Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 287 (2005) 416 – 424

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Pharmacologic approaches to butterfly wing patterning: Sulfated polysaccharides mimic or antagonize cold shock and alter the interpretation of gradients of positional information

Michael S. Serfas, Sean B. Carroll *

Howard Hughes Medical Institute, University of Wisconsin-Madison, 1525 Linden Drive, Madison WI 53706, USA

Received for publication 29 July 2005, revised 7 September 2005, accepted 10 September 2005
Available online 10 October 2005

Abstract

Butterflies produce complex and diverse wing patterns by mechanisms that are generally unknown. We have employed a pharmacological approach to explore the molecular mechanisms of pattern formation. In a screen of over 200 compounds injected into developing *Junonia coenia* pupae, we identified several specific sulfated polysaccharides that caused widespread, dose-dependent effects on adult wing patterns. These compounds were well tolerated and permitted butterflies to eclose normally and take flight at moderate levels of effect. Heparin and closely related chondroitin sulfates caused stage-specific expansion of distal and proximal band systems and reduction and repatterning of eyespots. Dextran sulfate and fucoidan, whose structures are widely divergent from heparin and one another, caused contraction of distal and proximal systems, but had no effect on eyespots. Nonsulfated or nonpolymeric saccharides were without effect. Pattern alterations were indistinguishable from those reported for extreme cold shock and exposure to sodium tungstate and "molsin". When administered after cold shock or coinjected with heparin, dextran sulfate reversed all patterning effects. We suggest that the primary effect of polysaccharide treatments is to alter the interpretation of gradients of positional information along the proximodistal axis of the pupal wing.

Keywords: Butterfly; Pattern formation; Heparin; Morphogen; Wing; Cold shock; Ground plan

Introduction

The vast diversity of butterfly wing patterns poses many questions for evolutionary and developmental biology. Most of over 13,000 species are uniquely identifiable by their wing pattern. Many occur in several forms with distinct patterns according to season or locality (Joron, 1998). The evolution of color patterns reflects a complex mixture of crypsis, misdirection of predators, sexual selection, aposematic coloration, and mimicry (reviewed in Beldade and Brakefield, 2002). Butterflies often concentrate toxic and distasteful alkaloids and warn using striking colors, but unpalatable butterflies and their mimics must be slaves to fashion: predators taste unfamiliar butterflies before learning to avoid them, imposing strong frequency-dependent selection (reviewed in Mallet, 1999). As a result, rapid divergence between populations and striking convergence between species occur simultaneously. The challenge for developmental biology has been to understand by what means the genome can "paint" a wing with a consistent pattern, yet allow it to change, seemingly to any model pattern ecology might demand, within a modest span of evolutionary time. Evolution of novel spatial patterns is crucial to the development of complex organisms, but rarely is the relevant feature so apparent to the eye.

Butterfly wing colors are produced by specialized cuticle scale-forming cells thought to be homologous to sensory bristles (Galant et al., 1998). These scale cells differentiate to form discrete colors according to the activity of three major pigment pathways, the iridescent ultrastructure of the cuticle, and the timing of cuticle hardening (Nijhout, 1991). The early pupal precursors of scale cells (pI homologues) emerge from an apparently homogenous wing epithelium, align into regular scale rows, and divide to form a pIIa homologue and a small cell that undergoes apoptosis, which is presumed to be homologous to the neuron/sheath precursor (Koch et al., 2003). The pIIa cells divide again to form socket and scale-forming cells, and emerging scale processes subsequently

* Corresponding author. Fax: +1 608 262 9343.
E-mail address: sbcarroll@wisc.edu (S.B. Carroll).

0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.09.014

emerge (Koch et al., 2003; Nijhout, 1980). Neither surgery, cold shock, nor drug treatment can alter wing pattern after 2 or more days of pupal development (Nijhout, 1980, 1984). Adult eyespot color motifs are foreshadowed by the expression patterns of the Spalt, Engrailed, and Distal-less (Dll) transcription factors and the ecdysone receptor (EcR) in the pIIa precursors (Brakefield et al., 1996; Brunetti et al., 2001; Keys et al., 1999). In these expression patterns and in the adult wing cuticle, boundaries between color elements are marked by the intermingling of scales of different types rather than formation of scales with intermediate identity, suggesting that discrete color fates are determined by the pIIa stage.

