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Distribution of Melanocytes in Feather Germs of a Plumage Mutant *Bh* (Black at Hatch) Embryo of Japanese Quail (*Coturnix coturnix japonica*)

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ABSTRACT—Development and distribution of melanocytes were histologically examined with the aid of dopa reaction in the feather germs of *Bh* (black at hatch) quail embryos. In the feather germs of 10-day wild-type embryos, two types of melanocytes were observed; strongly dopa reaction-positive melanocytes (with black pigment) and moderately positive melanocytes (with brown pigment), which were located in the black parts and yellow parts of the feather germs, respectively. These melanocytes were arranged in a regular pattern in the barb ridges of whole feather germs. By contrast, in the heterozygotes, the distribution of the two types of melanocytes were intermingled in the correspondent to the yellow parts of the wild-type feather germs. In the parts corresponding to the black parts of the wild-type feather germs, melanocytes with black pigment were mainly seen. The distribution of melanocytes in heterozygous feather germs resulted in the black coating of the heterozygotes. The homozygous feather germs mostly contained melanocytes with moderate staining of the dopa reaction and brown pigment, leading to the brown appearance of the feather germs. Development of homozygous feather germs was delayed 1 or 2 days compared to those of the wild-type and heterozygotes, but homozygous melanocytes were also arranged at regular intervals in the barb ridges of the feather germs, which resembled the pattern seen in the wild-type and heterozygous feather germs. Therefore, the distribution of the two types of melanocytes in the feather germs might determine plumage pigmentation patterns in *Bh* quail embryos.

INTRODUCTION

The *Bh* mutation in Japanese quail not only causes whole body hemorrhage and induces early death in homozygotes, but also changes plumage pigmentation patterns in both heterozygotes and homozygotes (Minezawa and Wakasugi, 1977; Ono and Wakasugi, 1983; Nakamura and Kaneko, 1993; Kubota *et al.*, 1995). Heterozygous and homozygous embryos exhibit black and brown coating, respectively, whereas wild-type embryos exhibit longitudinal black and yellow stripes in dorsal feather germs. Thus, the *Bh* embryo may be a good animal model to study mechanisms of pattern formation of avian plumage pigmentation. Because pigments of avian feathers are produced by melanocytes (Rawles, 1948, 1960), the number and distribution of melanocytes in the feather germs or type of pigment produced may be abnormal in both heterozygotes and homozygotes.

In the present study, we histologically examined the pigmentation pattern and distribution of melanocytes in embryonic feather germs of each *Bh* genotype with the aid of dopa reaction, which demonstrates tyrosinase activity in melanocytes (Mishima, 1960; Hirobe, 1978). We report here that there are two types of melanocytes in the dopa reaction in wild-type feather germs; strongly positive and moderately positive, and that the distribution of these two types of melanocytes is changed in the feather germs of heterozygotes and homozygotes.

MATERIALS AND METHODS

Materials

Embryos were obtained from matings of *Bh*/+ quail and incubated in an incubator at 37.8°C at more than 70% humidity. Chick embryos were obtained from a local breeder (Oohata Shever Co. Ltd., Yaizu, Japan).

Methods

Thigh and dorsal skins from *Bh* embryos at 7, 8, 9, 10, and 12 days of incubation were fixed in cold 10% formalin in phosphate buffer (pH 7.0) for 16–24 hr at 2°C. At least three embryos of each genotype at each time point were used except for 12-day *Bh*/*Bh* embryos, which were rarely observed. Fixed tissues were washed with distilled water

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and incubated with a 0.1% L-dopa (3,4-dihydroxyphenylalanine, Wako Pure Chemical Industries, Osaka, Japan) solution in phosphate buffer (pH 7.4) for 16–24 hr at 37°C (Mishima, 1960; Hirobe, 1978). The L-dopa solution was renewed 30 min after the start of incubation. Some of tissue samples were embedded in paraffin without incubation in the dopa solution. Serial sections 10 μm thick were deparaffinized and counterstained with eosin. For histology, some samples from dorsal and thigh skins were fixed in Bouin's fluid, and ordinary paraffin sections were prepared. Deparaffinized sections were stained with hematoxylin and eosin or azan. Each genotype of *Bh* embryo was confirmed by the pigmentation pattern on the feather germs under a dissection microscope after 10 days of incubation (Minezawa and Wakasugi, 1977; Kubota *et al.*, 1995). Genotypes of 7- to 9-day embryos were determined by grafting of their thigh skin onto the chorio-allantoic membrane of 9-day chick embryos (Kubota *et al.*, 1995).

