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Further studies on the melanophores of periodic albino mutant of *Xenopus laevis*

T. FUKUZAWA AND H. IDE
Biological Institute, Tohoku University, Aoba-yama, Sendai 980, Japan

SUMMARY

It is still unknown why dermal melanophores disappear during larval development, and why no or very few epidermal melanophores appear during and after metamorphosis, in *Xenopus laevis* showing periodic albinism (a^p). To elucidate these points, we investigated (1) the occurrence of depigmentation in mutant (a^p/a^p) melanophores during *in vitro* proliferation and (2) the incidence of melanophore differentiation from mutant melanoblasts in the skin *in vitro*.

During *in vitro* proliferation of mutant melanophores, a^p-type melanosomes decreased in number gradually and instead the number of premelanosomes increased in the cells, which caused depigmentation at the light microscopic level in the culture. Depigmentation was observed only in mutant melanophores, and not in wild-type (+/+) melanophores. These results suggest that autonomous depigmentation of mutant dermal melanophores is the cause of the disappearance of these cells *in vivo*.

Dopa-positive melanoblasts were demonstrated in both wild-type and mutant skins. However, the melanoblasts of metamorphosed mutant froglets did not differentiate *in vitro*, while those of wild-type froglets did. These results suggest that mutant melanoblasts in the skin of froglets lose the potency to differentiate into melanophores, and that this causes the lack of mutant melanophores in the froglets. The site of action of the a^p gene is also discussed.

INTRODUCTION

Periodic albinism (a^p) of *Xenopus laevis* was first described by Hoperskaya (1975). This mutant is characterized by (1) the complete absence of melanin in oocytes, (2) the appearance of melanin in the pigmented epithelium of the eyes and in skin melanophores at larval stages, and (3) the almost complete disappearance of melanin in metamorphosed animals. It is still in dispute whether the a^p gene acts directly in retinal pigment epithelium and melanophores (MacMillan, 1979, 1981) or acts in the environmental tissues to induce melanization in these cells (Hoperskaya, 1978, 1980, 1981, 1982*a*,*b*). Although the same experiment was done, the results of MacMillan (1981) differed from those of Hoperskaya (1978). This is partially due to the absence of a reliable criterion for distinguishing between wild-type (+/+) and mutant (a^p/a^p) melanophores. However, electron microscopic observations have revealed that there is a clear difference in melanosome structure between wild-type and mutant melanophores (Hoperskaya, 1981; Seldenrijk, Huijsman, Heussen & van der Veerdonk, 1982; Fukuzawa & Ide, 1983). This will be a useful criterion for determining mel-

Key words: melanophore, periodic albino, *Xenopus laevis*, mutation, epidermis, dermis, pigmentation.

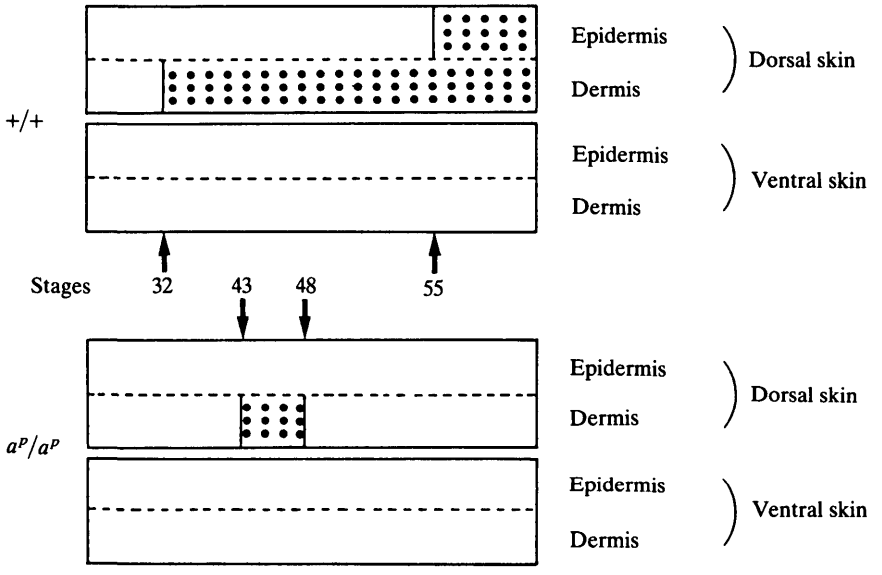


Fig. 1. Pigmentation and depigmentation in the trunk region of wild-type (+/+) and mutant (a^p/a^p) *Xenopus*. Dermal melanophores appear at stage 32 in wild-type larvae, and at stage 43 in mutant larvae. The mutant melanophores disappear at stage 48. Epidermal melanophores come to appear in wild-type stage-55 animals in caudalocephalic sequence, but not in mutant animals. Melanophores appear only in dorsal skin and not in ventral skin of the wild type.

anophore type. Further studies on mutant melanophores using this criterion seem likely to help in elucidating the action of the a^p gene.

As regards melanophore differentiation in wild-type and mutant *Xenopus*, there is a difference between dermal and epidermal melanophores, which has not been described by Hoperskaya (1975). While dermal melanophores in wild-type *Xenopus* differentiate at the larval stage, epidermal melanophores do not appear until the stage immediately before metamorphosis. It is still unknown (1) why dermal melanophores disappear during larval development, and (2) why epidermal melanophores do not appear during and after metamorphosis in periodic albinism (Fig. 1). To solve the former problem, mutant melanophores were cultured and examined electron microscopically. We have previously described the *in vitro* proliferation of melanophores of *Xenopus laevis*, and reported that mutant melanophores did not depigment at the light microscopic level during this proliferation (Fukuzawa & Ide, 1983). This has been re-examined electron microscopically in the present article and discussed further. If mutant melanophores depigment during culture, it is suggested that the a^p gene acts in melanophores and causes their disappearance at larval stages.