Butterflies pose substantial practical challenges for studies of their developmental mechanisms. While several mutants with striking alterations of wing pattern are known (Brakefield et al., 1996; Monteiro et al., 2003), genetic linkage maps have only recently become available (Jiggins et al., 2004), and it will be a formidable challenge to find color patterning genes by forward genetics. Germline transformation of the butterfly is also difficult (Marcus et al., 2004). Genetically normal butterflies have been modified by ectopic expression of genes using the Sindbis viral vector (Lewis et al., 1999), but this agent has a limited infectivity at the critical stage which compromises its utility for these studies (Lewis and Carroll, unpublished). Other approaches to manipulate gene expression, such as the administration of morpholino and 21-bp siRNA, have also proved ineffective (Serfas and Carroll, unpublished). Therefore, we have sought alternative approaches to gene-specific manipulations. Pharmacologic agents are desirable for much the same reasons that they are preeminent in human therapy: they are optimized for effectiveness in vivo; they can affect many gene products at once while exhibiting some degree of specificity; they alter development only after the time point of injection; and they can bypass functional redundancy within some pathways, thus offering a broad approach to uncovering functional pathways in the wing.

Some drugs are known that influence butterfly wing patterns; they appear to act by mimicking an altered climate. Early pupal injection of ecdysone or 20-hydroxyecdysone induces the summer morph of particular species such as *Araschnia levana* (Nijhout, 2003), *Bicyclus anynana* (Koch et al., 1996), and *Junonia coenia* (Nijhout, 1997). Early ecdysone peaks are characteristic of rapidly-developing summer pupae, but the color elements affected in different species are nonhomologous, suggesting that ecdysone affects the rate of development rather than a specific mechanism of wing patterning.

Unlike normal seasonal variation, cold shock of developing moths and butterflies causes a reproducible expansion of band systems from the proximal and distal wing at the expense of color patterns occurring at positions midway to the wing margin (Goldschmidt, 1938; Nijhout, 1984). The majority of aberrant butterflies collected from the wild exhibit such alterations (Nijhout, 1991), indicating that these patterns, though rare, are exposed to natural selection. Cold shock effects fall into a single graded series of severity (Nijhout, 1984), unlike the wide range of phenocopies exhibited by heat-shocked insects (Goldschmidt, 1938), suggesting that a single regulatory mechanism controls the cold shock effect. Injection of pupae with sodium tungstate, sodium molybdate (Otaki, 1998), or the crude *Aspergillus saitoi* protease extract "molsin" (Umebachi and Osanai, 2003) replicates this effect by an unknown mechanism. No reliable cold shock inhibitor or antagonist is known.

Here, we report a series of polysaccharides that, when injected, produced dramatic effects on wing patterning that either mimic or antagonize cold shock. These experiments demonstrate that drugs can produce consistent, compound-specific, dosage-dependent effects and are well tolerated in butterflies. The discrete, stage-dependent effects on several wing pattern elements along the proximodistal axis suggest that wing patterns are organized by gradients of positional information.

Materials and methods

Butterfly rearing

We maintained a colony of *J. coenia* at or above a population of 600 animals on a 15L:9D light cycle at 28°C using an artificial diet flavored with *Plantago lanceolata* (Carroll et al., 1994). These conditions favor development of the *linea* morph. The majority of prepupae molt to pupa 1–3 h after lights are turned on, and the midpoint of this interval is taken to be "0 h after pupation" for all pupae collected.

Injections

Injections were performed with Hamilton 10-μl syringes with 26 to 32 gauge needles with points filed to an approximately 45° angle. Pupae were injected near the base of the left wing, at the point where the crease in the cuticle behind the first thoracic segment passes between two small white projections on the anterior dorsal thorax. Pupae are surprisingly resistant to injury by a wide range of salts, solvents, and highly concentrated control compounds (Serfas and Carroll, in preparation) (Otaki, 1998), so the polysaccharides described were dissolved in distilled water without buffering or compensation for ionic strength. Reagents were purchased from Sigma Chemical (St. Louis), except dextran sulfate ~500 kDa from Dextran Products Ltd. (Ontario).

