from tail biopsies, tissue samples collected at autopsy, or skin melanocytes grown in culture as described (12–14). Genomic DNA was digested with Pst I to determine whether single or multiple integration of the transgene had occurred and to

Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen.
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distinguish between head-to-tail and tail-to-tail integration. The restriction fragments were resolved by gel electrophoresis, transferred to Nytran (Schleicher & Schuell), and hybridized with a SV40 T-antigen-specific RNA probe. Known amounts of the donor gene were used as a standard. Founder animals, which were hemizygous for the transgene, were in some cases mated to C57BL/6 controls.

RESULTS

Of the 13 original Tg(Tyr-SV40E) mice in the series, 6 had single-color coats, ranging from a light black or dark brownish-grey to pale grey; these were the founders of lines 5 and 9–13. The remaining 7, the founders or potential founders of lines 1–4 and 6–8, had coats of two colors (black or blackish-grey, two shades of grey, or grey and white) or three colors (black, grey, and white, or two shades of grey plus white). In addition, a new two-color case arose spontaneously in a descendant of the all-grey line 5; her coat had two shades of grey and she is included here as the potential founder of line 5a.

These eight multicolored cases are grouped in Table 1 according to three general classes representing different, or partly different, developmental modes of origin to which their patterns are here ascribed. The three groups are as follows: striped patterns with white, striped patterns without white, and headspot patterns. The cases will be individually characterized in the order in which they are listed.

**Striped Patterns with White (Lines 1–3).** The original mice designated as line 1 (a male) and line 2 (a female) were not previously reported; they were severely retarded and were sacrificed at 3–4 weeks of age. The line 3 animal was a sterile male with undescended testes and aspermia; his growth was somewhat retarded and he was killed at 15 weeks of age because of advanced metastatic ocular melanoma (1). Multiple copies of the transgene, from approximately 15 to 25, were present in these mice (Table 1). Integration had occurred in multiple sites, thus augmenting the chances for insertional mutagenesis. Each founder had tandem copies of the transgene oriented head-to-tail; tail-to-tail array may have occurred at some sites in lines 1 and 2 (data not shown).

The mouse of line 1, shown in Fig. 1A, had a medium grey coat with a “belt” consisting of a light grey stripe on each side, meeting at an angle at the dorsal midline; a faint transverse grey stripe at the level of the left shoulder; and a light grey stripe behind the right ear and on the left front face. The mouse of line 2 (Fig. 1B) was black with a grey stripe on the left side, flanked by two small white spots; and grey near the left ear and front of the face. The line 3 mouse (Fig. 1C) was pale grey with a sharp white stripe on each side, asymmetrically placed; he had ruby eyes. All three mice had a large white belly spot, merging in some places with the grey or white transverse stripes already noted, on one or both sides; and white feet and tail tip. A more detailed Southern blot analysis was carried out on DNAs of various tissues from the line 2 mouse, including skin, kidney, pancreas, spleen, thymus, brain, and tail. The results revealed genotypic mosaicism in these tissues, as

![Fig. 1. The coats of transgenic mice of lines 1, 2, and 3 display a few transverse stripes against a darker background. (A) The line 1 mouse was medium grey and had a "belt" of two light grey stripes, one on each side, meeting in the dorsal midline and merging ventrally with a large white belly spot; a barely visible transverse stripe at the level of the left shoulder; a light grey stripe behind the right ear and on the left front face; and white feet and tail tip. (B) The line 2 mouse had a black background with a prominent grey stripe on the left side, flanked by two small white spots, and merging ventrally with a large white belly spot; grey areas near the left ear and front of the face; and white feet and tail tip. (C) The line 3 mouse was light grey and had a sharp white stripe on each side, placed asymmetrically; a white belly spot; and white feet and tail tip.](image)

Table 1. Coat color patterns in Tg(Tyr-SV40E) transgenic mice of the C57BL/6 inbred strain

<table>
<thead>
<tr>
<th>Line no.</th>
<th>Approximate no. of transgene copies in founder</th>
<th>Integration site(s)</th>
<th>Coat colors</th>
<th>Pattern inheritance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>Multiple</td>
<td>Two greys, white</td>
<td>Invisible; not bred</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>Multiple</td>
<td>Black, grey, white</td>
<td>Somatic genotypic mosaic; invisible; not bred</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>Multiple</td>
<td>Grey, white</td>
<td>Sterile</td>
</tr>
<tr>
<td>5a</td>
<td>6</td>
<td>Single</td>
<td>Two greys</td>
<td>Pattern appeared in line 5; not yet bred</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>Single</td>
<td>Black, grey</td>
<td>Somatic and germinal genotypic mosaic</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>Single</td>
<td>&quot;Black,&quot; grey*</td>
<td>True-breeding phenotypic mosaic</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>Single</td>
<td>Two greys†</td>
<td>True-breeding phenotypic mosaic</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>Single</td>
<td>Two greys†</td>
<td>True-breeding phenotypic mosaic</td>
</tr>
</tbody>
</table>

