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Genetic Control of Adult Pigment Stripe Development in Zebrafish

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Adult zebrafish stripes are formed from stripes of gold iridophores alternating with stripes of black melanocytes lying beneath silver stripes. Analysis of defects in pigment pattern development caused by *sparse* (*spa*, *rose* (*ros*), and *leopard* (*leo*) single and double mutant combinations suggests that spa^+ and ros^+ functions are required for development of separate populations of pigment cells in the adult and that leo^+ functions to control assembly of melanocytes into stripes. Thus, between 2 and 3 weeks of zebrafish development, *spa*-dependent melanocytes differentiate throughout the flank, followed by *leo*-dependent assembly of these cells into stripes. Beginning at 3 weeks of development, a distinct *ros*-dependent population of melanocytes differentiates in the stripe. Both early and late differentiating melanocytes then affect the formation of the silver stripes, ensuring registration of melanocyte and iridophore stripes.

INTRODUCTION

Development of pigment pattern requires coordination of a variety of developmental mechanisms. In poikilotherms, such as fish or amphibia, the pigment pattern may consist of multiple pigment cell types (Bagnara and Hadley, 1973). Because pigment cells arise from the neural crest (DuShane, 1934; Rawles, 1944, 1948; Raible *et al.*, 1992), development of pigment pattern provides another opportunity to study how cells with different fates segregate from the pluripotent neural crest population (Weston, 1991). In many animals, pigment pattern may change during the transition from larval to adult stages (Niu and Twitty, 1950). How pigment pattern changes during the course of development is not clear, but may involve *de novo* differentiation of pigment cells from precursors set aside during embryonic development. In addition, the stereotyped sequence of development and positions of different pigment cells with respect to each other suggests that cell–cell interactions may be involved in coordinating the pattern of the various pigment cells (Goodrich and Nichols, 1931; Goodrich *et al.*, 1954; Epperlein and Löfberg, 1984).

The organization of zebrafish pigment cells into precise patterns has been described. (Kirschbaum, 1975). Pigment pattern in zebrafish consists of melanocytes (black cells containing melanin), gold or silver iridophores (iridescent cells containing either gold or silver reflecting platelets), and xanthophores (yellow cells containing pteridine pigments). Beginning at 2 weeks, melanocytes that ultimately contribute to adult stripe pattern arise in the dermis and are distributed evenly throughout the flank. During the next week, these and subsequently developing dermal melanocytes coalesce into stripes. Organization of differentiated dermal melanocytes into stripes, presumably via cell migration, requires the *leopard* (*leo*) gene product (Kirschbaum, 1975). In animals lacking *leo* gene function, dermal melanocytes remain dispersed throughout the flank, resulting in the appearance of melanocyte spots, rather than the normal stripes of the wild-type fish. Although the regulatory mechanisms that coordinate the morphogenetic behaviors of melanocytes and iridophores is obscure, the association of melanocytes with silver iridophores, and not gold iridophores, is retained in *leo* mutants.

Epidermal melanocytes contribute to a different component of adult pattern from dermal melanocytes. Thus, the epidermal melanocytes are restricted to the dorsal third of the body, imparting a dark aspect to the back of the fish. By 4 weeks of development, epidermal melanocytes are easily distinguished from dermal melanocytes by the association of the former with the scales (Kirschbaum, 1975). Because the pattern of epidermal melanocytes is not changed in *leo* mutants (Kirschbaum, 1975), the patterning of epidermal and dermal melanocytes is likely to be under different genetic control.

To begin to elucidate how different types and populations of pigment cells arise in the adult pattern, and how their morphogenetic behaviors are coordinated in development of stripes, we have begun a genetic dissection of adult pigment pattern development. Here, we report analysis of three mutations that disrupt development of adult pattern. Mutants in *sparse* (*spa*; Streisinger *et al.*,

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1986) and *rose* (*ros*) disrupt development of distinct classes of pigment cells, whereas *leo* mutants alter the distribution of differentiated pigment cells in the dermis. Analysis of single and double mutant combinations of these three genes suggest that the development of distinct classes of pigment cells, under different genetic control, contribute to the adult pattern. Pattern of residual classes of pigment cells in single or double mutants was then used to assess dependent relationships between pigment cells responsible for the coordinated development of the melanocyte stripe and the gold and silver iridophore stripes.