To solve the latter problem, larval and adult skins were cultured, and the incidence of melanophore differentiation from melanoblasts was examined. The ultrastructure of melanosomes in the newly differentiated melanophores was also examined. It has been previously reported that dopa-positive melanoblasts were

present in the ventral skin of larval *Xenopus* and differentiated *in vitro* (Ohsugi & Ide, 1983). It is useful, for the study of the action of the a^p gene, to determine whether wild-type and mutant melanophores differentiate from wild-type and mutant melanoblasts respectively, in the same culture conditions. If such is the case, it is suggested that the a^p gene acts directly in the melanoblasts and determines the phenotypes of melanophores. If mutant melanoblasts are present in adult skin, but do not differentiate *in vitro*, it is suggested that mutant melanoblasts lose the potency to differentiate into melanophores after metamorphosis, and this may cause the absence of epidermal melanophores in the mutant froglets.

In this article, differentiation and depigmentation of mutant melanophores are described and the site of the a^p gene action is discussed.

MATERIALS AND METHODS

Eggs of wild-type (+/+) and mutant (a^p/a^p) *Xenopus* were obtained and reared by the method of Gurdon (1967). Developmental stages were determined according to Nieuwkoop & Faber (1956).

Isolation and culture of melanophores

The tails of 50 tadpoles (stage 46) were cut off and washed several times with sterile Steinberg's solution (Jones & Elsdale, 1963). They were cut into small pieces with scissors and centrifuged at 100 r.p.m. for 5 min. The pellet was transferred to a plastic dish (Falcon, 3001) and cultured at 25 °C. Diluted L-15 medium supplemented with foetal calf serum and conditioned with chick neuroretina cells was used as the culture medium (Ide, 1974). Since the melanophores differentiated within 7 days, the other cells were removed completely with a tungsten needle. Only melanophores were collected after trypsinization, transferred to a plastic dish, and cultured in the medium containing 0·1 $\mu g\,ml^{-1}$ α-MSH (Fukuzawa & Ide, 1983). The medium was changed every 5 days.

Culture of skin explants

Dorsal and ventral skins at varying stages (stage 47–48, stage 52–53, stage 66) were dissected and washed several times with sterile Steinberg's solution. They were cut into small pieces with scissors, collected, and cultured by the same method as was used in melanophore culture, except that MSH-free medium was used. Wild-type dorsal skin (stage 66) was not used in this experiment, since dermal and epidermal melanophores pre-existing in the skin were not discriminated from the newly differentiated melanophores.

Dopa reaction

The dopa reaction was used for the detection of melanoblasts in the skin. The method was a modification of that reported by Mishima (1960). Whole bodies at larval stages and dissected dorsal and ventral skins of froglets were used. They were fixed with 15 % formalin in 0·05 M-phosphate buffer (pH 7·4) at 4 °C for 24 h, washed with the same buffer for 30 min, and incubated with 0·1 % 3,4-dihydroxyphenylalanin (dopa) in 0·1 M-phosphate buffer (pH 7·4) at 37 °C for 12 h. Then they were refixed in 10 % formalin and used for observation.

Electron microscopy

Cultured melanophores were fixed in 2 % glutaraldehyde and postfixed in 1 % OsO_4 at 3 °C for 1 h. Dehydration was performed with ethanol, and the specimens were embedded in Epon 812 and sectioned horizontally after removing the culture dish.

To establish the localization of the dopa-positive melanoblasts, transverse sections of the ventral skins (stage 66) were examined electron microscopically, after the dopa reaction. Small pieces of the skin were fixed in 2 % glutaraldehyde at 4 °C for 1 h, washed in 0·1 M-buffer for 2 h, and incubated in 0·1 % dopa solution at 37 °C for 4 h. Then the usual procedure was performed as described above.

RESULTS

Mutant melanophores

Mutant (a^p/a^p) melanophores, which had differentiated from unmelanized cells (probably melanoblasts) within 7 days after explantation, were collected and sparsely inoculated (Fig. 2). No cells other than melanophores were contained in the culture. These newly differentiated mutant melanophores were different from wild-type (+/+) melanophores in melanosome structure (Fig. 3). Most melanosomes in the mutant melanophores, a^p-type melanosomes, had a granular internal structure (Fig. 3B). Matured melanosomes, which were predominant in wild-type melanophores (Fig. 3A), were few in the mutant melanophores. These newly differentiated mutant melanophores had many a^p-type melanosomes and were observed to be melanizing light microscopically (Fig. 2A,B). As they proliferated, they gradually demelanized (Fig. 2C,D), and some of them almost completely depigmented (Fig. 2C,D, arrowheads). These depigmented cells had many premelanosomes with typical lamellar structures instead of a^p-type melanosomes (Fig. 3C). Completely depigmented cells appeared after about the 6th cell division, and no depigmentation was observed in non-proliferating melanophores.

While wild-type melanophores were also cultured under the same culture conditions, no depigmentation occurred. The melanophores contained many matured melanosomes after about 10th cell division as reported previously (Fukuzawa & Ide, 1983).

