The spatio-temporal framework of melanogenic induction in pigmented retinal cells of *Xenopus laevis*

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SUMMARY

Using methods involving transplantation of neuroectoderm and eye rudiments between \( a^p/a^p \) mutants and \(+/+\) embryos of *Xenopus laevis*, new aspects of melanogenic induction have been ascertained. The possibility of separate induction of a melanocyte cell type and of the product of terminal differentiation in it – melanosomes, has been demonstrated. Melanogenic induction starts at the late gastrula stage. It has an irreversible character which has been shown using the technique of double transplantation. Melanogenic factor (MgF) is produced only by the region of endomesoderm which is adjacent to eye rudiments in the course of normal development. Competence for melanogenic induction lasts till stage NF 30. Melanogenic induction appears to be species-specific; at least after transplantation of eye vesicles from \( a^p/a^p \) *Xenopus laevis* to *Rana temporaria* the melanoprotein synthesis cannot be switched on. L-Tyrosine, under the conditions of the experiments, is not a factor in the induction of melanogenesis.

INTRODUCTION

The mechanism of determination for melanoprotein production in cells, and thus of melanocyte differentiation, has not been studied. Most investigations have concerned cells already determined as melanocytes, but knowledge of early determinative events is of primary importance for complete understanding of cell differentiation (Whittaker, 1974).

Reactions by melanosomes within cells, synthesizing melanin itself, are governed by light, peptide hormones and cAMP (Fujii, Nakazawa & Fujii, 1973; Whittaker, 1974; Chen et al. 1974) and pigment cells of different origins show similar reactions (Burlakova, 1978). But the initial formation of melanosomes is primarily determined by inductive signals (Hoperskaya, 1978, 1980), with several groups of genes apparently participating (Doezema, 1973). The contribution of these genes can be studied by use of mutations affecting pigmentation (Vatti & Alekseevich, 1976), and this also allows us to discriminate between genes operative in the inducing tissue and those operative in the...
responding tissue. We have used embryos of the albino periodic mutant (ap/ap) of Xenopus laevis (Hoperskaya, 1975), which is a mutation of a regulatory gene, deficient in a specific inducing factor for melanogenesis (MgF) (Hoperskaya, 1980). Absence of this factor results in amelanotic development in both mutant and wild-type pigmented epithelium. The wild-type product is synthesized in endomesoderm and influences the character and numbers of melanosomes in the induced pigmented epithelial cells (Hoperskaya, 1977, 1978, 1980). In affecting primarily an inducing signal, the ap mutant thus resembles c (cardiac lethal) and o (ova deficient) in Ambystoma mexicanum (Briggs, 1973).

Adult ap/tp animals are practically albino, possessing very small numbers of melanosomes in dorsal regions only of the retinal pigmented epithelium. At early developmental stages melanosomes and premelanosomes are lacking (Bluemink & Hoperskaya, 1975) while at larval stages 39–40 (Nieuwkoop & Faber, 1956) melanosomes begin to form in the pigmented epithelium and also in the iris and the choroid coat (Hoperskaya, 1975). Some skin melanophores are pigmented as well. All the machinery of melanin synthesis is clearly available in the mutant and indeed tyrosinase, a key enzyme is more abundant in mutant oocytes and larval cells than in normals (Wyllie & De Robertis, 1976; Tompkins, Knight & Burke, 1977). The melanosomes initially formed are nevertheless abnormal and disappear at later stages (Hoperskaya, 1980, and unpublished).

Determination of the eye region within the neural plate starts in the late gastrula (Nieuwkoop, Oikawa & Boddingius, 1958) and we have evidence that induction of melanoprotein synthesis within this region may start around the same time. Following reciprocal transplantations of eye material at tail-bud stage (Nieuwkoop & Faber (1956) stage 21) only the ventral eye regions can still be affected (i.e. be induced or fail to be induced according to genotype of the mesodermal environment), implying that in other parts of the eye melanogenic induction has already occurred before eye-vesicle formation.

