

# Melanophore differentiation in *Xenopus laevis*, with special reference to dorsoventral pigment pattern formation

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## SUMMARY

During the development of *Xenopus laevis*, after stage 26, a large number of dopa-positive cells were observed in the ventral region. Electron microscopic observations revealed that these cells became localized in the epidermal layer and contained premelanosomes. In cultured ventral skin, fully matured melanophores appeared.

These results strongly suggest that a large number of melanoblasts are present in the ventral epidermis and remain there without final differentiation into melanized melanophores. Thus the positional difference of melanoblasts differentiation mainly contributes to dorsoventral pigment pattern formation of *Xenopus laevis*.

## INTRODUCTION

In many species of vertebrates, a common dorsoventral pigment pattern is observed; the dorsal region is dark in colour and the ventral region is light in colour. This pattern is mainly due to the fact that dark-coloured cells (melanophores or melanocytes) exist in the dorsal region and not in the ventral region. The purpose of the present article is to show how such a difference in pigment cell distribution occurs.

The first problem in analysing a general pigment pattern is whether it is formed by the intrinsic properties of melanoblasts or formed by interactions between melanoblasts and the tissue environment. Although a few investigations suggested the former interpretation (Twitty, 1936; Twitty & Bodenstein, 1939), many other investigations suggested the latter. If the latter interpretation is true, the second problem is how the tissue environments influence melanoblast development. Tissue-melanoblast interactions have been discussed by MacMillan (1976). The influence of the environment on melanoblast differentiation has been discussed by Twitty & Bodenstein (1939, 1944), Twitty (1944, 1945, 1953) and Lehman (1951); on melanoblast migration by Dalton (1950), Delanny (1941), Finnegan (1958), MacMillan (1976); on melanoblast proliferation and

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destruction by Finnegan (1955). Most of this previous work was based on transplantation, and little effort has been made to identify the melanoblasts.

In *Xenopus*, melanophores appear at about stage 34 on the dorsal surface of the head and in the upper flank region. As development progresses melanophores appear on the dorsal surface of the neural tube and dorsal ridges of the somites. These cells are dermal melanophores and no epidermal melanophore is observed in tadpole skin. The ventral skin, on the other hand, remains unmelanized at all stages. If the presence and distribution of melanoblasts in melanophore-free ventral skin were known and if the ability of these cells to differentiate into melanophores could be shown, we could establish the extent to which dorsoventral pigment pattern formation depends on an environmental control of melanoblast differentiation.

#### MATERIALS AND METHODS

Wild-type embryos of *Xenopus laevis* were obtained after Gurdon (1967). Stages of development were determined from the Normal Table (Nieuwkoop & Faber, 1956).

To detect melanoblasts, the dopa reaction was used. The method for light microscopy was a modification of that reported by Mishima (1960). Whole embryos and tadpoles at stages 20–50, and pieces of adult skin, were fixed with 15 % formalin in 0.05 M-phosphate buffer (pH 7.4) at 4 °C for 18 h. They were washed with the same buffer for 2 h and incubated with 0.1 % 3,4-dihydroxyphenylalanine (dopa) in 0.02 M-phosphate buffer (pH 7.4) at 37 °C for 12 h. Then they were refixed in 10 % formalin and used for observation.

Electron microscope studies to determine the tissue location and ultrastructure of dopa-positive cells were performed as follows. Tadpoles at stage 48 were fixed with 3 % glutaraldehyde in 0.05 M-phosphate buffer (pH 7.4) at 4 °C for 2 h and pieces of dorsal and ventral skin were removed from them. These pieces of skin were postfixed with 1 % osmium tetroxide at 4 °C for 1 h, dehydrated in an ethanol series, and embedded in Epon 812. Thin sections were cut and doubly stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). Some glutaraldehyde-fixed ventral skin was used for the dopa reaction. These pieces were washed with 0.05 M-phosphate buffer (pH 7.4) at 4 °C for 2 h to remove glutaraldehyde and incubated in the 0.1 % dopa solution at 37 °C for 9 h. After refixation with the same glutaraldehyde solution for 1 h, they were postfixed with 1 % osmium tetroxide at 4 °C for 1 h, and the same procedure for embedding was performed as described above.