MATERIALS AND METHODS

Stocks. Wild-type and mutant stocks were maintained as described (Westerfield, 1993). Initial mutant stocks were kindly provided by Dr. Charles Kimmel (*spa* and *ros*) and Dr. Janni Nusslein-Vollhard (*leo*). spa^{b5} (Streisinger *et al.*, 1986) and leo^{t1} (Kirschbaum, 1975) have been reported. ros^{b140} was isolated as a spontaneous mutation in the University of Oregon zebrafish colony and has not been previously reported. Initial mutant stocks were intercrossed with the clonally derived strain, C32 (Streisinger *et al.*, 1981), before phenotypic analysis. Double mutants were produced with the expected Mendelian ratios for unlinked or loosely linked genes. Genotypes of true-breeding double mutant stocks were confirmed by complementation testing.

leo^{t1} and spa^{b5} alleles are the most severe viable alleles available and appear to be complete loss-of-function alleles of their respective genes. Thus, trans-heterozygotes of leo^{t1} and a gamma-ray-induced deficiency of the corresponding region of linkage group I (A. Fritz, personal communication) results in animals with indistinguishable pigment pattern from that of leo^{t1} homozygotes (not shown). Recently, we have identified three ENU-induced *spa* alleles and one gamma-ray-induced allele by noncomplementation screens over the spa^{b5} allele. In each case, the trans-heterozygote of the new allele with the spa^{b5} allele resulted in an identical phenotype to that of spa^{b5} homozygotes. Additional alleles of *ros*, or deficiencies of the *ros* region (near the centromere of linkage group I; unpublished results), have not been identified.

Melanocyte counts. Fish were prepared for melanocyte counts by first incubating fish of various ages in 1 μg/ml epinephrine to cause contraction of melanosomes into the cell body (Goodrich and Nichols, 1931), followed by anesthesia and fixation in buffered formalin. For whole-body counts (Fig. 1), all melanocytes were counted, including, in adult stages, both stripe-associated (dermal) and scale-associated (epidermal) melanocytes. At 4 weeks, stripe melanocytes are easily distinguished from those that are associated with scale. Counts of stripe-associated (dermal) melanocytes at 4 weeks (Fig. 2) were restricted to one side of each fish, in a region delimited anteriorly by a line between the anteriormost rays of the anal and dorsal fins and posteriorly by the caudal peduncle.

Photography. Fish were prepared for photography by embedding in 1% agarose in a clear plastic petri dish, following anesthesia. Petri dishes were then inverted for photography. Illumination with diffuse daylight was used to reveal melanocytes and gold iridophores, or with an oblique tungsten source to reveal silver iridophores.

TABLE 1

PIGMENT CELL DEFICIENCIES IN ZEBRAFISH PIGMENT PATTERN MUTANT EMBRYOS OR LARVAE (2–14 DAYS) AND ADULT ANIMALS (4 WEEKS)

	spa	*ros*	*leo*
Embryo and larvae			
Melanocytes	–[a]	+	+
Iridophores	+	+	+
Xanthophores	+	+	+
Adult			
Melanocytes			
Fin melanocytes	+	+	±
Scale-associated melanocytes (epidermal)	–	+	+
Stripe-associated melanocytes (dermal)	±	±	±
Iridophores			
Gold stripe	+	–	+
Silver stripe	+	–	+
Eye (silver)	+	+	+
White cells[b] (dorsal and caudal fins)	–	+	+
Xanthophores	+	+	+

Note. + Indicates no gross deficiencies of cell type in mutant, ± indicates about 50% of cells not visible, – indicates severe deficiency in pigment cell type, compared to wild-type animals.

[a] In *spa* mutants, embryonic melanocytes disappear soon after differentiating (see Fig. 1).

[b] The white cells found at the tips of the dorsal and caudal fins are not clearly analogous to other types of pigment cells described in the literature. These cells are morphologically similar to melanocytes, but contain a white, noniridescent pigment.