The purposes of the present investigation were: (1) to determine the temporal characteristics of melanogenic induction; (2) to find out if the induction process can be reversed in cells of pigmented epithelium; (3) to show the possibility of separate induction for the melanocyte cell type itself and for the products of terminal differentiation within it; (4) to find out whether melanogenic factor is produced in parts of the endoderm and mesoderm other than those which contact eye material in normal development; (5) to study the duration of competence for melanogenic induction and the species specificity of the signal; (6) to find out whether L-tyrosine is an adequate inducer of the melanin-producing machinery.

MATERIALS AND METHODS

Wild-type (+/+ ) and white mutant (ap/ap) embryos of Xenopus laevis were obtained by gonadotrophin injection (Brown, 1970). Stages of development were determined from the Normal Table (Nieuwkoop & Faber, 1956; =NF).
Table 1. *Determination of the start of melanoprotein induction and its irreversibility in Xenopus laevis*

<table>
<thead>
<tr>
<th>Series</th>
<th>Type of experiment</th>
<th>Number of embryos</th>
<th>Operated</th>
<th>Survived</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>a(^p)/a(^p) → +/+ st. 10</td>
<td>25</td>
<td>15</td>
<td>Pigmented epithelium is heavily pigmented, and in most cells it is not distinguished from pigmented epithelium of +/+ embryos</td>
<td></td>
</tr>
<tr>
<td>Ib</td>
<td>+/+ st. 10 → a(^p)/a(^p) st. 10</td>
<td>30</td>
<td>18</td>
<td>Pigmented epithelium contains melanosomes only of oocyte origin</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>a(^p)/a(^p) → +/+ st. 10</td>
<td>25</td>
<td>19</td>
<td>Synthesis of melanoprotein induced at the gastrula stage remains very high but not uniform in all pigment cells</td>
<td></td>
</tr>
</tbody>
</table>

In series IV we also used embryos of *Rana temporaria*, obtained by injection of hypophyseal hormones, and staged according to Dabagyan & Sleptsova, 1975 (=DS). In series VI we also used embryos of *Ambystoma mexicanum*, staged according to Harrison’s Normal Table (Rugh, 1962; =H).

Operations were performed in Niu-Twitty saline, and the embryos transferred after 2–3 h to sterile water + antibiotics. Tadpoles were later kept in dechlorinated tap water at room temperature. In series VI early embryonic cells of *Ambystoma* were cultured in Holtfreter solution, while early embryonic cells of *Xenopus* were cultured in Niu-Twitty (Rugh, 1962).

L-Tyrosine was added to the culture medium in the following concentrations: 1 \(\mu\)g/ml, 10 \(\mu\)g/ml, 100 \(\mu\)g/ml. We treated with L-tyrosine explants of early gastrula ectoderm (NF 10) and eye vesicles (NF 21) of *Xenopus laevis*, and explants of late blastula ectoderm (H 8), and anterior neural plate (H 16) of *Ambystoma mexicanum*.

Material was fixed for histology in Bouin’s fluid, at intervals from 1 week to 6 months after operation. Serial sections, cut at 5 \(\mu\)m, were stained with azocarmine and counterstained according to Mallory.

**RESULTS**

I. Temporal characteristics and irreversibility of melanogenic induction

Pieces of neuroectoderm corresponding to future eye vesicles were reciprocally exchanged between wild-type and mutant embryo at early gastrula stages.
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(NF 10) (Fig. 1, Table 1, series Ia). The pieces were slightly larger than the implantation sites to promote good healing. Disturbance to the axial structures led to some decrease in the viability of embryos after these operations. Grafted regions subsequently formed eye vesicles, diencephalon and small pieces of head skin, all easily distinguishable from the host contribution because of the different content of egg melanosomes up to the stages of skin melanophore pigmentation (NF 35–40). Embryos where some part of the eyes had been formed from the host were excluded from the results.

Where transfer had been from white to wild-type embryos, the subsequent development of melanophore migration was normal, confirming previous observations (Hoperskaya & Golubeva, 1977; MacMillan, 1979). Melanoprotein synthesis was dramatically activated in the pigmented epithelium of the eyes of mutant genotype in these embryos, being indistinguishable from wild-type intensity in most regions (Fig. 3). However in some regions, perhaps as a consequence of imperfect contact between neuroectoderm and endomesoderm the pigmentation intensity remains typical of the mutant (Fig. 3).