Mutant melanoblasts

Dopa-positive melanoblasts were observed both in wild-type and mutant skins of metamorphosed frogs (stage 66) (Figs 4, 5). Wild-type and mutant melanoblasts resembled each other in size and shape. They also resembled epidermal melanophores (Fig. 4A), and were located in the epidermal layer (Fig. 6). The number of dopa-positive melanoblasts (130–200 per mm^2) was almost equal in wild-type and mutant skin, except that the number of these cells in wild-type dorsal skin (stage 66) was smaller than that in wild-type ventral skin (stage 66) by the number of differentiated epidermal melanophores in wild-type dorsal skin (stage 66). Dopa-positive melanoblasts were also present in wild-type and mutant skin at larval stages.

To investigate whether these melanoblasts have the potency to differentiate into matured melanophores, small pieces of skin explanted from tadpoles and froglets were cultured. Wild-type ventral and mutant skins, which contained no

melanophores (Fig. 1), were used. The explants attached to the dishes within 24 h, and fibroblastic and epithelial cells gradually migrated from the explants and proliferated (Figs 7A, 8A). After 7–20 days, differentiated melanophores appeared in the explants of tadpole skin (Figs 7B, 8B). The newly differentiated mutant melanophores were different from wild-type melanophores light

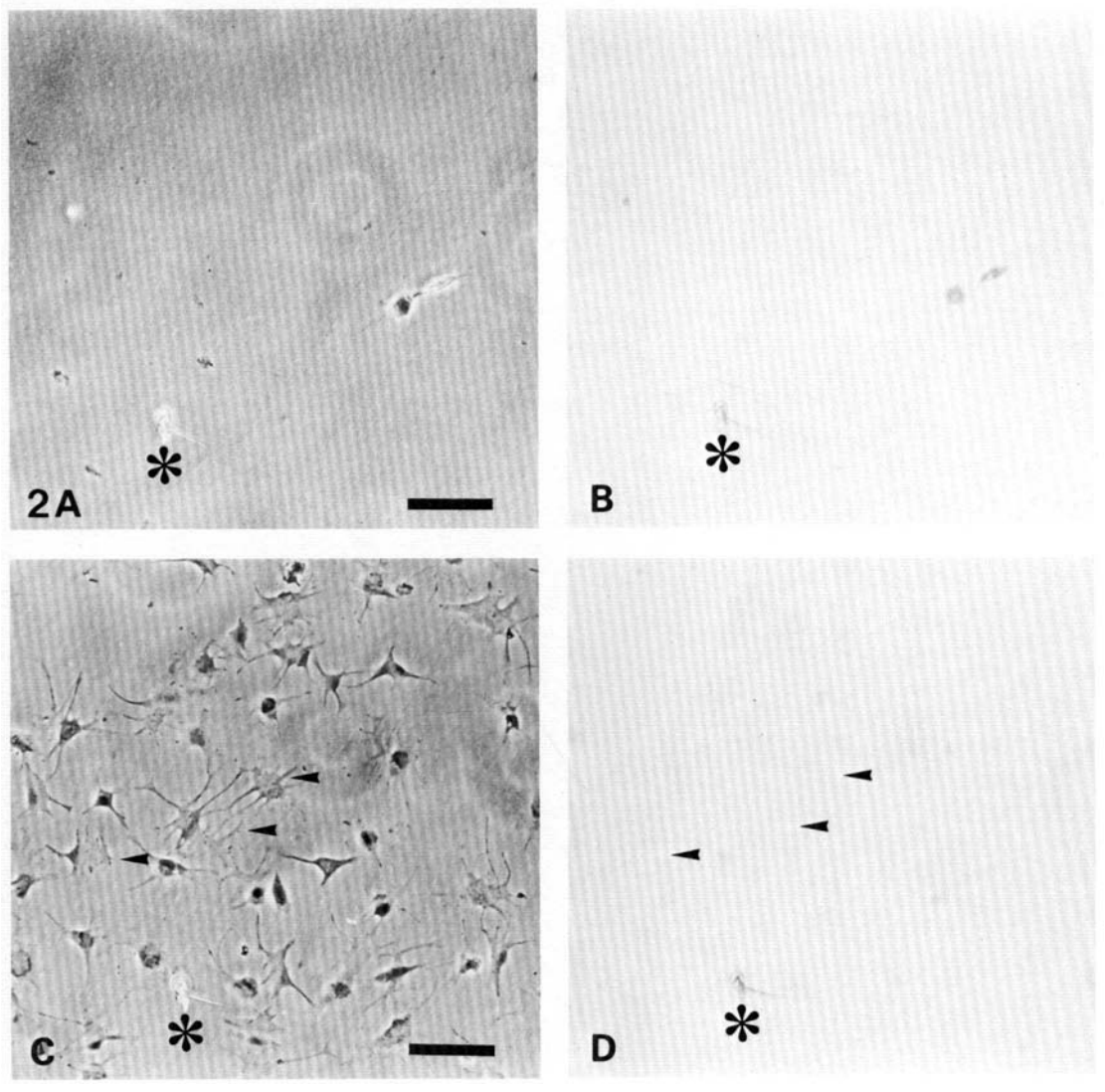
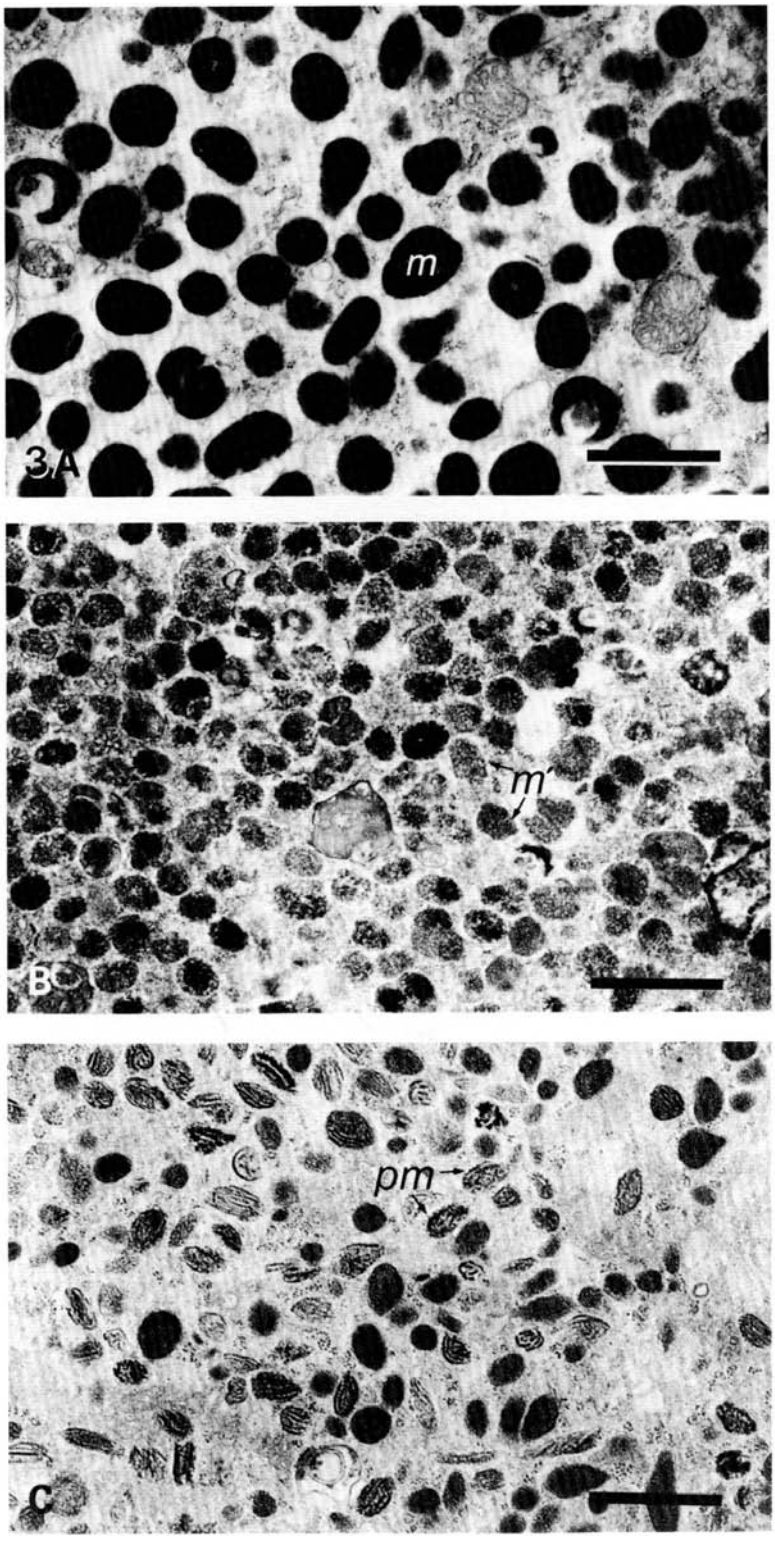