RESULTS AND DISCUSSION

Single Mutant Phenotypes

Table 1 summarizes qualitative assessment of pigment cell deficiencies in *spa*, *ros*, and *leo* mutants in embryonic, larval, and adult stages. Only *spa* mutants show defects in embryonic and larval pattern, whereas mutants for all three genes show defects in adult pattern. During larval stages (3 days to 2 weeks), melanocytes that differentiated in the embryo disappear from *spa* mutants (see Fig. 1), whereas *ros* and *leo* embryos are indistinguishable from wild-type embryos (not shown).

spa, *ros*, and *leo* cause distinct defects in adult pigment pattern. Mature *spa* mutants lack most or all the scale-

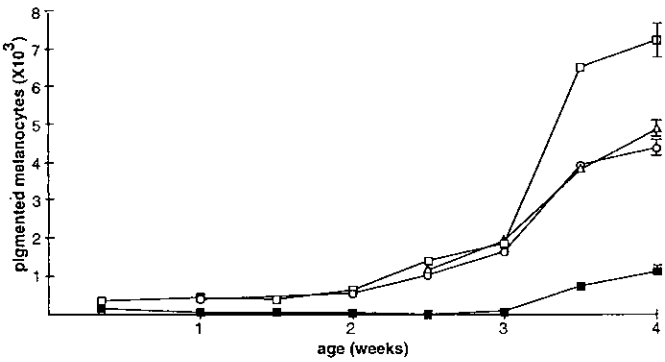

FIG. 1. Total melanocytes during the first 4 weeks of development in wild-type (open squares), *spa* (filled squares), *ros* (open circles), and *leo* (open triangles). Fish were incubated in 1 μg/ml epinephrine to concentrate melanosomes to the center of the cell (Goodrich and Nichols, 1931) and then fixed in formalin and counted with the aid of a Wild M3Z stereomicroscope. Between 3 and 10 fish were scored for each point. Error bars, shown for 4-week counts only, indicate standard deviation. Though *ros*, *leo*, and *spa* have nearly the same number of stripe melanocytes at four weeks, the fact that *spa* mutants lack scale-associated melanocytes, which are unaffected in *ros* and *leo* mutants, accounts for most of the difference in total number of melanocytes between *spa* and *ros* or *leo* mutants shown here.

associated (epidermal) melanocytes that are normally found in the dorsal third of the zebrafish (see Fig. 2), and lack the white pigment cells normally found at the tips of the dorsal and caudal fin (not shown). In contrast, mature *ros* mutants lack most dermal iridophores, retaining silver iridophores only around the eyes and a thin band of gold iridophores near the horizontal myoseptum. Thus, it seems unlikely that *ros* mutation results in a biosynthetic defect in iridophore reflecting platelet synthesis. Mutants for *leo* have all pigment cell types, but they are patterned in spots, rather than stripes. Quantitative analysis of the dermal melanocytes at 4 weeks, (below, see Table 2) indicates that *spa*, *ros*, and *leo* mutants each lack about 50% of the dermal or stripe-associated melanocytes found in wild-type animals.

Different phases of adult melanocyte development correspond to time of expression of spa, ros, and leo defects. To further explore the role of *spa*, *ros*, and *leo* in melanocyte pattern development, we assessed the number of melanocytes in wild-type and mutant fish at various ages of development through 4 weeks, when adult stripe pattern is established (Fig. 1). During the early larval period, from 3 days to 2 weeks of development, there is little increase in melanocyte number in wild-type fish. Melanocyte number then begins to increase starting at 2 weeks of development and starts to level off before 4 weeks. This increase appears to be biphasic—an initial increase in melanocyte number between 2 and 3 weeks of development, followed by a sharp increase in melanocyte number between 3 and 3.5 weeks. The inflections in the curve for melanocyte number in the wild-type animals matches the time that melanocyte deficits are most pronounced in *spa*, *ros*, or *leo* mutants. Mutants for *spa* have few or no melanocytes through 3 weeks development, suggesting that the *spa*$^+$ gene is required for the first phase of melanocyte increase. In contrast, *ros* or *leo* melanocyte deficiencies are not apparent until after 3 weeks of development, suggesting that *ros*$^+$ and *leo*$^+$ genes are required for a later phase of melanogenesis. Accumulation of dermal melanocytes during development of the stripes seems to parallel the findings from whole-fish melanocyte counts. In *spa*, dermal melanocytes are not observed until 3 weeks or later. In contrast, dermal melanocytes in *ros* and *leo* mutants appear indistinguishable from wild-type until after 3 weeks of development. By four weeks, *spa*, *ros*, and *leo* mutants have 48, 54, and 52% of wild-type dermal stripe melanocytes (Table 2). Together, these results raise the possibility that *spa* affects the development of one population of stripe melanocytes and *ros* and *leo* affect later development of a second population.

