Mutants highlight the modular control of butterfly eyespot patterns

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SUMMARY The eyespots on butterfly wings are thought to be serially homologous pattern elements. Yet eyespots differ greatly in number, shape, color, and size, within and among species. To what extent do these serially homologues have separate developmental identities, upon which selection acts to create diversity? We examined x-ray–induced mutations for the eyespots of the nymphalid butterfly Bicyclus anynana that highlight the modular control of these serially homologous wing pattern elements. These mutations reduce or eliminate individual eyespots, or groups of eyespots, with no further effect on the wing color pattern. The collection of mutants highlights a greater potential developmental repertoire than that observed across the genus Bicyclus. We studied in detail one such mutation, of codominant effect, that causes the elimination of two adjacent eyespots on the ventral hindwing. By analyzing the expression of genes known to be involved in eyespot formation, we found an alteration in the differentiation of the “organizing” cells at the eyespot’s center. No such cells differentiate in the wing subdivisions lacking the two eyespots in the mutants. We propose several developmental models, based on wing compartmentalization in Drosophila, that provide the first framework for thinking about the molecular evolution of butterfly wing pattern modularity.

INTRODUCTION

Butterflies display a bewildering array of wing color patterns. Patterns can develop independently on the dorsal and ventral surfaces as well as on fore- and hindwings. In many species it is possible to recognize elements of a ground plan (the Nymphalid Ground Plan, reviewed in Nijhout 1991) that consists of basal and central transversal bands across the wing, a series of marginal eyespots, a row of chevrons, and two narrow marginal bands. These pattern elements can be repeated in each wing cell (segment of the wing bordered by veins) and, in the case of the transversal bands, can be dislocated when crossing the wing veins (Nijhout 1991). Nijhout elaborated on earlier comparative morphological work to propose that it is the presence or absence, the shape, color, and size of each of these elements within each wing cell that gives rise to much of the diversity in butterfly wing patterns (Nijhout 1991, 1994, 2001). He proposed that a lack of physical communication between adjacent wing compartments might contribute to this individuality, especially when the pattern is shifted abruptly at the wing cell boundary (Nijhout 2001). To date, however, no further molecular or developmental framework has been put forward to explain the mechanisms behind such wing pattern modularity.

From a molecular and developmental perspective, eyespots are the best studied of the serially repeated wing pattern elements. They develop in the center of each wing cell and consist of concentric rings of colored scales. Eyespot development has been associated with the expression of Distalless (Carroll et al. 1994; Brakefield et al. 1996; Brunetti et al. 2001; Beldade et al. 2002a), a transcription factor that can be regulated by the Wnt signal transduction pathway (Cohen 1990), and with the expression of several members of the hedgehog (hh) signal transduction pathway (Keys et al. 1999; Brunetti et al. 2001). In the fly, these genes are involved in patterning the proximal–distal and anterior–posterior axis of the wing (reviewed in Carroll et al. 2001). In the butterfly, these genes are expressed in conserved patterns along the two wing axes and also in novel patterns in and around a circular group of wing epidermal cells, the focus, which specifies eyespot formation.

The temporal pattern of Dll expression in the nymphalid butterfly Bicyclus anynana, from early to late fifth larval instar, is initially of broad finger domains along the center of each wing cell extending from the distal margin, followed by a narrowing of the fingers and broadening of their tips in wing cells that will carry eyespots (Brakefield et al. 1996). The forewings of B. anynana that only carry two eyespots, for instance, display the Dll fingers in all wing cells, but the tips of the fingers only enlarge to produce the stable circular spots of expression in the two wing cells that produce eyespots (Brakefield et al. 1996).