Fig. 2. Depigmentation of mutant melanophores during *in vitro* proliferation. Two dark melanophores containing many melanosomes were cultured (A,B), and their daughter cells were traced for 50 days (C,D). B and D (ordinary light) are the photographs of the same field as A and C (phase-contrast), respectively. During the proliferation of the melanophores, the intensity of melanization decreased and some depigmented cells appeared (C,D, arrowheads). * is a crack on the dish used as a marker. Scale bar equals 100 μm.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microscopically in the intensity of melanization. The intensity of melanization in mutant cells was slightly lower than in wild-type cells. The newly differentiated wild-type melanophores contained many matured melanosomes, while the mutant melanophores contained many a^p-type melanosomes.

The number of newly differentiated melanophores was not easily determined, since the time of their appearance varied among skin explants and some of these cells commenced proliferation before the differentiation of all melanoblasts was completed.

The incidence of melanophore differentiation in the wild-type and mutant skin explants is summarized in Table 1. At stage 47–48, when most dermal melanophores disappeared in mutant larvae, melanophore differentiation occurred both in the dorsal and ventral skin explants of the mutant. The minimum culture time needed for the onset of melanophore differentiation in the mutant explants was slightly longer than that in the wild-type ventral explants. At stage 52–53, when dermal melanophores disappeared completely in mutant larvae, melanophore differentiation also occurred in the dorsal and ventral skin explants of the mutant. The minimum culture time in the mutant explants was almost equal to that in the wild-type explants. At stage 66, when metamorphosis was completed and epidermal melanophores appeared in wild-type dorsal skin, melanophore differentiation occurred in the ventral explants of the wild type. However, no melanophores appeared in either the ventral or dorsal explants of the mutant. The minimum culture time in the wild-type explants was considerably longer in the adult than at larval stages.