The number of barb ridges with black or dark brown pigment, with brown pigment, and with both pigments was counted through ten consecutive cross sections of five feather germs of each 10-day *Bh* embryo. The number of barb ridges with black melanocytes and with brown melanocytes after the dopa reaction was similarly examined.

RESULTS

Pigmentation in the feather germs on the dorsal skin started at 8 days of incubation. At 10 days, the feather germs on the dorsal skin, shoulder skin and thigh skin were pigmented, and their pigmentation patterns differed among genotypes. Rows of the feather germs of the wild-type dorsal skin showed longitudinal stripes of black and yellow; from the mid-dorsal region two rows of black feather germs, one row of feather germs with black and yellow stripes, and then two rows of yellow feather germs (Fig. 1). Histological analysis of eosin-stained sections and azan-stained sections demonstrated that melanocytes were present in the basal layer of the epidermis of the barb ridges in the feather germs (Figs. 2 and 3). They were pigmented and posessed pigmented dendrites and large nuclei. There were one to three melanocytes in the cross section of each barb ridge. Black or dark brown pigment was located in melanocytes and keratinocytes in the black part of the feather germs, and melanocytes in the yellow parts contained brown pigment (Figs. 2A, 2B, 2C, 3) (Table 1). The pigmentation pattern of each feather germ of the thigh skin was similar to that of the dorsal skin. Although the two types of melanocytes involved in pigmentation were mostly present in different barb ridges of the feather germs in cross section, they were sometimes present in the same barb ridges of the feather germs (Fig. 2C). In heterozygotes, the stripes of black and yellow in the rows of the feather germs of the dorsal skin were obscure, and each feather germ of the dorsal skin and thigh skin appeared to be black or "gray." The barb ridges on the first longitudinal row of the dorsal feather germs usually contained melanocytes with black pigment similar to those of the wild-type (Fig. 2D) (Table 1). However, both melanocytes with black pigment and melanocytes with brown pigment were observed in the parts corresponding to the yellow parts of the wild-type feather germs (Fig. 2E) (Table 1). Homozygotes, which were sometimes observed, developed brown feather germs and red feather germs on their skins. Histologically the homozygous feather germs mostly developed brown pigment

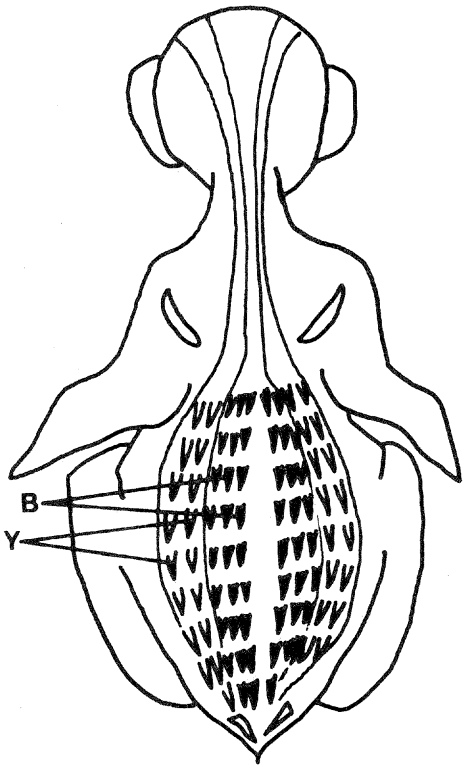

Fig. 1. Schematic drawing of the dorsal view of a 10-day +/+ quail embryo. Longitudinal rows of feather germs of the dorsal skin form stripes of black and yellow; two rows of black feather germs (B), one row of feather germs with black and yellow stripes, and then two rows of yellow feather germs (Y) are located longitudinally next to the two rows of short feather germs in the mid-dorsal region.