Here we describe the results of the first x-ray mutagenesis screen to be performed in butterflies. We concentrate on the
most common type of wing pattern mutation obtained—mutations in the number and size of eyespots on the hindwings of B. anynana. We look in detail at one such mutant, which we named “3+4.” We show that homozygotes for the mutant allele fail to develop eyespots numbers 3 and 4 (counted posteriorly along the row of seven hindwing eyespots), whereas heterozygotes have eyespots that develop to about half their normal size. This mutation has no visible effect on the other eyespots or on the rest of the wing pattern. We show that the mutation affects the process of focus establishment in these wing cells, interrupting early stages of eyespot specification. We finally propose several molecular and developmental models to explain how the serially homologous eyespots may have acquired different levels of developmental modularity and evolutionary independence. These models can account for the high diversity in the presence or absence of the serially repeated eyespots in different butterfly species (Nijhout 1991), as well as for the more gradual quantitative changes in eyespot morphology seen within populations and among species.

MATERIAL AND METHODS

X-ray mutagenesis
We used x-rays to induce wing pattern mutants. This mutagenic agent was chosen above chemical mutagens because ionizing radiation commonly causes more severe genetic defects, including deletions, duplications, and translocations (Goodenough 1984; Tothova and Marec 2001). We reasoned that the severity of the defects would lead to a greater proportion of dominant or codominant phenotypes detectable in the F1 offspring of irradiated butterflies when mated to nonirradiated butterflies. We performed a mutant screen on F1 adult individuals and isolated those showing any type of wing pattern alterations.

Virgin male butterflies, up to 2 weeks old, were placed in glassine envelopes with their wings folded. The envelopes were then placed under the x-ray source at a distance of approximately 20 cm and were irradiated through an aluminum filter (to block out the lower energy radiation) for a variable duration. Over several cohorts we applied a series of radiation intensities from 5 to 200 gray and aluminum filters of either 1 or 3 mm thickness. We irradiated approximately 100 males in every cohort. After irradiation, we placed the males in a population cage with the same number of virgin females. We collected eggs and reared the larvae on young maize plants. We scored freshly emergent butterflies for any alteration in their wing pattern.

Gene expression patterns
We dissected larval wing disks of both wild-type and mutant lines during the fifth (final) instar. We stained the disks simultaneously for the gene products of two transcription factors implicated in eyespot formation, Distal-less (Dll) and engrailed/invected (en) (Carroll et al. 1994; Keys et al. 1999), using a rabbit Dll polyclonal antibody (Panganiban et al. 1995) and a mouse engrailed/invected monoclonal antibody (Mab En4F11; Patel et al. 1989), respectively. We followed the staining protocol outlined in Brakefield et al. (1996). Primary antibody concentrations were 1:100 for Dll and 1:5 for en. We used donkey anti-mouse FITC and donkey anti-rabbit Texas Red as secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in a concentration of 1:200. We mounted the wings in Vectashield (Vector Laboratories, Burlingame, CA, USA), and captured the images on a BioRad MRC 1024 ES (Bio-Rad, Hercules, CA, USA) laser scanning confocal microscope.

Crosses
We set up a series of single pair crosses to examine the phenotypic effect in detail and to determine the mode of inheritance of the 3+4 mutation. We crossed mutant males and females to wild-type females and males, respectively, and crossed pairs of wild-type and mutant individuals as controls (Fig. 1). Males from the F1 hybrid crosses were individually back crossed to 3+4 mutant females (Fig. 1). We attempted to produce several replicates within each family by back crossing F1 fullsibs to separate 3+4 mutant females. The left hindwings of parents from each cross, as well as of all individuals from the F1 and back cross generations, were digitized with a charge coupled device (CCD) camera (High Technology Holland BV, The Netherlands). The diameters of all seven hindwing eyespots were measured on each left wing using Object Image 1.62 (www. simon.bio.uva.nl/object-image.html), along an axis parallel to the wing veins. Data were transferred to Microsoft Excel 8.0 for analysis. Detailed morphometric analysis of the 3+4 versus wild-type crosses was made to examine whether the 3+4 mutation only

Fig. 1. Crosses performed between 3+4 mutants and wild-type B. anynana. In the parental generation several mating cages (each with one male and two females) were set up for each male phenotype (left- and right-hand sides of diagram). Several males from each hybrid family were separately back crossed with individual 3+4 mutant females. Only the largest families are represented in the diagram: Families 7(1) and 7(3) represent the offspring of two male fullsibs (coded 1 and 3) from family 7.
affected eyespots 3 and 4, to quantify the effect of the mutation in heterozygotes, to examine the presence of any sex-linked effects, and to assess whether the segregation pattern in the back cross was consistent with that for a single polymorphic locus.