DISCUSSION

The nature of mutant melanophores

We have previously succeeded in obtaining the *in vitro* proliferation of melanophores from *Xenopus laevis*, and reported that mutant melanophores did not demelanize at the light microscopic level during the period of proliferation (Fukuzawa & Ide, 1983). In the present experiments, the reverse results were obtained. The mutant melanophores demelanized during *in vitro* proliferation. This discrepancy seems to be partially due to the difficulty of evaluating depigmentation. Since the intensity of melanization in the mutant melanophores differentiated *in vitro* varied considerably under light microscopy, even weakly melanized cells were regarded as pigmented cells in the previous article. To overcome this difficulty, newly differentiated mutant melanophores which contained many melanosomes were sparsely inoculated, traced during the proliferation, and

Fig. 3. Electron micrographs of wild-type (A) and mutant melanophores before (B) and after (C) depigmentation *in vitro*. Wild-type and mutant melanophores contained many matured (*m*) and a^p-type (*m'*) melanosomes, respectively. Depigmented mutant melanophores contained many premelanosomes (*pm*) instead of a^p-type melanosomes. Scale bar equals 1 μm.

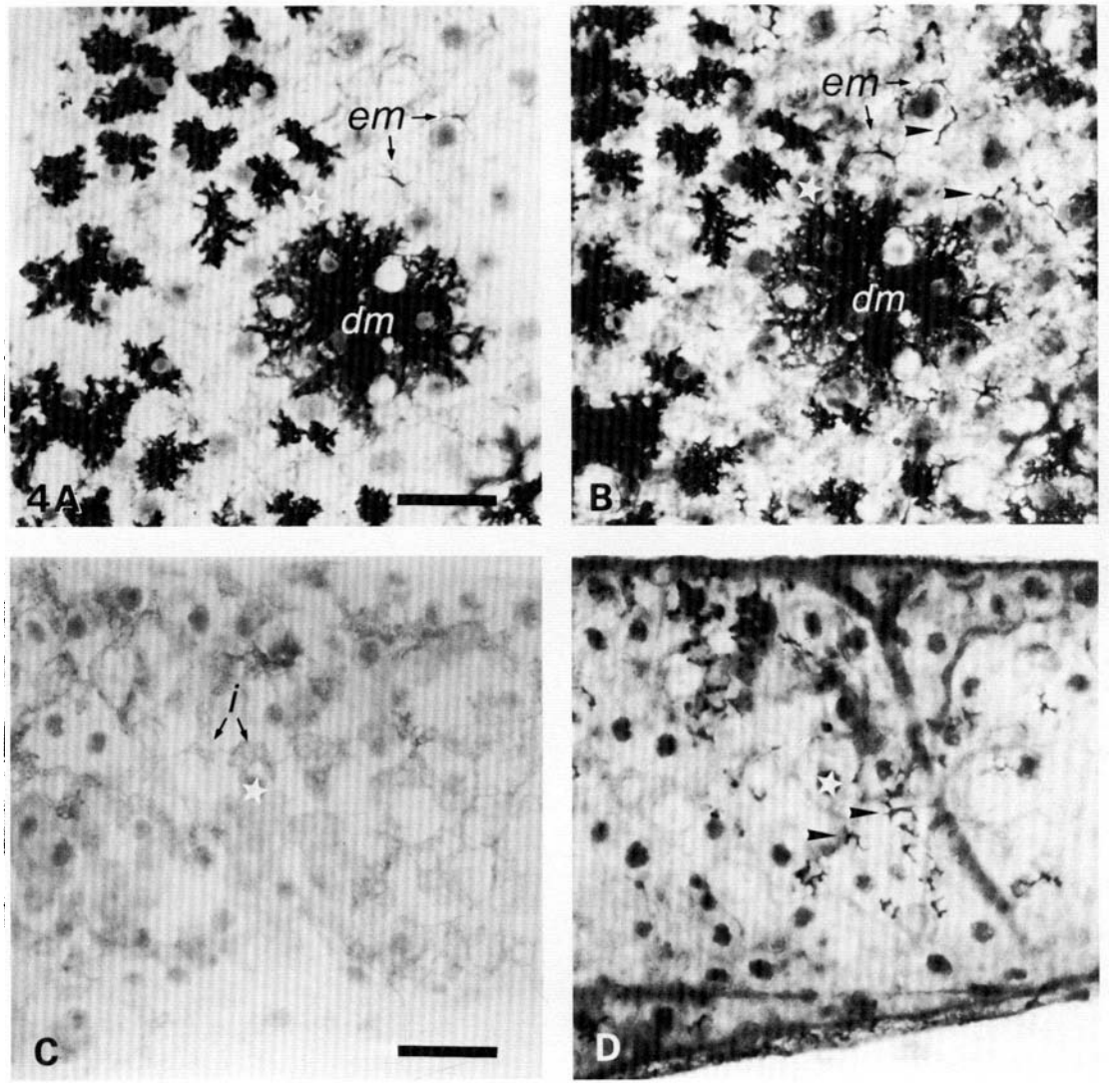

Fig. 4. Dopa-positive melanoblasts in the dorsal (A,B) and ventral (C,D) skin of metamorphosed wild-type *Xenopus* (stage 66). B and D are the photographs after dopa reaction of the same fields as A and C, respectively. Dopa-positive melanoblasts were present both in the dorsal (B, arrowheads) and ventral (D, arrowheads) skin. ☆, dermal gland; *dm*, dermal melanophore; *em*, epidermal melanophore; *i*, iridophore. Scale bar equals 100 μm.

compared with their daughter cells in relation to the degree of melanization. The melanosome structure was also examined. The results indicated that mutant melanophores depigmented gradually during proliferation and that the depigmented cells were filled with premelanosomes with typical lamellar structures instead of a^p-type melanosomes. The latter finding is consistent with the

observation that mutant melanophores of tadpoles (stage 50–54) contained many premelanosomes (Seldenrijk *et al.* 1982). It was also observed from *in vivo* study that the intensity of melanization became lessened with cell proliferation in mutant larvae (data not shown). From these results, the accumulation of premelanosomes may be considered as a cause for the disappearance of melanophores in the periodic albino. Although it is still unclear why a^p-type melanosomes decrease and premelanosomes increase in number in the mutant cells, it is possible that while