(Fig. 2F, G) (Table 1), though the blood vessels in the feather germs were often abnormally dilated and the development of the feather germs was impaired and delayed 1–2 days compared to the wild-type embryos and heterozygotes. Figure 3 schematically shows the distribution of the two types of melanocytes in the feather germs of the "yellow" row in 10-day *Bh* embryos.

A positive dopa reaction started to be seen in melanocytes of the feather germs at 8 days of incubation. The staining intensity and the number of positive melanocytes increased prominently at 9 days. The dopa reaction clearly demonstrated the contours of melanocytes, including their cell body and dendrites. However, each genotype could be determined from the staining pattern of melanocytes in the feather germs by the dopa reaction from 10 days. By the staining intensity of the dopa reaction two types of melanocytes were identified in the 10-day wild-type feather germs; strongly positive and moderately positive melanocytes, which were located in black parts and yellow parts of the feather germs, respectively (Fig. 4A, C). Because both types of melanocytes at this stage already possessed pigments, the difference in tyrosinase activity between the two types of melanocytes was unclear, though they were more darkly stained after the dopa reaction.

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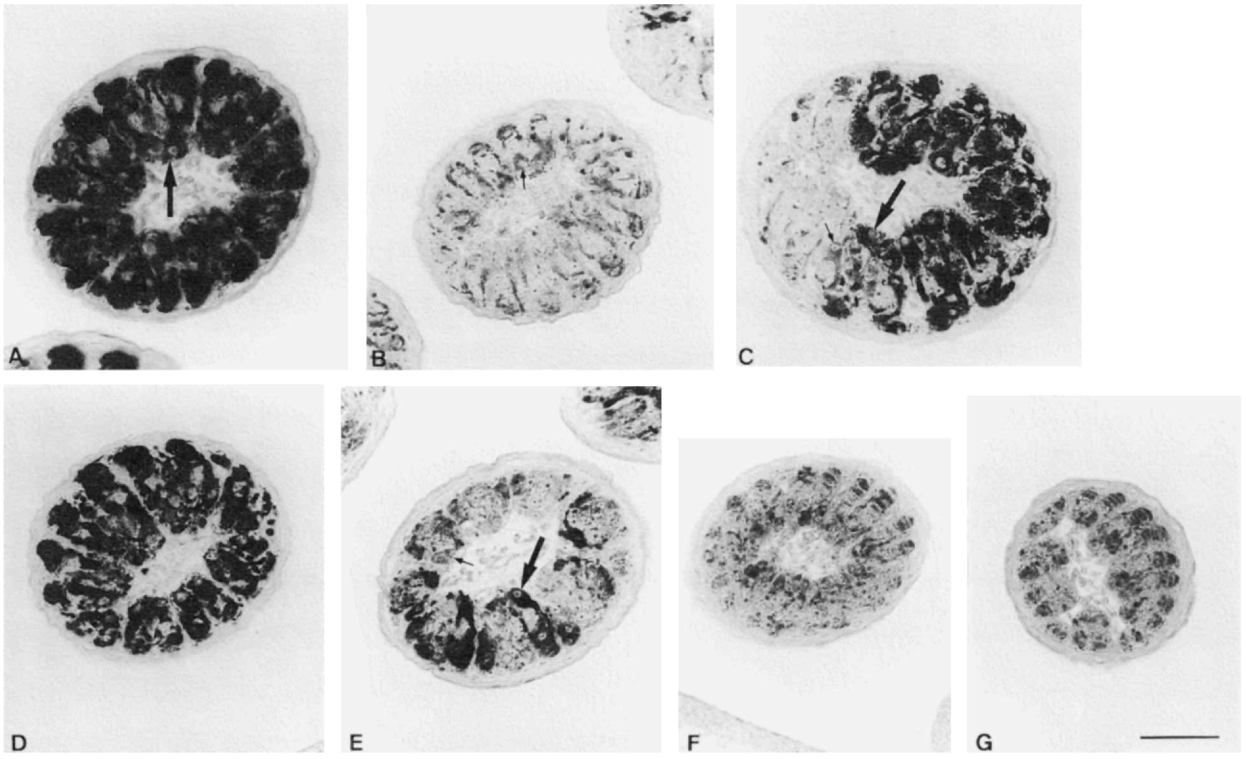