Imaging

Brightfield photographs were taken with a Kontron ProgRes 3012 digital camera mounted on a Wild Makroscop M420 dissecting microscope with ring illuminator. Because polysaccharide effects were symmetrical, panels were reversed as convenient to allow ready comparison between dorsal and ventral wing surfaces, left and right wings, or to present the less damaged of the two wings.

Results

Heparin injection alters wing pattern in a dose- and stage-dependent manner

The present work emerged from a screen for drugs that affect the development of adult wing patterns (Serfas and Carroll, in preparation). We targeted morphogenic signaling mechanisms with several drugs, as they were considered likely to be involved in eyespot formation. Morphogens are capable of patterning five or more different cell fates, each determined

by a narrow range of morphogen concentrations and separated from the next by a sharp threshold (Green et al., 1992). Thus, a single secreted molecule might pattern all the bands of an eyespot by forming a concentration gradient around its site of production. The known eyespot color fate markers Dll and Spalt are induced by the protein morphogens Wingless (Wg) and Decapentaplegic (Dpp) in the *Drosophila melanogaster* wing (McMillan et al., 2002). These proteins, like other known morphogens, move through the extracellular space or interact with receptors in a way that is highly dependent on interactions with the heparan sulfate glycosaminoglycan (HS-GAG) side chains of glypicans in the cell membranes of secreting, receiving, and intermediate cells (Belenkaya et al., 2004; Greco et al., 2001). In order to test whether morphogen interactions with HS-GAGs are important for color pattern formation in developing butterfly wings, pupae were injected with heparin, a fraction of highly sulfated HS-GAGs extracted from porcine liver.

Heparin proved to be a powerful modulator of wing pattern, and consistently affected particular wing pattern elements. Treatment with heparin caused a proximal displacement of wing marginal bands (most prominent in the dorsal hindwing), a "smearing" of orange proximal bands and their black margins in the dorsal and ventral forewing, and at higher dosages, a reduction and transformation of eyespot band motifs (Fig. 1). This effect was both dose-dependent and chemically specific. Heparin had no effect when injected at 0.3 μg/pupa, caused mild pattern changes at 0.9 μg, and produced progressively stronger alterations of wing patterns at higher dosages. The 0.9 μg dose should raise hemolymph heparin concentration to 0.5 U/ml, comparable to the serum level of 0.2–0.4 U/ml recommended for some patients (Kent et al., 2000). Apart from the polysaccharides and other compounds discussed herein, more than 160 other substances were injected into butterflies without comparable effect (Serfas and Carroll, in preparation). Heparin was fairly well tolerated by the pupae, which generally appeared to develop normally at doses up to 15 μg. Doses of 30 μg usually prevented pupae from eclosing properly but permitted pupal development.

The ability of heparin to affect several distinct color pattern elements allowed us to make a direct comparison of the timing with which each element passes through its heparin-sensitive stage. Pupae were injected with the strongest dose of heparin that permits the butterfly to eclose (15 μg) for each hour from 5–20 h after pupation (Fig. 2). Results for each injection time typically overlapped those ±1 h in either direction, in keeping with the 2-h interval over which pupation occurs. Surprisingly, the eyespots, which maintain developmental plasticity later than other elements when tested by surgery, appeared to become irreversibly committed much earlier as gauged by heparin refractivity. Overall eyespot size was sensitive to reduction between 5 and 9 h (Fig. 2). Particular eyespot color motifs became refractory to heparin at different times: black outer rings (<5 h); yellow rings (8–9 h); iridescent and black scales in the inner portion of the central field of the eyespot (8–12 h); and the orange and black scales of the outer portion of the central field (11–15 h) (see Fig. S2). Motifs in the remainder of the wing became refractory to heparin around 14–17 h in the hindwing margin and proximal forewing (Fig. 2). Additional tests found that the prominent white band of the forewing became refractory approximately 25–28 h. Curiously, when heparin was injected at this time, the ground scales of the white forewing band typically were more sensitive to heparin than the cover scales, giving the dorsal band a superficially