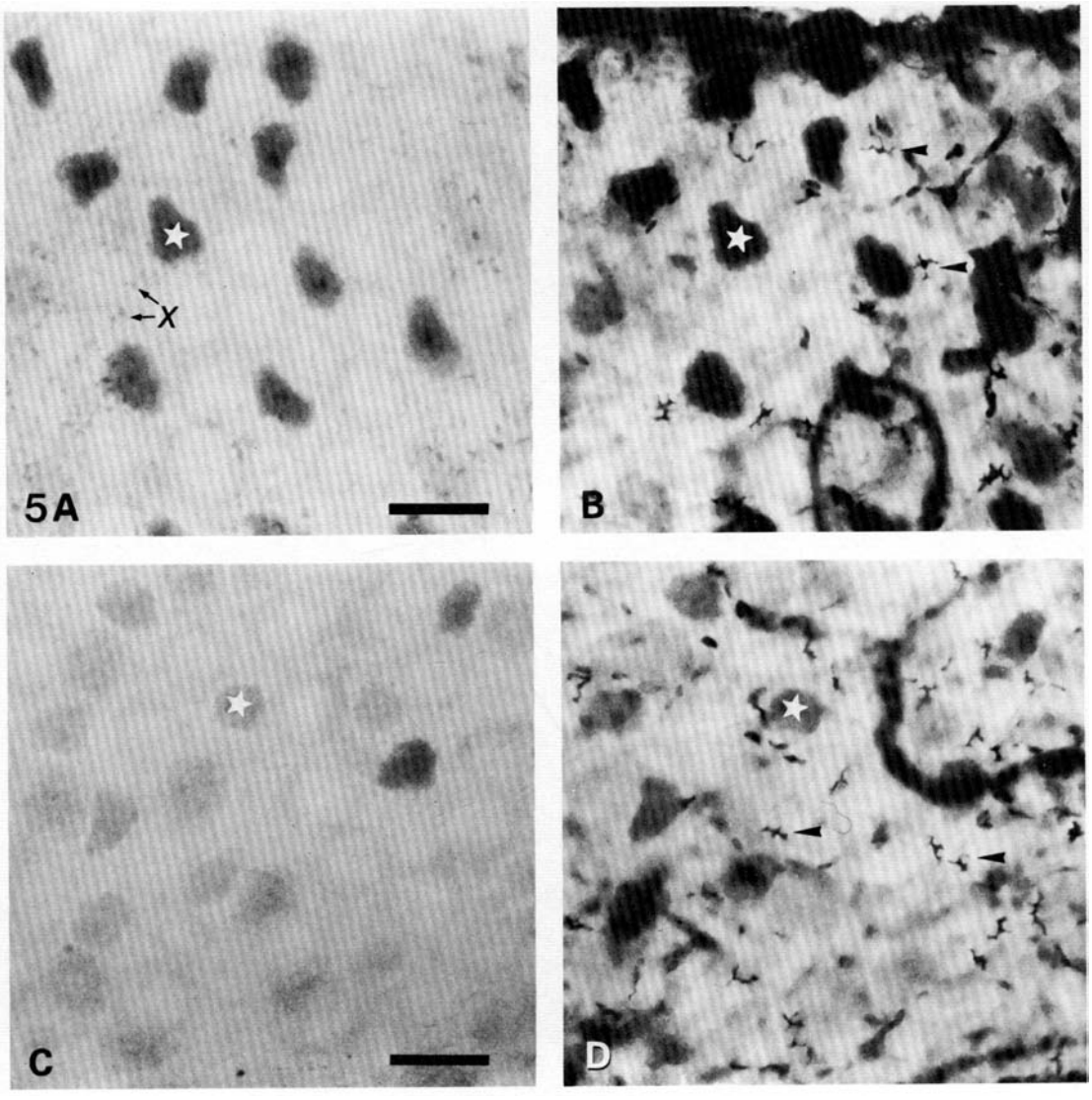

Fig. 5. Dopa-positive melanoblasts in the dorsal (A,B) and ventral (C,D) skin of metamorphosed mutant *Xenopus* (stage 66). B and D are the photographs after dopa reaction of the same fields as A and C, respectively. Dopa-positive melanoblasts were present both in the dorsal (B, arrowheads) and ventral (D, arrowheads) skin. ☆, dermal gland; *x*, xanthophore. Scale bar equals 100 μm.