Fig. 2. Pigmentation pattern of the feather germs on the dorsal skin of 10-day *Bh* embryos. A, B, C: wild-type. D, E: heterozygotes. F, G: homozygotes. A feather germ on the first row of the wild-type dorsal skin feather germs contains melanocytes with black pigment (A, large arrow), but melanocytes in a feather germ on the fourth row possess light brown pigment (B, small arrow). By contrast, the two types of the melanocytes are simultaneously observed in a feather germ on the fourth row in a heterozygote (E), while melanocytes with black pigment are present in a feather germ on the first row (D). Most homozygous melanocytes in the feather germs on the first row (F) and the fourth row (G) contain brown pigment. C shows melanocytes with black (large arrow) and brown (small arrow) pigment located in the same barb ridge of a feather germ on the third row of the wild-type dorsal feather germs. Eosin staining. Bar indicates 0.05 mm.

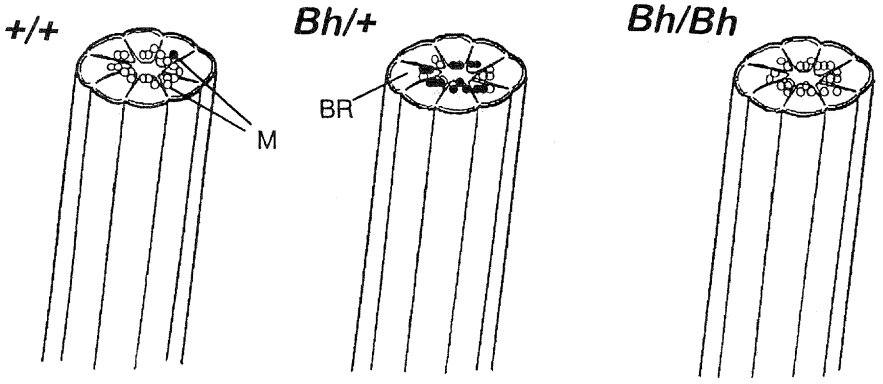

Fig. 3. Schematic drawing showing the distribution of the two types of melanocytes (M) in sections of the feather germs of the "yellow" row in 10-day *Bh* embryos. ●, melanocytes with black pigment; ○, melanocytes with brown pigment. BR, the barb ridge of the feather germ.

The strongly dopa reaction-positive melanocytes and moderately positive melanocytes corresponded to those with black pigment and with brown pigment, respectively. The strongly positive melanocytes had long well-developed dendrites, while those of moderately positive melanocytes were shorter and fewer in number (Fig. 4B). The distribution of the two types of melanocytes in the dorsal feather germs seen after the dopa reaction was similar to that of the two types of melanocyte pigmentation (data not shown). In addition, tangential sections of the feather germs showed that both types of melanocytes with tyrosinase activity were distributed at regular intervals in the epidermis of the barb ridges of the feather germs (Fig. 4C). In heterozygotes, both types of melanocytes were intermingled in some parts of the feather

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Table 1. Distribution of melanocytes with black pigment and with brown pigment in the barb ridges of the dorsal feather germs of 10-day *Bh* embryos

Genotype	Feather germs	No. of barb ridges with black pigment	brown pigment	both pigments
+/+	1st row[a]	7.8[c] ± 0.6[d]	0.3 ± 0.5	0.1 ± 0.2
	4th or 5th row[b]	0.2 ± 0.4	7.5 ± 0.7	0.4 ± 0.6
Bh/+	1st row	7.0 ± 0.9	0.6 ± 0.9	0.7 ± 0.8
	4th or 5th row	3.6 ± 1.7	2.3 ± 1.5	2.1 ± 1.7
Bh/*Bh*	1st row	0	8.6 ± 0.5	0
	4th or 5th row	0	8.0 ± 0.7	0

[a] The first of the longitudinal feather germ rows in the dorsal skin without counting the central feather germs which showed delayed development. The first row in wild-type embryos consisted of black feather germs.
[b] The fourth or fifth row of the feather germs was yellow in the dorsal skin of the wild-type embryos.
[c] The average number of barb ridges with black pigment, brown pigment, or both pigments in a cross section of the dorsal feather germ (at the yolk stalk level of the body). Seven to nine barb ridges were seen in the cross section of the feather germ.
[d] Standard deviation.