That *spa* on one hand and *ros* and *leo* on the other affect the time of appearance of melanocytes at distinct stages during development is not of itself a sufficient argument for distinct populations. An alternative to two distinct populations of melanocytes in the melanocyte stripe is that there is only a single population and that *spa* acts on its development at one stage and *ros* and *leo* act on its development at a later stage. For instance, the *spa* mutation might affect early proliferation of the dermal melanocyte precursors, acting in the mutant to halve the precursor population. Likewise, the *ros* and *leo* mutations might affect later stages of development, acting to prevent differentiation of approximately one-half the cells within a common population. As described below, such models for stochastic mutant affects, including affects on the same cells or cell lineages at different times during their development, can be tested by examining the phenotypes of double mutants.

Double Mutant Phenotypes

We propose that the *spa* mutation affects one population of dermal melanocytes, and *ros* and *leo* mutations affect a distinct population. We refer to the *spa*$^+$-dependent dermal melanocytes as early stripe melanocytes (ESM) and the *ros*$^+$/*leo*$^+$-dependent melanocytes as late stripe melanocytes (LSM). A reasonable alternative to distinct populations is that these mutations act stochastically to affect the development of some of the cells within the entire population of dermal melanocytes or their precursors. Thus, one dermal melanocyte is as likely to be affected by the *spa*, *ros*, or *leo* mutations as any other dermal melanocyte. To test the predictions that arise from these alternative hypothesis, as summarized in Table 2, we have examined the dermal melanocytes in double mutants. We reasoned