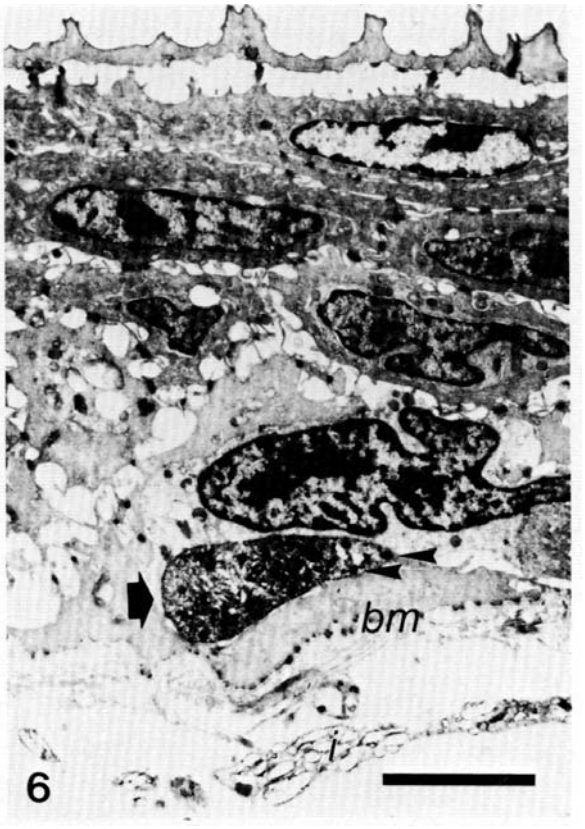

Fig. 6. Transverse section of the ventral skin of a metamorphosed wild-type *Xenopus* (stage 66) after dopa reaction. A dopa-positive melanoblast (arrow) containing many melanosomes (arrowheads) is located in the epidermal layer. *bm*, basement membrane; *i*, iridophore. Scale bar equals 5 μm.

a^p-type melanosomes are diluted among daughter cells, they are not newly synthesized from premelanosomes.

The nature of mutant melanoblasts

As reported by Ohsugi & Ide (1983), dopa-positive melanoblasts are present in larval and adult skin of wild-type *Xenopus*. The present experiments also demonstrate them in the larval and adult skin of the periodic albino mutant. The observations on the size and localization of the dopa-positive melanoblasts in the adult skins (Figs 4, 5, 6) indicate that these cells are epidermal melanoblasts. This is supported by the report by Ohsugi & Ide (1983) that the localization of dopa-positive melanoblasts shifted from the subepidermal layer to the epidermal layer during larval development. It is also suggested that the dopa-positive melanoblasts localized in the subepidermal layer at the tail-bud stage are dermal melanoblasts (Ohsugi & Ide, 1983). The number of dopa-positive melanoblasts in the ventral skin was almost equal in the wild-type and in the mutant, suggesting that the

a^p-gene did not affect the number of melanoblasts. This is in accordance with a previous report (MacMillan, 1980).

When small pieces of wild-type and mutant larval skin were cultured, wild-type and mutant melanophores differentiated, respectively. Thus, wild-type and mutant melanophores were considered to differentiate from wild-type and mutant melanoblasts, respectively. The minimum culture time needed for melanophore differentiation at larval stages was longer for mutant melanoblasts than for wild-

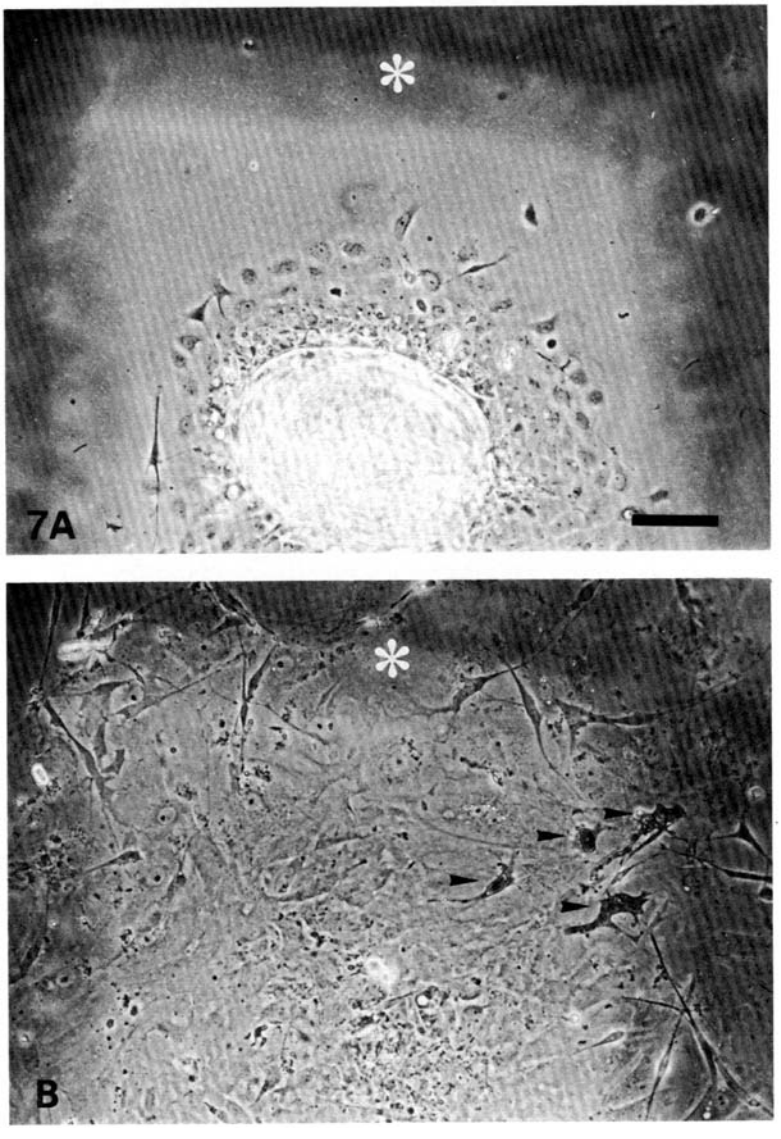

Fig. 7. Melanophore differentiation *in vitro* in an explant of wild-type skin. The wild-type ventral skin (stage 48), which contained no melanophores, was cultured (A), and wild-type melanophores appeared after 7 days (B, arrowheads). * is a marker under the dish. Scale bar equals 100 μm.