Tissue culture of the ventral skin was performed as follows to test for the presence of cells capable of differentiating into mature melanophores. Tadpoles at stage 48 were washed several times with sterile distilled water, transferred to Steinberg's solution and small fragments (0.4–2 mm<sup>2</sup>) of the ventral skin were removed from them. To stick these fragments sufficiently to the substratum, they

were inoculated onto a Falcon plastic dish containing a small amount of culture medium and the dish was placed in a humidified atmosphere at 25 °C for 8 h. The culture medium used was a mixture of five parts of L-15 medium (Leibovitz, 1963; Balls & Ruben, 1966) conditioned with chick neuroretina cells (Ide, 1974), three parts of doubly distilled water, two parts of foetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.1 µg/ml  $\alpha$ -MSH (melanocyte-stimulating hormone). Two ml of culture medium were added to the dish and the medium was exchanged every 5 days.

For counting the number of melanophores and dopa-positive melanoblasts in the skin, twenty tadpoles at stage 48 kept in natural light at room temperature were used. The number of these cells in 0.31 mm<sup>2</sup> were counted in four regions of dorsal skin (head and trunk), two regions of lateral skin (head) and four regions of ventral skin (head and trunk).

## RESULTS

### *Existence of dopa-positive cells in the ventral region*

During embryonic development, melanized melanophores could not be observed until stage 33 even in the dorsal region. However, after the dopa reaction, black-coloured cells appeared in the embryo at stage 26. After stage 28, these dopa-positive cells were clearly observed in the dorsal and ventral region (Fig. 1). They were distributed nearly evenly in both regions. After stage 33, dendritic melanophores appeared in the dorsal skin. In ventral skin, instead of the melanophores, a large number of dopa-positive cells were observed (Fig. 2). These cells were very small in size and the pigment was tightly aggregated in a punctate fashion. In the lateral region, both melanophores and dopa-positive cells were observed (Fig. 2B). When pieces of neural fold of stage 15/16 embryos were removed bilaterally, dorsal melanophores and ventral dopa-positive cells were not found in the corresponding region at stage 40 (data not shown). The localization of the dopa-positive cells observed in the cross sections of the ventral skin is as follows. At stage 28, dopa-positive cells were observed beneath the epidermal

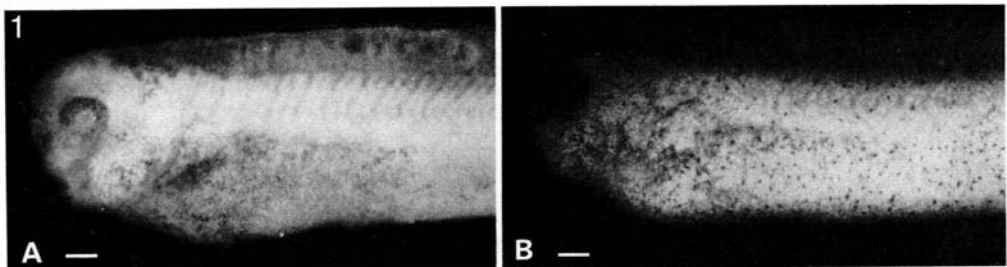


Fig. 1. Normal (A) and dopa-treated (B) embryos at stage 33/34. Black dopa-positive cells appeared in both dorsal and ventral regions after the treatment. Bar = 200 µm.

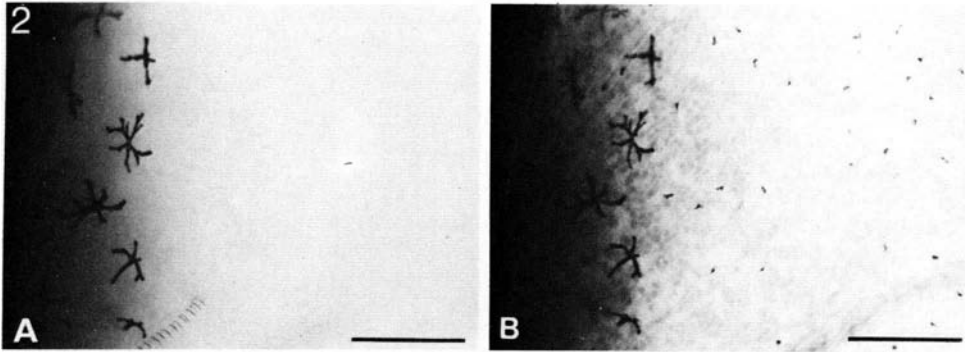


Fig. 2. Normal (A) and dopa-treated (B) lateral region of stage-48 tadpole. A large number of dopa-positive cells which were small in size appeared other than pre-existing large dendritic melanophores. left: dorsal, right: ventral. Bar = 200  $\mu\text{m}$ .