Where transfer had been from wild-type to white embryos, there was a failure of melanogenic induction, the pigmented epithelium of the wild-type dorsal iris containing even fewer newly synthesized melanosomes than are normally found in this region in mutants (cf. Figs. 2, 4 and 5), despite the normal morphogenesis which brought \( a^p/a^p \) lens epithelium into close contact with iris cells. Comparison of these specimens with normal ones allows quantitative assessment of the contributions to melanogenesis made by melanosomes of egg origin and those newly induced (Table 1, series Ib, Fig. 9).

**Figures 1–6**

Fig. 1. Experimental scheme of future eye region transplantation at early gastrula stage from mutant embryo to wild type; secondary transplantation of eye vesicle formed at the expense of mutant material at tail-bud stage. Grafted eye, which had been in contact with endomesoderm of \(+/+\) embryo, irreversibly preserves a high level of melanoprotein synthesis.

Abbreviations for this and later figures: Chc, choroid coat; endom, endomesoderm; EV, eye vesicle; I, iris; Int, interzone; IPE, induced pigmented epithelium; L, lens; n-f eyes, newly formed eyes; PE, pigmented epithelium; R, retina; UnII, uninduced iris; UnIPE, uninduced pigmented epithelium; VR, visual receptors.

Fig. 2. Mutant amelanotic pigmented epithelium (NF 47).

Fig. 3. Pigmented epithelium of a tadpole eye (NF 47), which has been formed from mutant eye material after its transplantation at early gastrula stage.

Fig. 4. Pigmented epithelium of a tadpole which developed from \(+/+\) eye material, transplanted to a mutant endomesoderm at early gastrula stage. Only individual melanosomes of oocyte origin are seen.

Fig. 5. Iris of tadpole eye totally free of melanosomes. It arose from \(+/+\) material after its grafting to mutant endomesoderm at early gastrula stage.

Fig. 6. Mutant eye after the secondary transplantation at stage NF 22. In those parts where induction occurred, it had an irreversible character.
Fig. 7. Absence of the pigmented epithelial cell type, appearing as a consequence of eye material transplantation from mutant to +/+ embryo at early gastrula stage.

Fig. 8. Aggregates of pigmented epithelial cells appeared from mutant eye material after its transplantation to +/+ embryo. Some cells are induced to melanoprotein synthesis, others are not.

Fig. 9 Group of pigmented epithelial cells which appeared from +/+ material after its transplantation to mutant embryo. Cells contain pigment granules only of oocyte origin.

Fig. 10. Inverted retina appearing in case of a disturbance of normal morphogenesis after presumptive eye material transplantation at the early gastrula stage. Choroid coat does not form.

In certain of these reciprocally chimaeric animals, eye morphogenesis was not normal, and cells of the pigmented epithelial layer were absent or were gathered in large groups, causing abnormal contact relationships between the various eye layers and the surroundings. All types of pigmented epithelial differentiation were, therefore, obtained with successful or unsuccessful melanogenic induction independent of the cells’ genotype (Fig. 11). Where the retinal layers themselves were inverted, the choroid coat was not formed at all (Fig. 10).
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This set of results demonstrates that subsequent to stage 10, selective induction of the pigmented epithelial zone of eye anlage, with its distinctive cell type, occurs, to be followed only later after further inductive influences by the activation of melanoprotein synthesis in this cell type. The influences of lens, ectomesenchymal cells and circulation (Lopashov, 1963) and arrangement of primary eye cells in a layer (Lopashov & Hoperskaya, 1967) formerly considered decisive factors in the determination of the pigmented epithelial cell type, now seem to be secondary influences governing the full expression of its differentiation (Hoperskaya & Golubeva, 1979).

Mutant eye vesicles which had developed in a wild-type environment up to stage NF 21, after a\(^p\)/a\(^p\) to wild-type grafts of the sort described above, were then back-transferred to mutant hosts to replace their own eye vesicles, removed via a dorsal slit. Eyes in such embryos developed much more intense pigmentation than that of control a\(^p\)/a\(^p\) eyes, showing that a substantial amount of irreversible melanogenic induction occurs by early-eye-vesicle stages, before embryonic melanoprotein synthesis actually starts (Fig. 6, Table 1, series II).