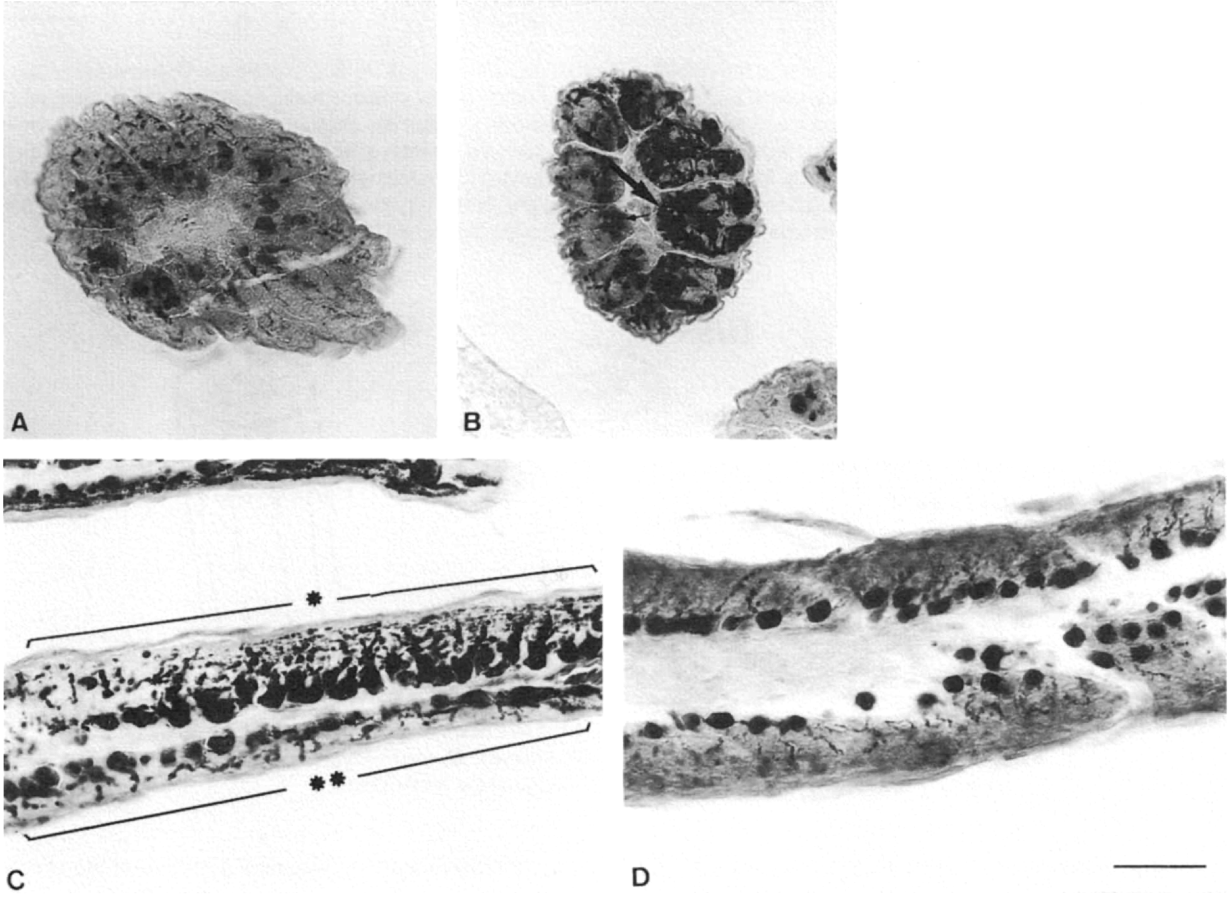

Fig. 4. Dopa reaction of melanocytes in the feather germs of 10-day *Bh* skin. A, C: wild-type. B: heterozygotes. D: homozygotes. Although in a dorsal feather germ of the fourth row only moderately dopa reaction-positive melanocytes are seen in the wild type (A), two types of melanocytes (strongly dopa reaction-positive [large arrow] and moderately positive melanocytes [small arrow]) are present in a heterozygote (B). Homozygous melanocytes in the feather germ are moderately dopa reaction-positive (D). Also note that melanocytes are distributed in a regular pattern in whole feather germs (tangential sections of the thigh skin, C, D). Dendrites in melanocytes of the black part (✱) of the feather germ are well developed. ✱✱, the yellow part of the feather germ. Bar indicates 0.05 mm.

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germs, which corresponded to yellow parts of the feather germs of the wild type (Fig. 4B), as seen in the distribution of two types of melanocyte pigmentation. Homozygous melanocytes in the feather germs were mostly moderately positive to dopa reactions, but they were distributed regularly in the whole feather germ (Fig. 4D). Black melanocytes sometimes developed on the base of the feather germs of 12-day homozygotes, which were rarely seen.

Melanocytes in the dermis and subcutaneous tissue of the dorsal skin were all black, independent of genotype. However, melanocytes were more numerously (approximately 1.5 times) in the dermis and subcutaneous tissue in the 10-day homozygotes than in wild-type embryos and heterozygotes.

DISCUSSION

The present study demonstrated that there were two types of melanocytes in the feather germs of 10-day quail wild-type embryos; strongly dopa reaction-positive melanocytes with black pigment, and moderately positive melanocytes with brown pigment, which were located in the black parts and yellow parts of the feather germs, respectively. The boundary between the black parts and the yellow parts in the feather germs was distinct in the wild type. In the heterozygous feather germs, the distribution of the two types of melanocytes was irregular, and they were intermingled, especially in what corresponded to the yellow parts of the wild-type feather germs. Homozygous feather germs whose development was usually delayed mostly contained moderately positive melanocytes with brown