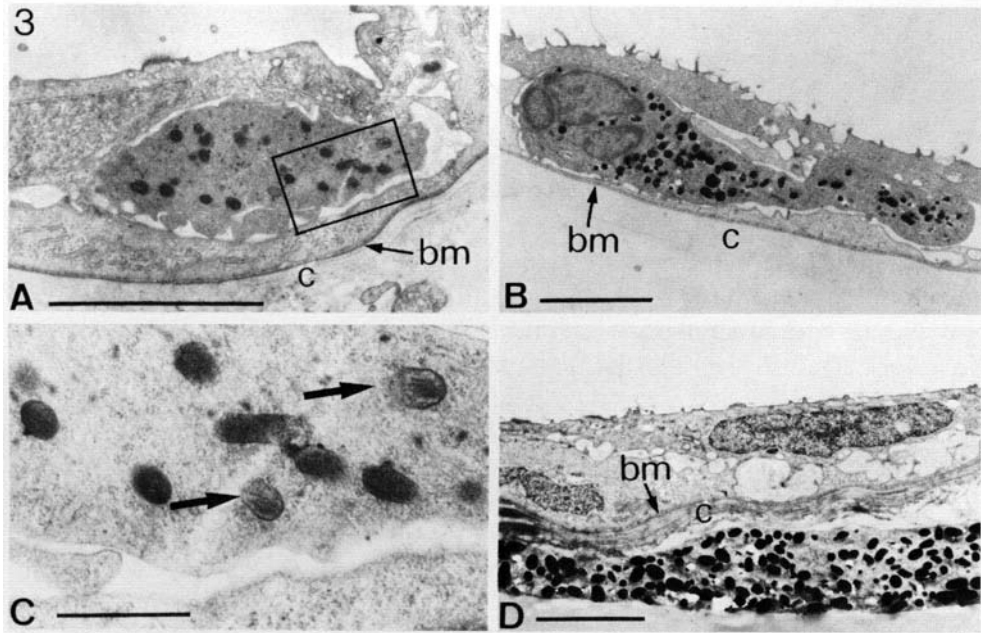


Fig. 3. (A) Normal ventral skin. Melanoblast laden with electron-dense granules is localized between two epidermal layers. bm, basement membrane, c, collagen layer. Bar = 5  $\mu\text{m}$ . (B) Dopa-treated ventral skin. Melanized cell with many melanosomes appeared between two layers of epidermal cells. bm, basement membrane, c, collagen layer. Bar = 5  $\mu\text{m}$ . (C) Enlargement of box in (A) showing premelanosomes (arrows) with lamellar structures in the melanoblast. Bar = 1  $\mu\text{m}$ . (D) Normal dorsal skin of stage-48 tadpole. The matured melanophore is localized beneath the collagen layer (c). bm, basement membrane. Bar = 5  $\mu\text{m}$ .

layer (subepidermis). At stage 44, some of them remained beneath the epidermal layer and others were observed in the epidermal layer. Some dopa-positive cells in the epidermal layer retained their processes in the subepidermal layer,

suggesting migration from the subepidermal layer to the epidermal layer. At stage 48, all dopa-positive cells became to be localized in the epidermal layer (see next section). Dopa-positive cells could be observed in the ventral region at all stages examined from stage 26 to adult frog.