II. Duration of competence of melanocytes for melanogenic induction, and species-specificity of the signal

Eye rudiments from stages NF 30 and 32 were grafted to replace the eye vesicles of stage-21 embryos, thus contacting their inducing endomesoderms. Small regions only of the pigmented epithelium of a\(^p\)/a\(^p\) rudiments, grafted to wild-type hosts in this way, were activated to form melanosomes (Table 2, series IIIa). Five hours later, at stage 32, induction of melanogenesis failed completely (Table 2, series IIIb) (Fig. 12), showing that pigmented cells are by this time impermeable, or in some other way resistant, to the inducing signal.

We did not observe melanogenesis following transplantation of eye vesicles from *Xenopus* to *Rana temporaria* (Fig. 13, Table 2, series IVa) although transplantation to +/+ *Xenopus* at the same stages causes dramatic induction of melanin synthesis (Hoperskaya, 1977, 1978). This apparent species-specificity of the induction process may relate to (1) qualitative specificity of MgF; (2) inability of this factor to interact across species with other factors affecting competence, or (3) absence of an MgF synthesis in endomesodermal cells adjacent to *Rana temporaria* eye vesicles at the stage concerned (DS 26). This situation may be analogous to the quantitative variations in distribution of lens inducing capacity in head endomesoderms and eye vesicles as between different amphibian species (Tahara, 1962a, b; Lopashov, 1963).

Following implantation of heterospecific eyes, host ectomesenchyme cells migrate only poorly from *R. temporaria* host onto the grafted eye vesicles, at those morphological stages where this migration is still intensive in *Xenopus* (Hoperskaya, 1978). The timing of processes crucial to melanogenic induction may differ in the two species.

The reciprocal eye vesicle transplantation to the above, i.e. stage-26 (DS) *R.
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temporaria to ap/ap Xenopus stage 21, gives eyes whose pigmentation is hardly distinguishable from that of control Rana eyes (Fig. 14, Table 2, series IVb). Since ap/ap Xenopus endomesoderm cannot induce melanoprotein synthesis we can conclude that melanogenic induction of future retinal pigmented epithelium in Rana has already occurred by DS stage 26.

III. Heterotopic transplantation of eye vesicles and anterior neural plate pieces to the belly region

Our aim, in the transplantation of eye vesicles and eye-forming regions of the neural plate to ectopic sites, was to ascertain whether mesoderm or endoderm other than that normally coming to be adjacent to the eye possesses melanogenic inducing capacity. Data in Table 3 show that such tissues located ventral and caudal to the normal region possess no such capacity, since mutant material transplanted from eye vesicle or antero-lateral stage-16 neural plate under the belly epidermis of stage-21 +/+ embryos, remained ap/ap in pigmentation. In the reciprocal transplantation, the fully melanized pigmented zones of the ectopic eye structures developed (Figs. 16, 17) showed that melanogenic induction, as well as the induction of the pigmented epithelial cell type (see part I), had occurred in the donor by stage 16 (Table 3, series Va).

In one series of operations, ap/ap stage-21 vesicles were grafted into contact with the anterior side of the left vesicle of a +/+ host of the same stage. The transfer of the induced state, i.e. melanization of the ap/ap epithelium contacting the host eye and brain but devoid of contact with endomesoderm, was never observed (Figs. 18, 19). We conclude that induced wild-type eye vesicle, or brain, cannot itself furnish the inductive signal (Table 3, series Vc, d).

Figures 11–14

Fig. 11. Schematic representation of all possibilities for pigmented epithelium differentiation independently of the cell genotype as a result of presumptive eye material transplantation at early gastrula stage; absence of pigmented epithelium cell type at all; pigmented epithelium in a form of aggregate, amelanotic; pigmented epithelium in a form of monolayer, amelanotic; pigmented epithelium in a form of aggregates, overloaded with melanosomes; pigmented epithelium in a form of monolayer, overloaded with melanosomes.