Fig. 2. Time dependence of heparin-induced color pattern changes. *Junonia coenia* pupae were injected with 15 μg of heparin at the times indicated. Representative ventral forewings and dorsal hindwings are shown for each injection time. Arrows note typical features: a weak black ring surrounded by wing background coloration (5 h), ectopic pale and black scales (7 h), wing background color within the eyespot (10 h), refractory dark scales surrounded by ectopic light scales within the eyespot central field (12 h), greater retention of wing margin bands in the posterior eyespot (15 h). Expansion of proximal bands of the forewing is restricted to a proximal domain delimited by the position of the eyespots (left of dotted line), with the exception of a triangular domain that is never affected by heparin (arrow).

Fig. 1. Concentration dependence of heparin-induced color pattern changes. *Junonia coenia* pupae were injected with the indicated dose of heparin (μg) at 5 h after pupation. Dorsal hindwings (top) and ventral forewings (bottom) are shown for each dosage. Formation of the third dark band inward from the wing margin (arrows), or more distal motifs, consistently appears to be associated with a suppression of eyespot expansion and a repatterning of the color scheme within the eyespot.

yellowish appearance as normal white scales overlaid darkened ground scales (data not shown). This suggests that at least in this region at this time, the cover scales passed through the heparin-sensitive stage more rapidly than the adjacent ground scales.

Sulfated polysaccharides either mimic the effects of cold shock or antagonize them

The effects of heparin administration closely resembled those described for cold shock of *J. coenia* (Nijhout, 1984). Pupal injection of high concentrations of sodium tungstate, molybdate (Otaki, 1998), or the crude *A. saitoi* protease extract "molsin" (Umebachi and Osanai, 2003) has been described to produce cold shock-like effects in other species. In order to compare the effects of these regimens directly, we directly compared the effects of cold shock with injection of sodium tungstate, "molsin", and heparin. The range of effects produced by each treatment were similar to those shown in Fig. 1, although heparin and molsin seemed to be less toxic, and allowed more severely affected animals to eclose properly (Figs. 3B–E).

Because very simple compounds and stimuli induced pattern changes indistinguishable from those produced by heparin, we considered whether the effects of heparin are general consequences of treatment with sugars or polyanions, or instead depend upon specific features of the molecular structure of heparin (Fig. 4). While heparan sulfate and heparin were equally potent, progressively higher dosages were required for equal or lesser effects from injections of keratan sulfate, which is polymerized via a different saccharide linkage, and chondroitin sulfates C and A, which also lack the epimerization of the carboxyl group (Figs. 3F–I). The nonsulfated polysaccharide counterparts of heparan and dextran sulfate, hyaluronic acid, and dextran, had no effect on wing pattern at all (data not shown). Other compounds without effect on wing pattern included glycogen (an unsulfated polysaccharide); beta-gentiobiose and lacto-N-biose (unsulfated disaccharides); glucosamine 2-sulfate and glucosamine 6-sulfate (sulfated monosaccharides); glucosamine 6-phosphate