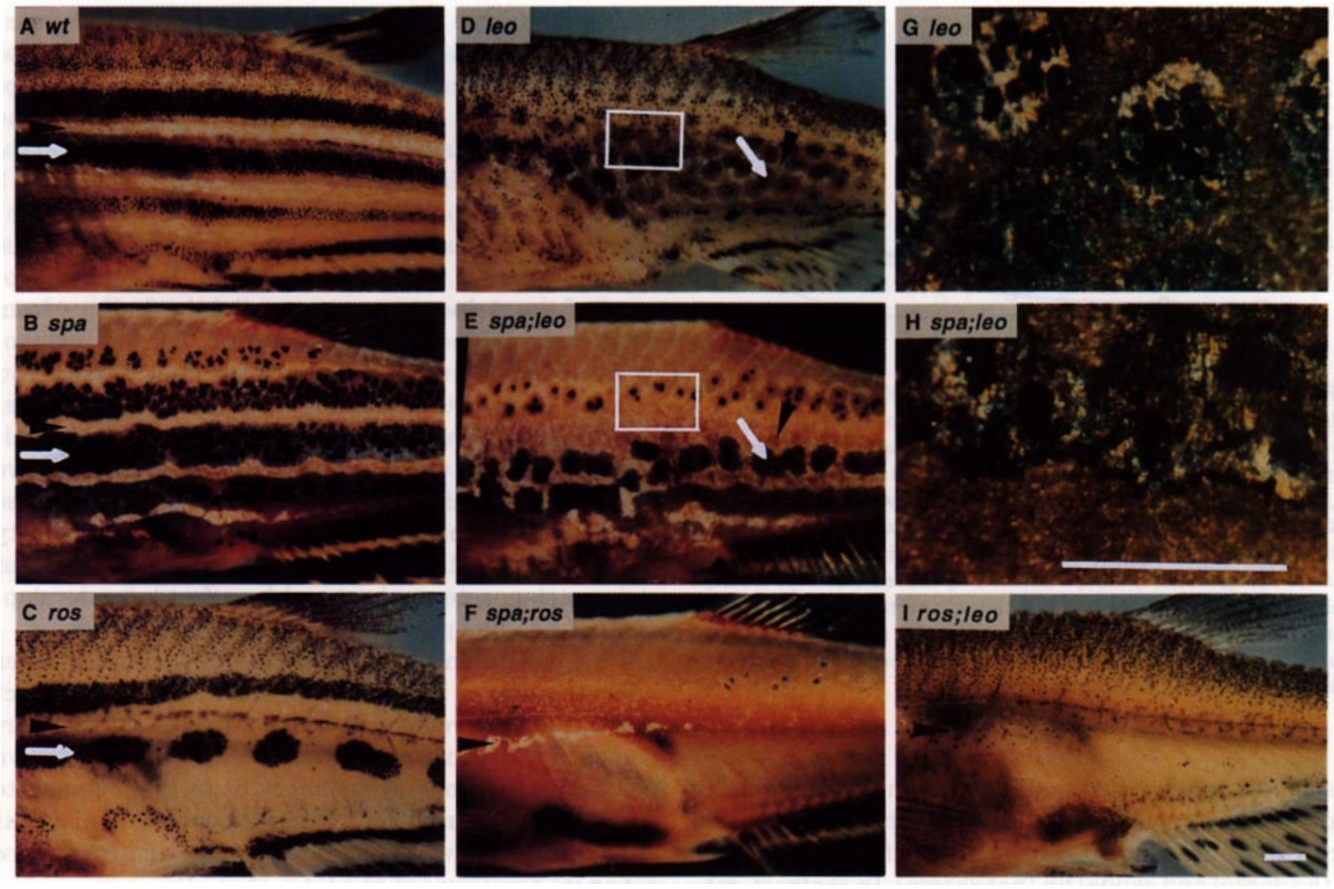

FIG. 2. Pigment cell patterns in single and double mutant adult zebrafish. Genotypes are indicated in the upper lefthand corner of each panel. Black arrowheads point to gold iridophore stripes, or gold iridophore regions in the case of *leo* mutants; white arrows point to melanocyte/silver iridophore stripes, or spots in *leo* mutants. Subjects in A–F and I were photographed with indirect daylight (to reduce glare), but which also fails to clearly illuminate silver iridophores. Reflection of a nearby tungsten source in G and H allowed for better visualization of silver iridophores. Regions shown are higher magnification of boxed regions in D and E, but from different individuals. Note absence of scale-associated (epidermal) melanocytes in dorsal third of *spa* mutants (B, E, F) and absence in *ros* mutants of most iridophores (C, F, and I, arrowhead indicates single band of gold iridophores that persist in *ros* mutants). Scale bar, 1 mm (panels H and I).

that if *ros* or *leo* were disrupting the development of a distinct subpopulation of the dermal melanocytes, then we would expect no additional deficit in dermal melanocytes in the *ros;leo* double mutant. Alternatively, if *ros* and *leo* each acted stochastically to affect development of cells within the entire dermal melanocyte population, then we would expect to find a more severe deficit in the double mutants than in either single mutant. The result that *ros;leo* double mutants retain nearly as many melanocytes as *leo* mutants alone (44% of wild-type, 85% of *leo* melanocytes, see Table 2) excludes the model that *ros* and *leo* act stochastically to affect the development of cells from the entire population of dermal melanocytes and supports our model that the *ros* and *leo* mutations affect the development of a distinct subpopulation of dermal melanocytes (LSM).

The melanocytes that persist in *ros;leo* double mutants identify a second subpopulation of dermal melanocytes. To determine whether these melanocytes correspond to the melanocytes that are affected in the *spa* mutants, we examined double mutants for *spa;ros* and *spa;leo*. We reasoned that if *spa* affected a second, distinct population of dermal melanocytes, then double mutants of *spa* with *ros* or with *leo* should show additive affects, resulting in severe deficiencies in dermal melanocytes. Alternatively, if *spa* acts stochastically to affect the development of some of the cells in the entire population, then we would expect overlap in its combined affect in double mutants with *ros* or *leo*. In this case, we would predict *spa;ros* double mutant to retain 26% and *spa;leo* double mutants to retain 25% of the wild-type number of dermal melanocytes. Our observation that *spa;ros* and *spa;leo* double mutants lack nearly all dermal melanocytes (3 and 8% of wild-type melanocytes, respectively, see Table 2) indicates that the population of dermal melanocytes that are unaffected by *ros* and *leo* mutations are affected by the *spa* mutation. The most parsimonious explanation is that the population of mel-