*Electron microscopic observations*

Although normal ventral skin is light microscopically transparent in tadpoles, cells laden with electron-dense granules could be observed in some sections. These cells were localized only in the epidermal layer (Fig. 3A). The granules appeared to be typical premelanosomes with lamellar structures (Fig. 3C). After the dopa reaction, cells containing many melanosomes appeared in the same region of the epidermal layer (Fig. 3B). These observations revealed the

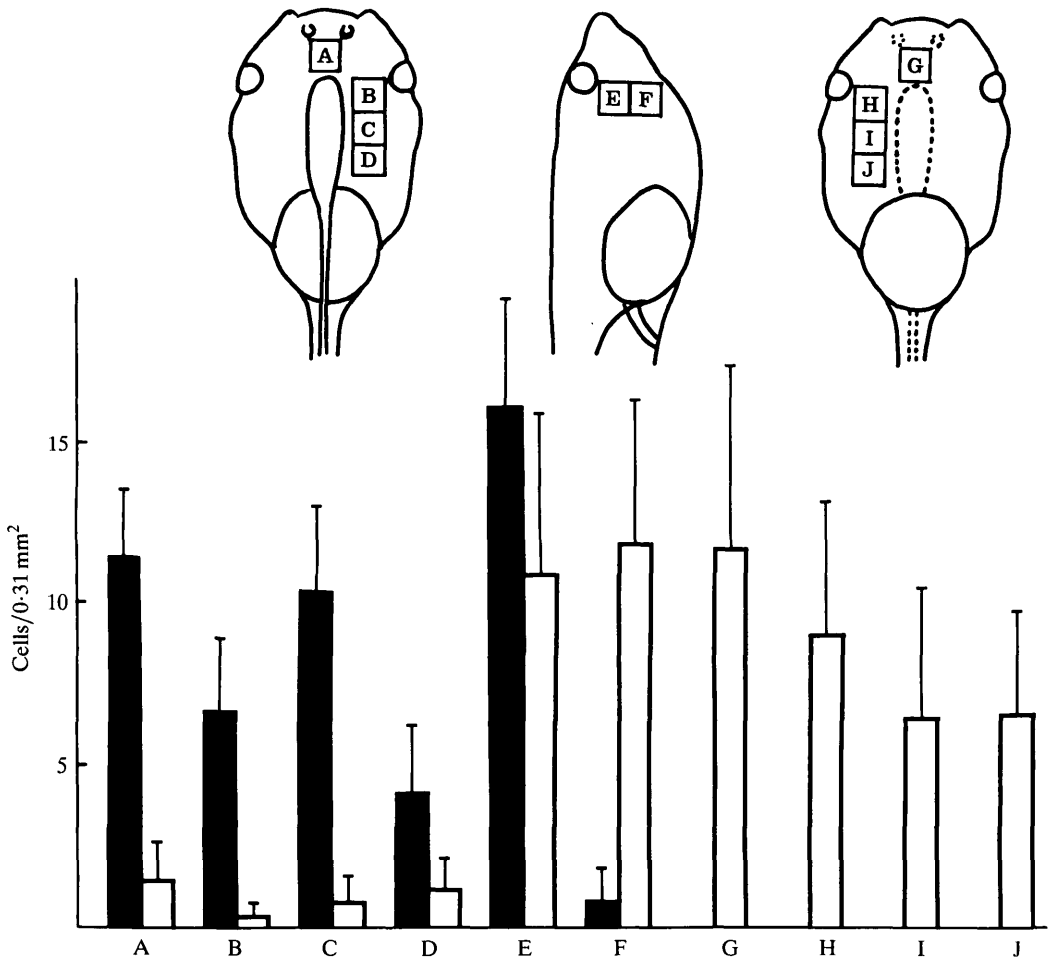


Fig. 4. The number of melanophores (black columns) and dopa-positive melanoblasts (white columns) in ten skin regions (0.31 mm<sup>2</sup>). A-D, dorsal region; E and F, lateral region; G-J, ventral region. Vertical lines = standard deviations.