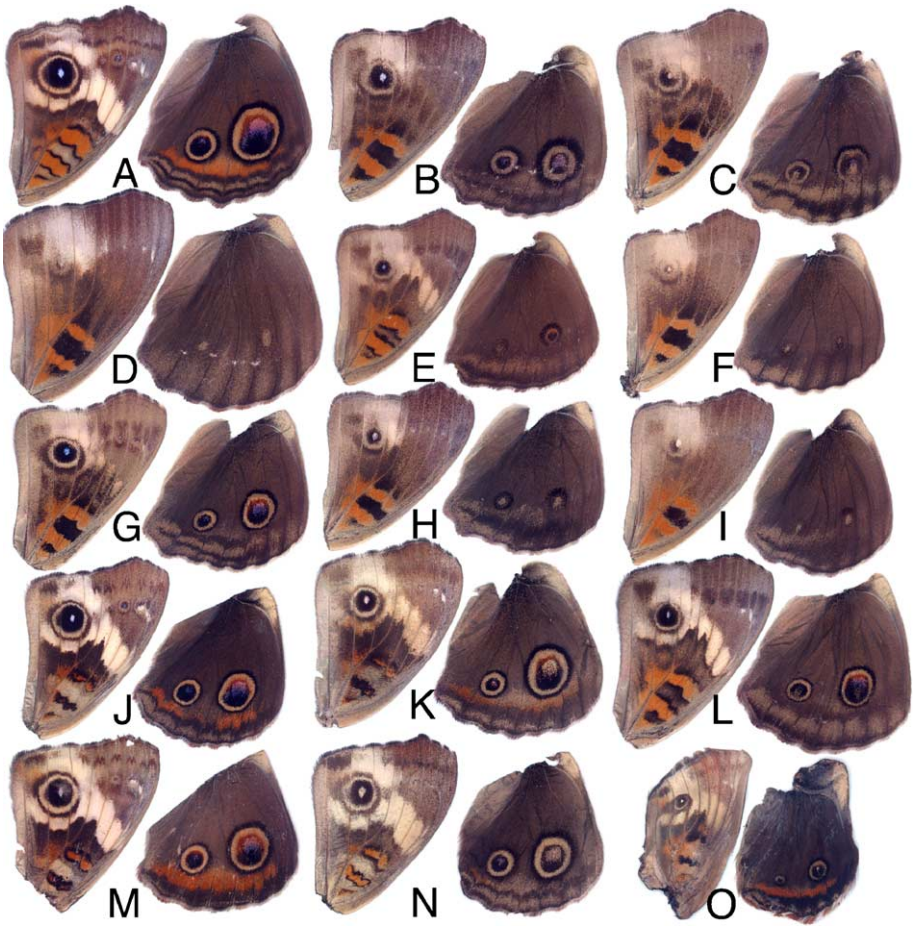

Fig. 3. Pharmacological replication and antagonism of cold shock induced wing patterns. Ventral forewings and dorsal hindwings are shown for pupae injected with 2–3 μl of aqueous solution at the indicated times after pupation. (A) Control (3 h). (B) Cold shock of lab-raised *Junonia coenia* (9 h) for 5 days at −7°C (uninjected). (C) Sodium tungstate, 70 μg (1 h). (D) Crude *Aspergillus saitoi* protease extract "molsin", 120 μg (4 h). (E) Heparin, 3 μg (5 h). (F) Heparan sulfate, 10 μg (5 h). (G) Chondroitin sulfate A, 100 μg (2 h). (H) Keratan sulfate (Chondroitin sulfate B), 20 μg (5 h). (I) Chondroitin sulfate C, 100 μg (2 h). (J) Dextran sulfate, 18 μg (3 h). (K) Fucoidan, 40 μg (2 h). (L) Mixture of 10 μg heparin and 10 μg dextran sulfate (1 h). (M) Combined treatment with 70 μg sodium tungstate and 10 g/l dextran sulfate (1 h). (N) Cold shock (−7°C, 3 days) immediately followed by dextran sulfate, 10 μg (10 h). (O) Low molecular weight (~6000) heparin, 15 μg (4 h) followed by dextran sulfate, 50 μg (8 h). This pupa did not eclose, and is shown at a higher magnification; these scale lengths and wing shape are normal for uneclosed wings, but eyespot sizes are 60% of normal.