pigment. Thus, the *Bh* gene might disturb normal distribution of the two types of melanocytes in the feather germs and increase the number of melanocytes with black pigment in heterozygotes and with brown pigment in homozygotes. It is unknown at present how the *Bh* gene affects growth, differentiation and arrangement of the two types of melanocytes in the feather germs, or controls pigment production in melanocytes. Whether or not the same plumage melanocyte can produce both black and yellow pigments in quail is an important question in considering mechanisms of the *Bh* gene action, but remains to be revealed. The same melanocyte in mouse has been shown to synthesize both pigments responding to the hormonal stimuli (Tamate and Takeuchi, 1981; Hirobe, 1992).

We have shown that in homozygotes dilation of blood vessels in the feather germs and subcutaneous blood vessels, and poor development of dermis often occur (Kubota *et al.*, 1995), implying that the *Bh* gene might be expressed in the environment surrounding melanocytes and change the plumage pigmentation pattern. Although we have not found histological abnormalities in the heterozygotes other than the plumage change (Kubota *et al.*, 1995), subtle changes may also occur in the heterozygous feather germs. Because both types of melanocytes were sometimes closely adjacent in the same barb ridges of the wild-type and heterozygous feather germs, as shown in the present study, the differentiation of "black" and "yellow" melanocytes, or production of the two types of pigments by melanocytes must be controlled very locally. Melanocytes may be a heterogeneous cell population in pigment production, and may produce different pigment in the same microenvironment. It is also important to chemically determine the pigments in feather germs of *Bh* embryos. This is one of the questions we hope to resolve in the near future.

We observed that many melanocytes differentiated in the dermis and subcutaneous tissue of the homozygotes, suggesting that migration of melanoblasts might be abnormal in the homozygotes in which poor development of the dermis and dilation of subcutaneous blood vessels occur. Melanocytes in the body skin always contained black pigment at all genotypes. The control mechanisms of their pigment productions might be different from those of plumage melanocytes.

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