TABLE 2
EPISTASIS ANALYSIS OF MUTATIONS THAT AFFECT DEVELOPMENT OF STRIPE MELANOCYTES

Genotype	Percent of wild-type stripe melanocytes	Predictions for double mutants			Inferred defects in stripe melanocyte populations
		Epistatic	Multiplicative	Additive	
spa	48 ± 9	—	—	—	ESM development
ros	54 ± 1	—	—	—	LSM development
leo	52 ± 1	—	—	—	LSM development[b]
spa;ros	3 ± 0.4	48	26	2[a]	Additive (ESM + LSM)
spa;leo	8 ± 4	48	25	0[a]	Additive (ESM + LSM)[b]
ros;leo	44 ± 6	52[a]	28	6	Epistatic (LSM)[b]

Note. Four-week-old mutant and wild-type fish were treated with epinephrine (1 μg/ml) (Goodrich and Nichols, 1931) to aid in visualizing individual melanocytes and then fixed in formalin. Melanocytes from three or more fish reared on each of two or more occasions were counted for each genotype. Percent of wild-type stripe melanocytes was calculated by dividing melanocyte counts in mutants by the average number of melanocytes counted in three or more wild-type fish grown simultaneously under identical conditions and multiplied by 100. Percent of wild-type melanocytes from mutant fish reared on two or more occasions were averaged to give final percent of wild-type melanocytes shown for each mutant (± standard deviation). Predictions for melanocytes in double mutant interactions were calculated as follows: Epistatic (mutations affect a common developmental mechanism or pathway in the same population of melanocytes), expect the more severe of the two mutant phenotypes; Multiplicative (mutations affect different pathways of development in a common population of melanocytes), expect 100× (fraction wild-type melanocytes for mutant 1× fraction of wild-type melanocytes for mutant 2); Additive (mutations affect distinct and independent populations of melanocytes), expect 100 − (100 − percent of wild-type melanocytes for mutant 1) − (100 − percent of wild-type melanocytes for mutant 2).

[a] $P < 0.05$.

[b] *leo* also has affects on migration of ESM into the stripes.

anocytes affected by *spa* in the *spa;ros* or *spa;leo* double mutant correspond to the same subpopulation of dermal melanocytes that are affected in *spa* single mutants.

The interpretation outlined above for the *spa;ros* and *spa;leo* double mutant phenotypes assumes that the dermal melanocytes that persist in the single mutants are completely normal. However, mutations acting singly might have affects that in some cells are greater than the threshold required to disrupt development (accounting for the dermal melanocyte deficiencies), and in other cells, these affects might fall below the threshold required to disrupt development. In double mutants, combination of such subthreshold affects from two mutations might exceed the threshold required to disrupt development. If accumulation of subthreshold affects were responsible for the severe dermal melanocyte deficiency in *spa;ros* and *spa;leo* double mutants, then we might also expect to find interaction of subthreshold affects in *ros;leo* double mutants, resulting in severe dermal melanocyte deficiencies in these fish as well. As noted above, *ros;leo* double mutants have nearly as many melanocytes as *leo* or *ros* single mutants. Thus, the specificity of the interaction of *spa* with *ros* and *leo* argues against the accumulation of subthreshold affects, adding support to our model that *spa* affects a distinct population of dermal melanocytes from that affected by *ros* and *leo*.

It should be noted that we refer in this discussion to dermal melanocytes in the body region (the trunk and tail), ignoring melanocytes that develop in the stripes of the caudal and anal fins. An interesting finding from these experiments is that *spa;ros* double mutants retain the caudal and anal fin stripes (not shown). Following the general logic used above, retention of melanocyte stripes in the fins of *spa;ros* double mutants argues for at least one more population of stripe melanocytes, under distinct genetic control, in the adult zebrafish. We have not counted fin melanocytes in detail, so we can not yet exclude some role for spa^+-dependent or ros^+-dependent melanocytes in the fins. Appearance of melanocytes in the fin in *spa* mutants seems to be somewhat delayed (not shown), suggesting some role for spa^+-dependent melanocytes in development of the fin stripes as well.