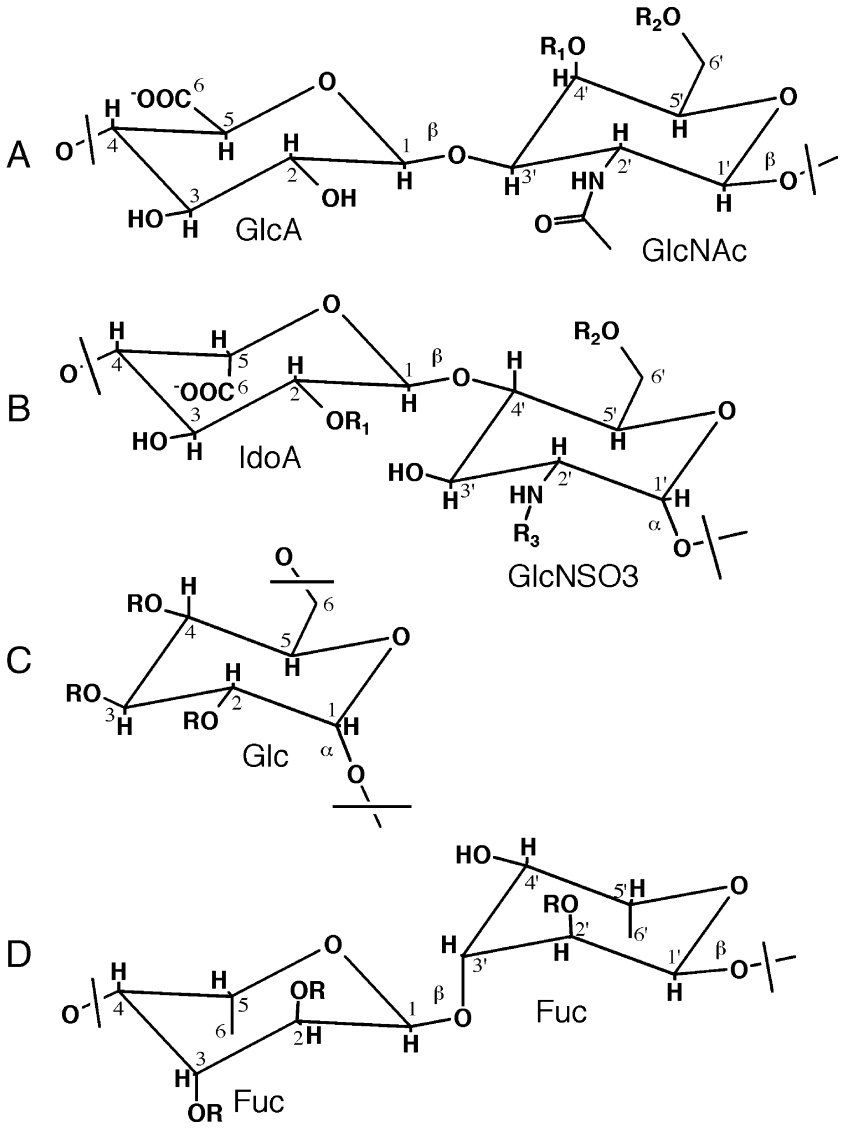

Fig. 4. Comparison of polysaccharide structures. (A) Chondroitin sulfates. A sulfate group is present at R1 in chondroitin sulfate A, or R2 in chondroitin sulfates B and C. Additionally, chondroitin sulfate B (keratan sulfate) is epimerized at carbon 6 to resemble the heparin structure. (B) Heparan sulfates differ by the disaccharide linkages and epimerization. Heparin proper is highly sulfated at positions R1, R2, and R3, while heparan sulfate is more variably sulfated. (C) Dextran sulfate is a simple glucose polymer with sulfate added synthetically at open positions R. (D) Fucoidan is a mixture of fucose polymers. This structure is a consensus for *Fucus vesiculosis*, the source of our injection stock. Sulfate is present variably at R positions.

and sorbitol (unsulfated monosaccharides). This suggests that both sulfation and a general polymer structure are required, while features such as epimerization and saccharide linkage are contributory to full activity.

In contrast, the most structurally diverged sulfated polysaccharides, dextran sulfate (Fig. 3J) and fucoidan (Fig. 3K), affected wing pattern in a way that was largely the opposite of what was observed from heparin treatment. Both compounds caused outward displacement of hindwing marginal bands and a reduction in the size of the orange proximal bands and their black margins in the forewing. Very rare individuals have been reported possessing weakly opposite modifications from those seen in cold shock, but no method has been known for their induction (Otaki and Yamamoto, 2004). In order to determine whether dextran sulfate can act as an antagonist of compounds with the opposite activity, it was coinjected with heparin or sodium tungstate. In both cases, the effects of these agents on wing pattern were blocked by dextran sulfate (Figs. 3L, M). Depending on the relative dosages used, the wing pattern produced by combined treatment resembled that seen from a lower dosage of heparin (Fig. 3L) or dextran sulfate (Fig. 3M). To determine whether cold shock treatment is subject to similar antagonism, dextran sulfate was injected immediately following incubation of pupae at −7°C for 3 days. Once again, patterning defects characteristic of cold shock treatment were suppressed (Fig. 3N). Accordingly, we term this drug a “cold shock antagonist”.