RESULTS

X-ray mutagenesis

We screened 9028 F1 individuals distributed over 26 separate cohorts at several irradiation intensities. The most common type of mutant phenotype found (179 individuals) involved a reduction in the size or complete disappearance of eyespots on the hindwing (Table 1, Fig. 2). At the highest irradiation intensities, we obtained up to 4 mutant individuals per 100 screened. Most of these mutations affected individual eyespots with no effect on the other eyespots. Eyespot 3 (third eyespot from anterior margin; Fig. 2) showed the largest number of hits, followed by eyespot 1 and eyespot 7 (Table 1). We observed, however, that neither eyespot 4 or 5 were affected in isolation. Effects on more than one eyespot were found in many different combinations (Table 1, Fig. 2). Nearly all mutants were infertile, most likely because of deleterious mutations caused by the x-rays. The 3+4 mutation appeared independently in several cohorts, either as reduced size (nine individuals) or as complete absence of eyespots 3 and 4 (one individual; Table 1, Fig. 2). After a series of failed attempts to breed each of these mutants separately with wild-type butterflies of the opposite sex, we were able to establish one fertile line from several of these individuals mating together with wild-type butterflies in a population cage. This population bred true after some generations of out-breeding followed by selection for the 3+4 mutant phenotype (although the line has since been lost).

Gene expression patterns

Dll and En protein, which are markers for the organizing centers of future eyespots, were present in all eyespot foci of

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<tr>
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Numbers of mutants are given for the various radiation intensities. Of the 9028 screened offspring, 6045 were from males irradiated with ≤50 Gy, 1924 from males irradiated with 80 or 100 Gy, and 1059 from males irradiated with 150 or 200 Gy. r, eyespot reduced in size; a, eyespot absent. Shaded rows indicate individuals represented in Figure 2. 1Males irradiated with 100 Gy with a 4-mm aluminum filter.
wild-type individuals (Fig. 3, A and D). In the 3 + 4 mutant individuals there was no focal expression of Dll or En in the hindwing wing cells that lack the eyespots (Fig. 3, B, C, E, and F). Fingers of Dll expression, however, remained visible in all wing cells of the mutant wing disks (Fig. 3C).

**Crosses**

The only eyespots that differed in size in the offspring of families, where either one or both parents carried the mutation, were eyespot numbers 3 and 4 (Fig. 4). Thus, there was no indication that this mutation affected the size of any other eyespot. In addition, eyespots 3 and 4 were of intermediate size in heterozygotes (Fig. 4), indicating a codominant effect. There was no detectable evidence of sex linkage because offspring of the mutant reciprocal crosses produced similar results (Fig. 4). Back crosses between the F1 heterozygous males and 3 + 4 mutant females yielded, in most families, a 1:1 segregation pattern with half of the offspring showing no eyespots at positions 3 and 4 and the other half carrying small eyespots at these positions (Table 2). In two families, however, the segregation deviated significantly from the 1:1 ratio (Table 2). This variation may be due to the multiple founding of this line with mutagenized individuals. The observed discrete phenotypic classes and the 1:1 segregation ratio observed in most of the families suggest that a single mutant codominant allele of major effect is primarily responsible for the 3 + 4 phenotype.

**DISCUSSION**

The 3 + 4 mutation, together with the others shown in Figure 2 and listed in Table 1, highlight the modular control of the serially homologous eyespots on butterfly wings. Through x-rays we were able to eliminate most of the individual hindwing eyespots in different specimens (with the exception of eyespots 4 and 5), that is, without disrupting the normal development of the other eyespots or of the other wing pattern elements. The rich phenotypic repertoire achieved in the *B. anynana* mutants is unparalleled among the 80 members of the genus *Bicyclus* (Condamin 1973) and could not have been deduced by the simple examination of pattern variation among these species. With a few exceptions, namely three closely related species that do not develop eyespots 3 and 4 (Monteiro and Pierce 2001) and three others that do not develop eyespot 4, all species develop seven eyespots on their ventral hindwings. The degree of potential developmental modularity underlying the row of hindwing eyespots of *Bicyclus* butterflies seems to be far greater than that sorted by natural selection within this genus. Although it is fascinating...
to inquire why this should be the case, here we focus on the putative molecular and developmental mechanisms behind such modularity.