Fig. 12. Pigmented epithelium of ap/ap embryo after eye rudiment transplantation (NF 32) to endomesoderm of wild-type embryo (NF 21). Absence of melanogenesis.

Fig. 13. Pigmented epithelium of Xenopus laevis, transplanted at st. NF 21 to Rana temporaria st. DS 26. Activation of melanogenesis does not proceed.

Fig. 14. Pigmented epithelium of Rana temporaria eye transplanted at st. DS 26 to Xenopus laevis st. NF 21.
Table 2. Duration of melanocyte competence and species-specificity of melanogenic induction

<table>
<thead>
<tr>
<th>Series</th>
<th>Type of experiment</th>
<th>Number of embryos</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Operated</td>
<td>Survived</td>
</tr>
<tr>
<td>IIIa</td>
<td>Transplantations between embryos of various age</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>IIIb</td>
<td>$a^+ / a^+$ st. 32 $\rightarrow$ $a^+ / a^+$ st. 21</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>IVa</td>
<td>$X.l. a^+ / a^+$ st. 21 $\rightarrow$ $R.t.$ st. 26</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>IVb</td>
<td>$R.t.$ st. 26 $\rightarrow$ $X.l. a^+ / a^+$ st. 21</td>
<td></td>
<td></td>
</tr>
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</table>

IV. Test of L-tyrosine as a possible specific melanogenic inductor

Landström & Løvtrup (1978) have suggested that L-tyrosine might be the melanogenic inducer in the embryo. It is an initial substrate for melanin synthesis (Riley, 1977), and may be an activator for melanogenesis in certain cells already differentiated to produce melanin (Gilbert et al., 1973; van Woert, 1973). In two series of experiments we have incubated the early gastrula ectoderm cells of *Ambystoma mexicanum* and *Xenopus laevis* in L-tyrosine solutions. In large groups of *A. mexicanum* cells we did not observe the appearance of pigment granules, whereas in small groups of early embryonic cells, melanin granules were found approximately to the seventh day of cultivation.

In cultures of various age cells of *Xenopus laevis*, treated with L-tyrosine even...
Table 3. Heterotopic transplantations of eye material (to ascertain the localization of melanogenic activity on Xenopus embryos)

<table>
<thead>
<tr>
<th>Series</th>
<th>Type of experiment</th>
<th>Number of embryos</th>
<th>Operated</th>
<th>Survived</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vb</td>
<td></td>
<td>15</td>
<td>14</td>
<td>Synthesis of melanoprotein does not change, i.e. corresponds to +/+ type</td>
</tr>
<tr>
<td></td>
<td>Vc</td>
<td></td>
<td>15</td>
<td>12</td>
<td>Synthesis of melanoprotein corresponds to +/+ type</td>
</tr>
<tr>
<td></td>
<td>Vd</td>
<td></td>
<td>12</td>
<td>11</td>
<td>Activation of melanoprotein synthesis does not take place</td>
</tr>
</tbody>
</table>

in the highest concentration, we did not observe any signs of melanization. This suggests that the inducer of melanogenesis cannot be such an elementary compound as L-tyrosine. Apparently without L-tyrosine (or L-phenylalanine) the process of melanogenesis in cells cannot proceed. Therefore, the availability of these amino acids is an obligatory but insufficient condition for melanogenesis. Moreover, it is unlikely that the \( a^p \) mutation, which is quite viable, could be for a synthetic step in amino-acid metabolism, and also unlikely that so ubiquitous a molecule as tyrosine should be topographically restricted, as our results show that the melanogenic inducing signal is.

**DISCUSSION**

Our results have given us insight into the timing and positioning of melanogenic induction. This starts to a considerable extent at the middle–late gastrula stage, evidently as a component of primary embryonic induction. During this stage the pigmented epithelium cell type emerges and melanogenesis in it starts, but these processes are not identical and do not automatically follow each other. The induction of a selective cell type of retinal pigmented epithelium can be separated from the induction of terminal differentiation in it. As a result of such separation the amelanotic pigmented epithelium both in the form of a layer and in the form of aggregates can arise, independently of the genotype of reactive tissue.