Despite their broadly opposing effects and mutual antagonism, heparin and dextran sulfate did not have direct opposite effects on the eyespot. Dextran sulfate did not affect the eyespots, while the effects of heparin exhibited a sharp dosage threshold of effect only for this motif. Heparin reduced eyespot

size and altered the color and shape of individual eyespot bands, but only at doses that displace the most proximal dark wing margin band to the position of the eyespots or beyond (Fig. 1, arrows). These effects varied independently of one another between treated individuals (compare Figs. 3E, H). The observed reduction of eyespot size suggests that the morphogenic induction of the eyespot was impaired, while changes of band coloration suggest that the interpretation of the morphogen gradient was altered. In order to determine whether heparin treatment could prevent the morphogenic induction of the eyespot when the eyespot color scheme develops normally, heparin was injected at 4 h after pupation, and dextran sulfate was injected 4 h later to neutralize its effects. The resulting wings produced eyespots with normal band color patterns but much smaller size (Fig. 3O), suggesting that morphogenic induction of eyespots can be inhibited during this time interval. The role of heparin signalling on morphogens was also explored at the level of Dll expression, which may be regulated by candidate morphogens (Brunetti et al., 2001). Injection of heparin at 5 h substantially reduced the size of the region expressing high levels of nuclear Dll at 26 h after pupation (Fig. S1).

Polysaccharide band shifts are closely associated with proximodistal identity

Polysaccharide treatment altered the position and width of the hindwing marginal bands, but appeared to affect only the proximodistal dimension of these motifs (Fig. 5). Strikingly, the displaced bands retained many aspects of their normal structure, such as the presence of breaks surrounding wing veins and much stronger expression posteriorly in the wing. Analysis of wings treated with varying dosages suggested that bands can be shifted by varying degrees, but there is a boundary at the approximate position of the eyespot foci beyond which proximal or distal pattern elements cannot be displaced (Fig. 5, dotted line). Hindwing bands shifted "beyond" this boundary appeared truncated or were lost from the wing altogether. A similar phenomenon was noted for the proximal color motifs of the forewing as they approached the corresponding position from the proximal side (see Fig. 2). In both forewing and hindwing, this boundary divides the wing into proximal and distal domains with distinct color schemes, and pattern elements are lost near the boundary in heparin-treated pupae.

Heparin affects the proximal forewing independently of morphogen signaling

The effects of polysaccharides on the proximal forewing band pattern can be described as an overall expansion or contraction of concentric motifs centered on the orange bands. Bands of black, tan, and dark grey scales surrounding the orange bands were widened and displaced as would be expected if they are patterned by a morphogen produced at the position of the future orange bands, whose mobility or stability is altered by the treatments. Paradoxically, grafting or