The C57BL/6 inbred strain is black nonagouti. Lines 1 and 2 have not previously been described. Tumors and some other features, but not the coat patterns, of lines 3–8 (excluding 5a) have been described (1, 2). Tandem copies of the transgene are arrayed head-to-tail in all lines; tail-to-tail array in some integration sites may have occurred in lines 1 and 2.

*The darker stripes are designated "black" because they may be slightly less than full color.
†These coats had a brownish grey cast.
documented by different proportions of hybridizing bands of higher molecular weight representing flanking sequences (data not shown).

Genetic mosaicism in the line 2 animal was also demonstrated in pigment cells of the coat. Hair bulb melanocytes from skin of the black area and of the grey stripe were separately explanted. The two cultures yielded black and grey cells, respectively (Fig. 2A); the latter had far fewer pigment granules (Fig. 2B). Southern blot hybridization of the DNAs indicated that the transgene, with the complete set of integrations, was present only in the grey cells (Fig. 2C). Cells from each of the cultures were then tested by immunohistochemistry for fluorescence with the monoclonal anti-T-antigen antibody, pAB 419 (a gift prepared by E. Harlow), together with tests of a known negative control culture (wild-type C57BL/6 melanocytes) and a known positive control (transformed fibroblasts producing SV40 T antigen). The positive staining in the nucleus of the grey cells and not of the black cells (data not shown) was consistent with transgene presence only in the grey cells and proved that the T-antigen gene was in fact expressed in those cells.

Striped Patterns Without White (Lines 5a, 8, and 4). The founder of line 5 was a nonpatterned light grey male (shown in figure 1 of ref. 2) with approximately six tandemized head-to-tail copies of the transgene integrated in a single site (data not shown). In matings to C57BL/6, he produced a number of all-grey offspring resembling himself. To our surprise, a striped female descendant recently arose in the fourth generation; she has been designated the potential founder of a new line, 5a (Table 1). Slightly paler grey transverse stripes (with a yellowish cast) were present on each side of the body, asymetrically. The Southern hybridization pattern of the tail DNA resembles that of its line 5 forebears (data not shown); this does not rule out the possibility of a small deletion or substitution in its DNA or of somatic mosaicism. The 5a mouse succumbed to melanoma, and her oocytes were grafted to the ovarian capsules of C57BL/6 nontransgenic hosts. Graft-derived progeny have not yet been obtained.

The founder of line 8 (a female) was dramatically striped in black and grey throughout the length of the head and body (Fig. 3A). However, matings with C57BL/6 yielded only all-grey transgenic and all-black nontransgenic offspring (Fig. 3B). Analysis of the DNAs disclosed that the founder’s transgene (comprising two copies at a single integration site) was present only in the grey progeny (Fig. 3C) and not in the black ones (data not shown). The founder was thus a germline as well as a somatic genotypic mosaic, most likely due to late integration of the transgene. The pervasiveness of the pattern suggests that integration nevertheless occurred at an early embryo stage. The all-grey hemizygous offspring have produced transgenic homozygotes; these are a lighter grey than the hemizygotes.

The female founder of line 4 was also transversely striped throughout the head and body (Fig. 4A) and bore a striking resemblance to the line 8 founder (Fig. 3A); the “black” component in this case is so designated because it may be slightly less than full-color. In contrast to line 8, the line 4 mouse proved to be true-breeding. All her transgenic offspring of both sexes (from matings to C57BL/6) were patterned in “black” and grey like the founder (Fig. 4A and B).

The same basic pattern was inherited, with individual epigenetic differences. Examples of individuality are seen in the specific locations of dark and light stripes and in the left–right asymmetries at the dorsal midline (Fig. 4B). In a Southern analysis, the hybridizing profile of the founder, who had

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**Fig. 2.** The grey and black coat areas of the line 2 mouse (shown in Fig. 1B) comprised separate genotypic populations of pigment cells, as determined by Southern analysis of DNAs from cultured skin melanocytes. (A) Suspensions of cultured pigment cells (8 × 10⁶ cells per ml) obtained from a black (Left) and a grey (Right) area. (B) Lightly pigmented cells in the culture from the grey area with pigment granules especially visible in some dendrites (phase contrast). (C) Southern blot analysis of DNAs from the cultured grey cells (g) and black cells (b) showing the transgene sequences present only in the grey cells.