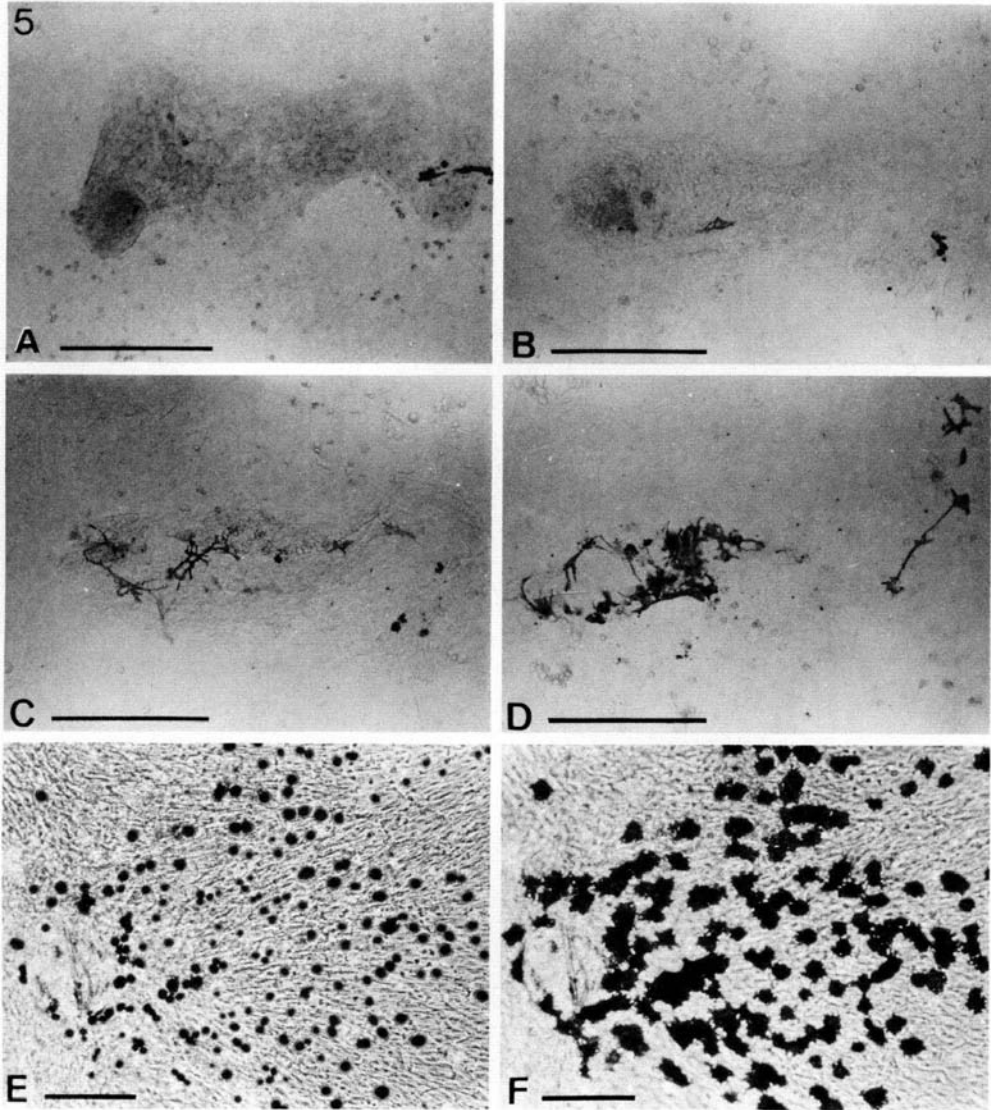


Fig. 5. (A–D) Serial photographs showing the differentiation of melanophores in cultured ventral skin. The black figures (right) in (A) and (B) are not of melanized cells but floating debris in the medium. Bar = 500  $\mu\text{m}$ . (A) after 1 day, no melanized cells appeared. (B) after 5 days, one slightly pigmented melanophore (centre) appeared. (C) after 9 days, many dendritic melanophores appeared, some of which were fully melanized. (D) after 15 days, the number of melanophores further increased. (E) and (F) Response of differentiated and proliferated melanophores to MSH stimulation. 64 days culture. Phase contrast. Bar = 200  $\mu\text{m}$ . (E) melanophores with concentrated melanosomes after the removal of MSH. (F) melanophores with dispersed melanosomes 90 min after the addition of MSH.

presence of dopa-positive melanoblasts, in the ventral epidermis, which remained unmelanized in the course of embryonic and postembryonic development. In dorsal skin, on the other hand, fully matured melanophores were localized beneath the collagen layer (subdermis, Fig. 3D).

*Dorsoventral distribution of melanophores and dopa-positive melanoblasts*

The numbers of melanophores and dopa-positive melanoblasts respectively were counted in ten regions of the body surface of stage-48 tadpoles. The results are summarized in Fig. 4. Although some variation in the cell number occurred, the predominant distribution of melanophores and melanoblasts in dorsal and ventral skin respectively was demonstrated. The number of dopa-positive cells in the ventral region was approximately equal to that of melanophores plus dopa-positive cells in the corresponding dorsal region. This indicates that melanoblasts were nearly evenly distributed in both regions, although the proliferation and migration of the blast cells may change the number slightly.