We conclude that two distinct populations of melanocytes exist in the dermal pigment stripes. The development of these melanocytes, together with other pigment cells that contribute to adult pattern, are affected by different genes; *spa* affects the ESM, epidermal melanocytes, and white pigment cells in the fins, *ros* affects LSM and stripe iridophores. Presumably, neural crest cells that arise during embryonic development give rise to these different classes of pigment cells. Although it is tempting to speculate that the ESM and LSM are lineage-specified when their precursors migrate from the neural crest, as Raible and Eisen (1994) have shown for neural crest cells that differentiate during embryonic development, our data are equally consistent with a model in which both early and late stripe melanocytes share a common precursor as late as 2 to 3 weeks in development. Further molecular genetic characterization of the *spa* and *ros* genes may help clarify these issues.

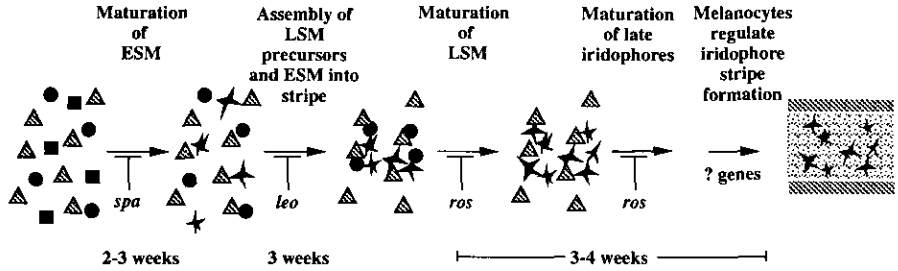

FIG. 3. Model for development of adult pigment stripe. Squares represent early stripe melanocyte (ESM) precursors, circles represent late stripe melanocyte (LSM) precursors, and stippled triangles represent iridophore precursors. Mature melanocytes are shown by black stars and gold and silver iridophores are represented by heavy and light stippling in the last panel. For simplicity in presenting the cartoon, we indicate roles for spa^+ and ros^+ in final differentiation of pigment cells, but possible roles earlier in the development of pigment cell lineages are also consistent with the data presented.

Melanocytes Regulate Morphogenetic Behavior of Iridophore Stripes

Knowledge of which classes of pigment cells are absent from each mutant or double mutant, coupled with analysis of pattern of remaining pigment cells in these mutants should allow us to infer the role of the different classes of pigment cells in coordinating the morphogenesis of melanocyte and iridophore stripes. Three regulatory pathways might lead to coordination of melanocyte and iridophore stripe development. First, the morphogenesis of each stripe could be independent of the other's development. This model predicts that in reciprocal ablations of each pigment cell type, the residual pigment cells will be unchanged from wild-type pattern. Second, morphogenesis of one type of pigment stripe might be dependent on the other type. If this were the case, then removal of one cell type should disrupt morphogenesis of the other, but in the reciprocal ablation, the residual pigment cells should develop normally. The third regulatory mechanism would be that morphogenesis of melanocyte and iridophore stripes is interdependent, so that in each ablation, morphogenesis of the residual cell type is perturbed.

To discriminate among these alternatives, we compared *ros* mutants, which lack most iridophores, yet retain ESM, and *spa;leo* double mutants, which lack most melanocytes (both ESM and LSM) but retain gold and silver iridophores. Melanocyte stripes are formed in *ros* mutants, despite the absence of iridophores. Since *ros* mutants appear to lack the stripe iridophores, this suggests that stripe iridophores have no role in assembly of melanocytes (ESM) into stripes. The pattern of melanocytes in *ros;leo* double mutants supports the idea that iridophores have no role in assembly of ESM into stripes. As in *leo* mutants (Fig. 2D), melanocytes in *ros;leo* mutants (Fig. 2I) are dispersed throughout the flank of the fish, retaining the initial ESM pattern seen at 3 weeks of development in wild-type, *ros*, *leo*, or *ros;leo* fish (not shown). These results are consistent with previous findings that leo^+ is required for assembly of dispersed melanocytes (ESM) into stripes (Kirschbaum, 1975). Close examination of *ros* mutants shows some gaps in the melanocyte stripe and absence of the ventralmost melanocyte stripe (Fig. 2C). We do not know whether this is due to the fact that *ros* mutants lack half of the melanocytes that contribute to the stripe pattern or that *ros* affects some other aspect of pattern formation. However, our results do allow us to conclude that iridophores are not required for the leo^+ function on ESM. Whether iridophores have a role in stripe formation of LSM is not clear, since the mutation (*ros*) that ablates iridophores also ablates the LSM.