**Fig. 3.** The striped founder of line 8 is shown with transmission and DNA evidence of genotypic cellular mosaicism. (A) The founder was completely patterned on head and body with black and grey transverse stripes. (B) Progeny (from matings with C57BL/6) were all-grey transgenics or all-black nontransgenics. (C) Southern blot analysis of DNA from the striped founder and three all-grey progeny shows the transgene present in the grey progeny. (It was absent in the all-black offspring; data not shown.) The 5.2-kb band results from head-to-tail tandem transgene copies; bands of higher molecular weight represent genomic flanking regions.
The striped founder of line 4 is shown with transmission and DNA evidence of true-breeding phenotypic mosaicism. (A) The founder is patterned throughout head and body with black and grey transverse stripes. (B) Transgenic progeny (from matings with C57BL/6) all have the same basic pattern as the founder, with individual differences. (C) Southern blot analysis of DNAs demonstrates inheritance of the same hybridizing profile in striped parent and offspring. The head-to-tail tandem transgene copies integrated in a single site yield the band at 5.2 kb, and the genomic flanking regions are contained within this signal.

approximately eight tandem copies of the transgene at one chromosomal site, continued to be stably transmitted to the transgenic offspring (Fig. 4C). Mating of a striped line 4 male with a C57BL/6 female yielded some striped males (as well as females), thus establishing that the transgene was not integrated on the X chromosome. Line 4 came close to extinction, due to early-onset malignant melanoma (1), and was rescued by grafting ovaries to C57BL/6 hosts, which then bore graft-derived transgens among their progeny. It is not yet known whether viable transgenic homozygotes can be derived.

Cell culture lines of the dark and the light skin melanocytes from line 4 have been established. Preliminary observations demonstrate, by immunofluorescence, much greater expression of the SV40 T antigen in the light cell cultures (L.L. and B.M., unpublished results).

Headspot Patterns (Lines 6 and 7). The founders of lines 6 and 7 (a male and female, respectively) were not striped. They had a slight overall reduction in coat color, with one or more small lighter (not white) areas visible, chiefly on the crown of the head in the position where a white headspot occurs in naturally white-spotted genotypes such as W/+ (4). This coat pattern persisted in the transgenic progeny (Fig. 5A). The founders of lines 6 and 7 each had approximately four transgene copies at a unique integration site (Fig. 5B) and the hybridization profile remained stable in their transgenic offspring (data not shown). Both lines have progressed to homozygosity. The homozygotes are lighter than the hemizygotes and have the same coat patterns.

Melanocytes in Skin and Hair. Plucked and mounted hair preparations, and histological sections of fur-bearing body skin, were examined microscopically from mice of many of the lines in order to define the differences between lightly colored, darkly colored, and white areas. In both lightly and darkly pigmented areas, melanocytes were localized in the hair bulbs and were present in numbers roughly comparable to C57BL/6 controls. However, in grey areas the melanocytes contained notably fewer pigment granules than in darker areas of the same mouse or in controls. Moreover, there was often a focal distribution of more melanized and less melanized cells or groups of cells within the same hair bulb (Fig. 6). The grey hairs themselves had far fewer pigment granules than normal, and light grey hairs had even fewer than dark grey hairs. There were occasional larger clumps of granules within a hair, with no detectable periodicity; these may have been contributed by the more heavily pigmented melanocytes in the hair bulb. No pigment cells or granules were seen in white skin and hairs.

A composite showing enlargements of typical melanocytes in hair bulbs of transgenic body skin from a grey-hair stripe. Melanocyte immaturity is evident from the subnormal numbers of pigment granules and small clumps of cells melanized to very different extents within the same hair bulb. (×230.)
The pattern of transverse stripes, independently on left and right sides, in the transgenic mice of lines 8, 5a, and 4 is indistinguishable from the basic pattern of coat melanocytes previously seen in allogeneic mice produced by aggregating early-embryo cells of different color strains (5). The exact patterns in transgenic lines 1, 2, and 3 are clearly partial versions of this pattern, with minor aberrations. The allogeneic patterns enabled the developmental history of coat melanocytes to be visualized. They led to the conclusion that the stripes that could be independently resolved represent clones, each mitotically descended from a single melanoblast. The separate identity of the stripes, and their unrelatedness to genotypes of cells in the skin, were demonstrated by means of strain-specific genetic markers (10). In the present study, the same clonal model of melanocyte development is literally applicable to the striped mice of transgenic lines 2 and 8, which are also cellular genetic mosaics, and possibly to lines 1 and 3, which have multiple sites of transgene integration. Genetic mosaicism in these transgenics arises, however, by late integration of the transgene at one or more chromosomal sites (or secondary loss from some cells).