*The appearance of fully matured melanophores in cultured ventral skin*

To test the differentiative potency of the ventral melanoblasts, ventral skin was cultured in conditions which permitted the growth of bullfrog melanophores (Fig. 5A). Fibroblastic and epithelial cells began to migrate from the explants within 12 h. Within 2 to 5 days, slightly pigmented melanophores appeared in the explants in many cases (Fig. 5B). The number of melanophores increased gradually and melanization of the cells also progressed (Fig. 5C and 5D). In some cases, these melanophores migrated from the explants and proliferated (Fig. 5E and 5F). The differentiated melanophores responded to MSH stimulation. If MSH was removed from the culture medium, these cells contracted (Fig. 5E). Upon the addition of the hormone, they expanded (melanin dispersion) again (Fig. 5F). These results show that the melanoblasts existing in the ventral skin have the ability to differentiate into fully matured melanophores *in vitro*.

DISCUSSION

It has been previously demonstrated that the primary source of vertebrate pigment cells is the neural crest (DuShane, 1934, 1935; see Weston, 1970 for review). This has been confirmed in *Xenopus laevis* (Stevens, 1954). In the present experiment, after neural fold removal dorsal melanophores and ventral dopa-positive cells were not found. Thus the dopa-positive melanoblasts are of neural crest origin. The presence of melanoblasts in the ventral region indicates that a considerable number of neural crest cells can migrate to the ventral region, differentiate into melanoblasts and remain there without final development to melanized melanophores.

These results are inconsistent with the explanation of MacMillan (1976) who

interpreted the loss of melanophores in the ventral skin of *Xenopus laevis* by the low affinity between the melanoblasts and surrounding ventral tissues. Our present results showed that melanoblasts were not lost in the ventral skin and thus the melanogenesis of the ventral melanoblasts may be influenced by their surrounding tissues. However, the possibility of upward migration from the ventral region can not be excluded completely since the number of melanophores plus melanoblasts in the lateral region was large, although this could also be caused by proliferation of melanoblasts in that region.

It has been shown previously that when both the eyes of adult *Xenopus laevis* were removed surgically, the black pattern extended even to the ventral skin (Imai & Takahashi, 1971). This suggested the possibility of the presence of melanogenic cells in the ventral skin. Our present results proved that melanoblasts really existed in the ventral skin, especially in the epidermis and showed their number, distribution pattern and degree of differentiation. The number of ventral melanoblasts seems to be enough to make the colour of ventral skin as dark as that of dorsal skin if they differentiated completely into melanophores. Although these melanoblasts contained premelanosomes and tyrosinase activity, they remained unmelanized during the course of development. The ventral epidermis especially seems to make them remain in that state. Further studies are necessary to determine whether something in the ventral epidermis inhibits melanoblast differentiation, or whether something is lacking in the ventral epidermis. It is, however, at least certain that these ventral melanoblasts can differentiate into complete melanophores in some conditions.

In any case, the dorsoventral pigment pattern of *Xenopus laevis* tadpoles is formed as follows. The melanoblasts migrate from the neural crest region and, in dorsal regions, become distributed in the dermis and differentiate into melanophores. In ventral regions, they become distributed in the epidermis and remain unmelanized. Whether the melanoblasts differentiate into melanophores or not seems to be controlled by the information which exist in the dermis of dorsal skin or the epidermis of ventral skin. We can interpret such positional difference of information in terms of positional value (Wolpert, 1981). That is, melanoblasts recognize the difference in positional value of these regions and determine whether they should become black or not.

We also confirmed the presence of dopa-positive melanoblasts in the ventral region of the Japanese newt, *Cynopus pyrrhogaster*, just after hatching (unpublished). In flatfish, the presence of melanoblasts in the ventral region has also been reported (Osborn, 1941a). In salamander and catfish, the presence of cells with melanizing potency in the ventral region is also suggested (Lehman & Youngs, 1959; Osborn, 1941b). Further, much previous work suggests that the pigment pattern is formed by environmental influences on melanoblast differentiation (see INTRODUCTION). Thus in many fishes and amphibians, the dorsoventral pigment pattern may be formed in the same manner as described above.



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