Eyespot formation starts with the differentiation of the focal cells at its center, a process normally marked by \textit{Dll} and \textit{en} expression (Carroll et al. 1994; Keys et al. 1999). We found that in homozygous \textit{3/H11001} individuals the lack of eyespots in two of the wing cells was associated with the absence of foci in these wing cells. The antibody stainings suggest that the mutation affects the process of focal establishment by preventing the up-regulation of both \textit{Dll} and \textit{en} in wing cells 3 and 4, before or during the fifth larval instar. In the eyespot prepattern model outlined in Brakefield et al. (1996), this disruption occurs after the \textit{Dll} fingers are established but before the \textit{Dll} spots are up-regulated at the tips of the fingers, in the late fifth instar larva. This pattern is also likely to apply to the expression patterns of other focal markers such as \textit{spalt}, \textit{cubitus interruptus}, and \textit{patched} (Keys et al. 1999; Brunetti et al. 2001). A single wild-type allele, however, is sufficient to induce small eyespots in these wing cells. It is unclear whether this single allele dose effect results in a smaller group of cells differentiating as focal cells or whether the signaling properties of a similarly sized focus are affected by the lower dose effect.

Multiple explanations can account for the mutant phenotypes we observed. Here we explore three putative developmental scenarios with increasing likelihood and a combination of the last two scenarios. First, we can envisage multiple copies of one or more focus regulator genes involved in the establishment of the foci, each copy controlling focus differentiation in a subset of the wing cells. When any of these copies are mutated, the respective foci do not differentiate. This model, however, is not consistent with observations in well-studied developmental systems, where a small number of transcription factors belonging to a few dozen gene families and remarkably conserved across taxa control most developmental pathways and operate many times during an organism’s ontogeny (Carroll et al. 2001; Davidson 2001).

Alternatively, we can envisage a set of single copy focus differentiating genes, potentially expressed in all wing cells but regulated independently in each wing cell by a discrete

![Fig. 4. Mean eyespot diameter for the F1 (female) offspring of each family (males show a similar distribution). Filled circles correspond to the family means of wild-type × wild-type crosses (families 16, 17, 19, 20, and 51), open circles to \textit{3+4/H11001} crosses (family 3), upward pointing triangles to wild-type (males) × \textit{3+4/H11003} crosses (families 7, 8, 9, and 41), and downward pointing triangles to \textit{3+4/H11001} crosses (families 13 and 36). See also Figure 1.](image)

![Table 2. Frequency distribution of offspring from back crosses carrying either no eyespots 3 and 4 or small reduced eyespots](table)

**Table 2. Frequency distribution of offspring from back crosses carrying either no eyespots 3 and 4 or small reduced eyespots**