What is particularly striking is that the founder of line 4, with the same clonal pattern as in the allogeneics, is not a genotypic mosaic; her striped pattern and transgene are faithfully transmitted to all transgenic progeny. The line 4 transgenics are thus phenotypic, but not genotypic, mosaics in which the same transgene is being expressed differently in some pigment cell clones than in others. Moreover, the variations in expression must have been present among the initiator cells of the coat melanoblast population in order for the specific phenotype to be clonally perpetuated. The color pattern in line 4 therefore exemplifies the formation of phenoclines, defined as phenotypically different clones among cells of the same type that correspond to the developmental lineages of which that type is comprised (6–8). As the transgene in line 4 is autosomal, it would not be expected to produce the pattern in hemizygotes by random activation only in some clones. If homozygotes are ultimately obtained in line 4 and are similarly patterned, this would argue against single-allele activity per cell as an explanation of two melanocyte phenotypes.

A pattern of light (grey or whitish) transverse stripes in a dark coat has also occasionally been observed in aging (6) and has been reported in viral infection (15). In the latter, high levels of maternally transmitted murine leukemia virus were found to be localized in cells of the grey stripes, apparently dating from the time the clones were established, and resulting in melanocyte dysfunction.

The skin and hair bulbs were examined histologically from the white stripes of line 3 and were found to lack melanocytes, as in naturally white-spotted genotypes (4). A possible explanation is that one of the multiple transgene integrations in this line (and in line 2) may have occurred late and caused the preprogrammed death of a clone or part of a clone (6) by insertional mutagenesis in a critical host locus, resulting in a white area.

Although the striped components of the patterns in lines 1, 2, and 3 must have been established early in the developmental origin of coat melanocytes from neural crest, the white belly spot, feet, and tail tip are likely to have resulted from later events in development. The lightly pigmented headspot in line 6 and line 7 is also best explained by a later event. As proposed elsewhere (6, 7), areas of late closure, such as the umbilicus and the anterior neuropore, may be devoid of melanoblasts, or may receive very few—producing a light belly spot and headspot, respectively—because the cells are by then less proliferative. Other, distally located, areas are at a similar disadvantage; these are the feet and tail tip.

Hypopigmentation in the grey clones clearly results from decreased melanin synthesis in the melanocytes of the hair bulbs, rather than from loss or migratory failure of the cells themselves, and is intrinsic to the melanocytes. This is evident from the approximately normal numbers of pigment cells, but reduced numbers of melanin granules in them, in the hair bulbs (Fig. 6). Reduction in melanin granules also occurs autonomously in the isolated cells in culture (Fig. 2B). Both eumelanin and phaeomelanin pigments are diminished, as observed in matings to C57BL/6-A/A as well as C57BL/6 (1).

The light phenotype of the affected transgenic cells is consistent with incomplete or blocked differentiation. Inasmuch as proliferative potential generally declines with terminal differentiation (3), these transgenic cells may still possess a greater capacity for proliferation than do fully differentiated melanocytes. It has been proposed that transformation occurs when cells such as melanocytes are moving toward differentiation and wrongly retain rather than decrease their proliferative activities (16). As this would predict, some of the Tg(Tyr-SV40E) transgenic mice have in fact already developed skin melanomas (1).

The true-breeding striped mice of line 4 provide an exceptional source of material with which to examine the molecular basis and consequences of clonal variation in gene expression. Melanocytes of the respective colors can be isolated and cultured, and the different expressions of the transgene can be characterized; as the transgene sequence is known, the flanking sequences at the single integration site can be isolated. The results may then furnish a model for the many naturally patterned autosomal genotypes at other loci, such as the chimera-mottled mutant at the tyrosinase locus, with the same basic coat color pattern. In addition, the fate of clones of genetically identical melanocytes with quantitatively or qualitatively different expressions of an oncogene, the T antigen, can now be followed under defined conditions in culture, and the role of systemic influences, or of therapeutic regimens, in oncosogenesis can be assayed on the two clonal phenotypes exposed to identical conditions in the organism.

Note Added in Proof. Putative homozygotes obtained in line 4 are patterned like the hemizygotes.

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