These two different aspects of the induction of the retinal pigmented epithelial
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cells are mediated by different influences coming from the same sources. As far as concerns the retinal pigmented epithelium there are two sources of melanogenic induction. The first source is prechordal plate underlying eye material in the course of gastrulation and neurulation and the second source is an endomesodermal region situated behind the eye vesicles at tail-bud stage (Fig. 15). Transplantations of eye material into alien surroundings shows that only these zones possess melanogenic activity.

Experiments involving neural plate isolation show that up to the stage of neurulation the process of melanogenesis in future melanocytes has already been switched on earlier, at middle-late gastrula stage, but apparently not in all cells. The process of melanogenic induction continues until tail-bud stages. It has been shown (Hoperskaya, 1978) that mutant eye vesicles, transplanted at st. NF 21 to +/+ endomesoderm, always contain retinal pigmented epithelium which has been induced to melanogenesis, either in all cells or only in the most ventral cells. The inherited amelanotic state of these cells was overcome by the endomesodermal influences of +/+ embryos. In reciprocal eye transplantations at this stage only the ventral parts of +/+ pigmented epithelium were amelanotic, whereas those parts which were subjected to melanogenic induction at earlier stages were unable to reverse their melanized state. These results clearly show that the ventral part of the retinal pigmented epithelium undergoes melanogenic induction last.

Therefore, the temporal framework of melanogenic induction in normal development stretches only between the stages of middle-late gastrula and tail bud and depends on two sources of equivalent strength. The results of the present investigation show that competence of eye material for melanogenic induction lasts till st. NF 30–32.

Grafting of presumptive eye material of +/+ embryos to an a^p/a^ environment causes failure of embryonic melanoprotein induction in pigmented epithelial cells, and thus allows us to estimate quantitatively the contribution of pigment granules of egg origin to the overall population of melanosomes.

Like many other induction processes, that for melanogenesis, and its expression as melanin synthesis, is irreversible once accomplished, demonstrating the switch-like character of this cellular response in development.

The selective induction of the pigmented epithelial cell type may be better understood if we consider the mutation of eyeless (e) in Ambystoma. The eyeless mutation, which is expressed, among other peculiarities, in the complete absence of eyes, affects the competence of neuroectodermal material (van Deusen, 1973; Epp, 1978). If we suppose that a certain competence might depend on the synthesis of specific proteins (Grunz, 1970), the absence of the protein synthesis, coded for by gene e, located in neuroectoderm would make the formation of eye material impossible. In our experiments with a^p mutants the competence for eye material formation is available, but the absence of the factors mediating melanogenic induction may result in the absence of the
product of terminal differentiation within cells of the normally pigmented retinal epithelium. Generally speaking we can suppose that the formation of the pigmented epithelial cell type might depend on the interaction of factors determining competence, and localized in neuroectodermal cells, with factors determining induction and entering these cells from their environment. Investigation of the process of melanocyte differentiation can serve as the starting point for deeper insight into mechanisms of the correlation between induction and competence in the emergence of individual cell types.

One of the most intriguing questions is that of the nature of the factors mediating melanogenic induction. The present results show that L-tyrosine cannot be the activator of melanogenesis, and apparently does not play such a role in normal development.

The emergence of melanin-containing cells among the early embryonic cells of *Ambystoma* treated with L-tyrosine or some other chemicals (Landström & Løvtrup, 1978; Barth & Barth, 1974) tells us nothing of the normal induction of melanogenesis. It is known that living embryonic cells contain many more potential-inducing factors than those which are normally active (Born, Tiedemann & Tiedemann, 1972; Tiedemann, 1975). The release of inducing factors which are normally inactive, together with the ease with which *Ambystoma* embryonic cells in cultures can deteriorate may account for unspecific inductions without inducers.

The approach developed in the present communication suggests how the true determination of melanocytes arises, how an individual melanocyte cell type is brought about by different inductive actions, and how it is possible to turn on or off melanin-biosynthesizing machinery in cells at certain stages of development by the action of a melanogenic factor, localized in endomesoderm.

We express here our gratitude to Professor G. V. Lopashov for his advice and critical reading of the manuscript, and to Dr Jonathan Cooke for his editorial work.

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*(Received 20 November 1979, revised 12 May 1980)*