<table>
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<th>Family no.</th>
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This table tests whether the frequencies differ significantly from a 1:1 ratio. We highlighted in gray the two families 7(1) and 7(3) that show deviations from the 1:1 segregation pattern expected with the presence of a single mutant allele. Significance \(P\) values from Chi-square tests (all \(df = 1\)): *\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\); \(P > 0.05\) = NS.
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combination of regional regulator genes expressed in only subsets of the wing cells. Research in *Drosophila* demonstrates that wing veins form at boundaries of expression of regional regulator genes (Sturtevant et al. 1997; Biehs et al. 1998). The domains of expression of these genes are determined by signaling mechanisms derived from the anterior–posterior boundary, set up very early in wing development. It is likely that similar vein positioning mechanisms are conserved in butterflies as the anterior–posterior boundary, as well as the expression of some regional regulators, have already been identified in conserved domains in butterfly wings (Keys et al. 1999) (Fig. 5A; see also *en* expression in the posterior wing domain in Fig. 3, D–F). Each regional regulator gene domain may cover one or more wing cells, as in *Drosophila* (Fig. 5B), and the combinatorial overlap of the different regional regulator gene domains throughout the anterior–posterior axis will give each wing cell a unique genetic identity. The resulting genetic subdivision of the wing, which changes genetic composition at vein boundaries, can also function to modify the process of focus differentiation in each wing cell. Mutations affecting the production or concentration of these regional regulator genes may affect focus establishment in only a subset of the wing cells that require these signals for gene activation or repression. This model, however, suffers from the problem that if the regional regulators themselves are mutated, other developmental processes using the same genes, such as vein positioning, could also be affected. With the exception of a mutant lacking all eyespots, we never detected any vein alterations in the eyespot number mutants. On the other hand, it could be that the process of focus determination is a less buffered mechanism relative to that of vein positioning, where slight to moderate changes in the production of any of these regional regulators cause abrupt shifts in the balance of focus activators and repressors but do not affect the process of vein formation.

A third possibility is that the *cis*-regulatory region of one or more genes involved in focus differentiation is organized in modules (Fig. 5E), each module regulating gene expression in a single focus or a subset of foci. The patterns of eyespot elimination could reflect the linear arrangement of these modules on the DNA if mutations delete continuous sequences of *cis*-regulatory DNA of variable length, covering one or more regulatory modules of a single gene. This is consistent with the observed patterns of coelimination of eyespots, which can be interpreted as following from an ordering of the modules in the following sequence with respect to eyespot identity: -6-7-1-3-4-2- (where the module for focus 5 can be placed at either end, and the whole sequence oriented in reverse; Fig. 5E). This leads to one of the two most

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**Fig. 5.** A developmental model for butterfly wing patterns. Genes identified in the figure are already known to be expressed at the given location in butterfly wings of either *Precis coenia* or *B. anynana*. The rest of the diagram is hypothetical. (A) The anterior–posterior boundary (set up very early in embryogenesis) is thought to function, like in *Drosophila*, as the initial source of signals to pattern the anterior–posterior axis of the wing. Through several steps, these signals may subdivide the butterfly wing into separate genetic subdivisions, each expressing a different combination of regional regulator genes (B). (C) Vein markers (in purple) may become expressed at such boundaries to define the future position of the veins. In turn, several other genes may be turned on in and around the future vein tissue. These regulatory cascades, which may involve signaling from the presumptive veins and wing margin and lateral inhibition processes, are repeated in each wing cell. (D) In the last larval instar, the wing disks of eyespot bearing species differentiate a central group of cells, the focus, involved in eyespot formation. Complex gene regulatory cascades, with the involvement of genes from the *hh* and *wnt* pathways, are probably responsible for patterning each wing cell and for differentiating eyespot foci. (E) We propose, among other things (see text), that the differentiation of foci in each wing cell is controlled by one or more focus regulator genes that are turned on in each wing cell via the input of the regional regulator genes that bind to discrete modules on their *cis*-regulatory domains. Each module is controlling gene expression within a single wing cell. When any of these modules are deleted, transcription of the focus regulator gene is affected only in the corresponding wing cells (F). (G) In wing cell 3, for example, only “red” and “yellow” regional regulator gene products are present, leading to the activation of a single *cis*-regulatory module and expression of the gene in wing cell 3. The interpretation of the positional information, laid out by the prepattern genes in steps A to D by specific pigment production pathways, gives rise to the adult wing pattern—in this case displaying the 3+4 mutation (F). The genes/gene products are abbreviated as follows: *cubitus interruptus*, Ci; *Distal-less*, Dll; *decapentaplegic*, Dpp; *engrailed* or *inverted*, En; *hedgehog*, Hh; *patched*, Ptc; *wingless*, Wg. (From Carroll et al. 1994; Keys et al. 1999).
parsimonious explanations for all observed mutations (the other is 2-7-1-3-4-6). When one or more cis-regulatory modules is disrupted this would cause the disappearance of the gene’s expression pattern in the corresponding foci, leaving intact the modules controlling gene expression in the rest of the foci. A few of the mutant patterns we obtained, however, would need to be explained through multiple hits on the same gene. Alternatively, different focus differentiation genes with modular enhancer regions might be present, such that multiple hits then reflect different modules mutated in each of these genes.