In contrast to the finding that melanocyte stripes form independently of iridophores, iridophore stripe development seems dependent on the development of melanocytes. If melanocytes had no role in patterning iridophores, we would predict that stripes (in leo^+ animals) or spots (in *leo* mutants) of silver iridophores would persist in the absence of melanocytes. When *spa;leo* mutants (which lack most melanocytes, yet retain both classes of iridophores) are compared to *leo* mutants (which have ESM), it is clear that regions in the flank that have melanocyte/silver iridophore spots in *leo* mutants lack melanocytes and appear uniformly gold in the *spa;leo* double mutant. In fact, silver iridophores are found only in the vicinity of the few melanocytes that persist in *spa;leo* double mutants (see Figs. 2E and 2H). Thus, melanocytes must be present for the silver iridophore stripe (or spot) to appear.

An alternative explanation for the failure of silver iridophore development in *spa;leo* mutants is that spa^+ function is required independently of melanocytes. This does not seem likely, since *spa* mutants have a silver iridophore stripe (in association with the melanocyte stripe) in regions that lack silver iridophores in *spa;leo* double mutants. Additionally, chimera analysis indicates that spa^+ acts autonomously to promote melanocyte localization and survival in the embryo (S. Johnson, unpublished results). Though the chimera experiments

do not exclude a cell autonomous role for *spa* in iridophore development, the finding that spa^+ function is required cell autonomously in embryonic melanocytes adds credence to our model that the spa^+ dependence of silver iridophore formation in *leo* mutants is indirect, requiring spa^+ for melanocyte development, which, in turn, affects silver iridophore development. Presumably, in wild-type stripe development, the melanocyte stripe forms first and subsequently affects the development of the silver stripe. The mechanism by which melanocytes interact with iridophores to control their appearance and morphogenetic behavior remains to be elucidated.

The inferences we have derived from mutational analysis of pigment stripe development are summarized in Fig. 3. We suggest that distinct precursors of adult melanocytes and iridophores are set aside in the embryo for later development of the adult pigment stripes. At 2 weeks, ESM precursors, dependent on spa^+ function, begin to differentiate, followed by leo^+-dependent assembly into stripes. At 3 weeks, LSM precursors, dependent on ros^+, also begin to differentiate in the stripes. Subsequent morphogenesis of the silver and gold iridophore stripes depends on the development of the melanocyte stripes, thereby ensuring the coordinated development of these different types of pigment cells.

Experiments with mutations that alter pigment pattern have led us to suggest that two distinct populations of stripe-forming melanocyte precursors are present in the zebrafish dermis and that the development of these distinct subpopulations is affected by different genetic regulatory mechanisms. The molecular (functional) basis for the different genetic regulatory mechanisms that control the development of distinct populations of pigment cells is presently unknown. Such molecular mechanisms must now be elucidated not only to confirm the existence of distinct melanocyte populations, but also to understand the nature of the regulatory mechanisms themselves. Accordingly, it will be necessary to identify the products of the genes that, when mutated, cause the observed phenotypes. This requires that the genes be mapped and then cloned. We have recently constructed a genetic map for zebrafish (Postlethwait *et al.*, 1994), locating *leo* to linkage group 1, *spa* to linkage group 20 (Postlethwait *et al.*, 1994), and *ros* to the centromere of linkage group 1 (about 30 cm from the *leo* locus; S. Johnson, unpublished results). More recently, we have constructed high marker density maps around the *spa* and *leo* loci (S. Johnson, unpublished results) that should facilitate the isolation of these genes and their molecular characterization.

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