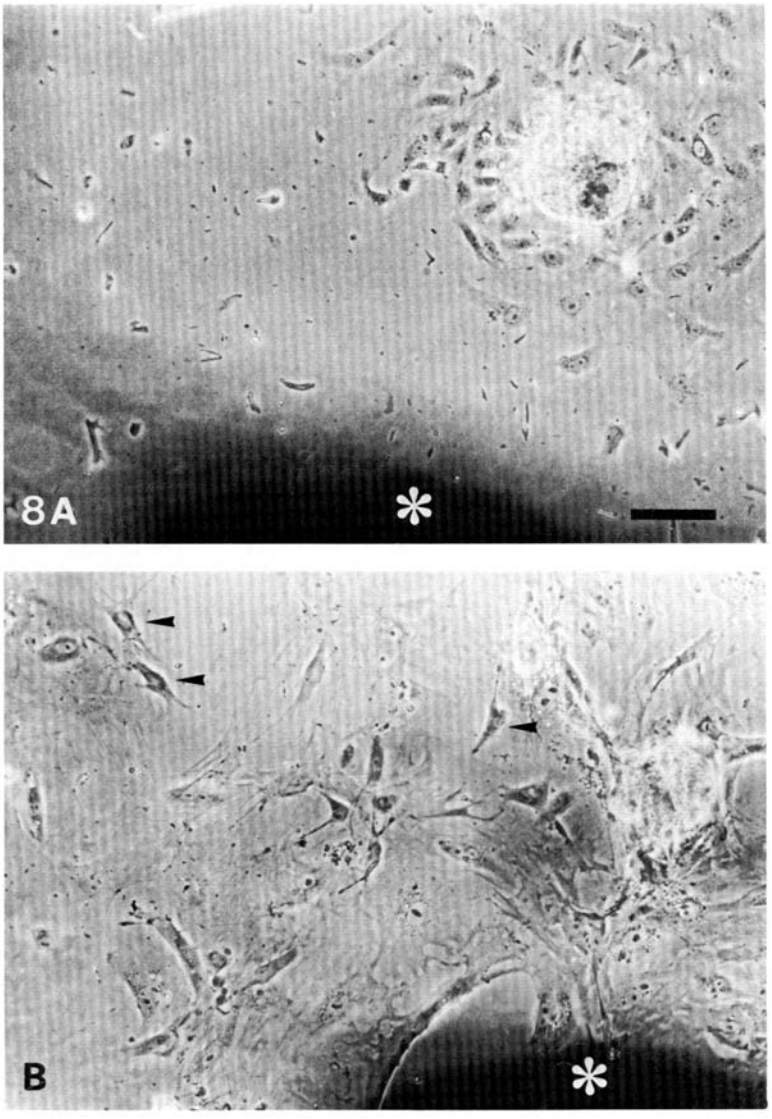

Fig. 8. Melanophore differentiation *in vitro* in an explant of mutant skin. The mutant dorsal skin (stage 48), which contained no melanophores, was cultured (A), and mutant type melanophores appeared after 20 days (B, arrowheads). * is a marker under the dish. Scale bar equals 100 μm.

type ones. This might reflect the delay of dermal melanophore differentiation in mutant larvae. These results strongly suggest that the a^p-gene acts in melanoblasts and determines both the structure of melanosomes and the timing of melanophore differentiation.

Neither melanization of melanoblasts nor remelanization of depigmented melanophores of mutant adult frogs occurred in the present culture conditions.

Table 1. *Incidence of melanophore differentiation* in vitro

Explants		Stage 47–48	Stage 52–53	Stage 66
+/+	Ventral skin	$\frac{14}{44}$* (7 days†)	$\frac{8}{44}$ (12 days)	$\frac{5}{43}$ (45 days)
a^p/a^p	Dorsal skin	$\frac{13}{75}$ (10 days)	$\frac{3}{55}$ (11 days)	$\frac{0}{88}$
	Ventral skin	$\frac{9}{44}$ (10 days)	$\frac{7}{39}$ (11 days)	$\frac{0}{151}$

* Number of explants with differentiated melanophores/total number of explants.
† Minimum time needed for melanophore differentiation.

This result suggests that although dopa-positive melanoblasts are present in the mutant adult skin, they almost lose the potency to differentiate into melanophores. Depigmentation of dermal melanophores during larval stages seems to be irreversible. These findings explain partially why epidermal melanophores were seldom found after metamorphosis in the periodic albino mutant.

The present results are consistent with the reports by MacMillan (1979, 1981) that the a^p-gene acts directly in melanoblasts and melanophores. Hoperskaya (1982) argued that the melanization of melanophores and of retinal pigment epithelium was induced by a melanogenic factor (MGF) at early stages. However, the possibility that MGF acts on melanoblasts and affects the structure of melanosomes is inconsistent with the present finding that wild-type and mutant melanophores differentiated *in vitro* from wild-type and mutant melanoblasts respectively, in the same culture conditions. This is because if MGF is contained in the culture medium and if the MGF acts on melanoblasts qualitatively and/or quantitatively to determine the structure of melanosomes, the phenotypes of melanophores which differentiate *in vitro* from both wild-type and mutant melanoblasts should be equal. If MGF is not contained in the culture medium, the possibility will be ruled out that MGF is involved in the melanization of melanophores from melanoblasts. Thus, the present results support the view that the a^p-gene directly affects melanoblasts and melanophores without the intervention of environmental factor(s), although it still remains possible that MGF may act on gastrula ectoderm or neural crest cells to determine the structure of melanosomes.

MacMillan (1980) also suggested the presence of a ventral trunk factor which may play a role in the differentiation of mutant melanophores. However, the role of such a factor is not so clear as that in the case of MGF.

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