This last model, combined with a discrete distribution of regional regulators mentioned in the second model, mimics the regulation of the seven even skipped (eve) stripes in Drosophila. Each stripe is under the control of a different set of transcription factors (e.g., Stanoevich et al. 1991). Each transcription factor has a specific domain of expression in the embryo, and the binding sites for the set of transcription factors regulating each stripe are clustered together as a module in the cis-regulatory DNA of eve (Harding et al. 1989; Sackerson et al. 1999).

Given the latter model, a likely molecular explanation for the 3+4 mutation is a deletion or disruption of the control module(s) of eyespots 3 and 4 in the target cis-regulatory region of a focus regulator gene. Mutating this cis-regulatory region would prevent this gene from being activated in the two wing cells in question, preventing focus differentiation and subsequent eyespot differentiation. The back crosses of two families suggest that more than one mutant gene or 3+4 allele was segregating in our mutant population. This could be explained by mutations of variable length/degree affecting the cis-regulatory region of the same gene or of other genes also involved in focus differentiation. From the eve studies in Drosophila (Sackerson et al. 1999), it is known that the modular cis-regulatory regions necessary for stripe expression are not sufficient for full gene activity. There are other sequences adjacent to the region sufficient for proper spatial expression that augment the eve signal.

Our last model addresses the ability of eyespots to appear or disappear from the wing with little or no effect on the other eyespots, but it can also address how each eyespot acquires its own characteristic size. In B. anynana, selection experiments have shown that a quantitative trait like eyespot size can be gradually changed by artificial selection over a number of generations (Monteiro et al. 1994). Tissue grafting experiments with foci of these size selected lines showed that eyespot size is dependent to a large extent on properties of the focal cells (Monteiro et al. 1994). Recent selection experiments have also shown that single eyespots display a large amount of developmental independence from the other serial homologous eyespots present on the same wing. For example, it is possible to select for a large anterior eyespot and a small posterior eyespot on the same wing with the same relative ease as selecting for a simultaneous increase or decrease of both eyespots (Beldade et al. 2002b,c; Beldade and Brakefield 2003, this issue). This developmental independence could be achieved by changes in the number and type of binding sites at these spatially segregated regulatory modules, which would allow the evolution and fine tuning of the properties of individual foci, leading to eventual differences in eyespot size (see Fig. 5).

We can also apply this joint model to explain the evolution of modularity of serially homologous pattern elements across the Lepidoptera. Nijhout (2001) recognized that early moth lineages showed little morphological divergence between serially homologous pattern elements relative to later lineages of moths and butterflies. This suggests that serially homologous patterns may have evolved increased levels of individuality and modularity through time. If we assume a conserved mechanism of vein differentiation shared between flies and butterflies, then it is likely that a different combinatorial distribution of wing regional regulator genes was already present in the ancestral Lepidoptera. Evolution of individuality among serially homologous pattern elements could then involve some combination of the following processes:

1. The gradual co-option of regional regulator genes into the regulation of color pattern genes via the appearance of specific binding domains in the cis-regulatory regions of the latter, leading to their differential regulation in the subset of wing cells where the regional regulators are expressed;
2. The duplication and diversification of these cis-regulatory domains (modules) such that the regulation of genes involved in color pattern development could now evolve in a largely independent manner within a particular wing cell, or a subset of wing cells;
3. The co-option of color patterning genes possessing a modular cis-regulatory domain that originally evolved for a different function (e.g., the pair-rule genes in Drosophila) of which eve is an example. Such a gene could gradually evolve and modify its existing cis-regulatory modules to co-opt several of the regional regulators already present in the wing.

The goal of future research should be to resolve the mechanisms of modularity in a single model species, such as B. anynana, and then to use this knowledge to understand how these mechanisms have evolved to produce different patterns across the Lepidoptera.

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