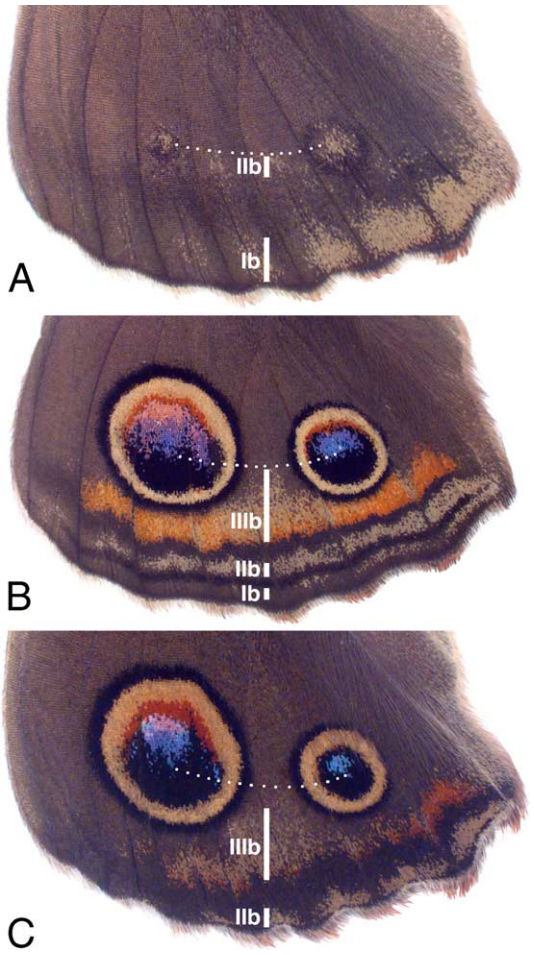

Fig. 5. Displacement of hindwing marginal bands by pupal injections of heparan sulfate (A, 10 μg, 5 h), lacto-N-biose (B, 40 μg, 4 h), and dextran sulfate (C, 18 μg, 3 h). Heparan sulfate generally causes bands to widen and shift inward from the wing margin, while dextran sulfate has the opposite effect. Lacto-N-biose lacks activity. The yellow marginal bands (Ib, IIb) and orange parafocal element (IIIb) are indicated. Following injection of either heparin or dextran sulfate, marginal bands are restricted to the distal wing domain between wing margin and the dotted line at the position of the eyespot centers. Bands that would otherwise stray from this region are truncated or lost, as are IIb (A) and Ib (C). The orange parafocal element IIIb is lost as distal bands overrun it (A), but does not shift distally with the other bands following dextran sulfate injection (C).

cautery of these bands in the pupa does not change the proximal band pattern, as would be expected if morphogen signaling was occurring (French and Brakefield, 1995; Nijhout, 1980, 1991). However, it is possible that polysaccharides lead to the secretion or mobilization of a morphogen at a time when normally it is no longer received. In order to determine whether morphogens contribute to the alteration of proximal band patterns by heparin, surgeries were performed prior to heparin injection to separate the apparent morphogen sources from recipient tissues. Forewings were subjected to the autograft of a 180° rotated strip of wing tissue at 6 h after pupation, and heparin was subsequently injected at 15 h. The dorsal surface of the forewing was used for this procedure because it adheres to the pupal cuticle, facilitating surgical manipulation. The autograft region was chosen so that in heparin-treated butterflies it included a small region of black scales associated

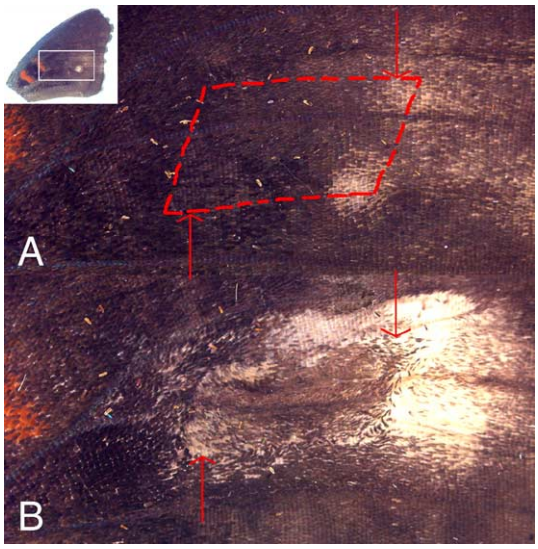

Fig. 6. Heparin-induced color changes do not require long range morphogen signalling. (A) Unoperated right forewing (mirror image) injected with 10 μg heparin at 15 h after pupation. The region corresponding to the autograft is marked. (B) Left forewing of the same butterfly, in which this region was excised and rotated 180° at 6 h after pupation. Arrows mark dark and light scales that have been rotated to opposite ends of the graft. Other effects seen occur more generally in grafted wings, including a somewhat reduced intensity of pigmentation (presumably due to reduced pigment precursor or oxygen circulation), a partial reduction in the degree of effectiveness of heparin treatment within and surrounding the graft, and the occurrence of regions without wing scales at some of the graft margins where healing is incomplete.

with proximal band pattern at one end and the remnant of the proximal white forewing band at the other end (Fig. 6A). If morphogens were responsible for repatterning, then the effect of heparin should be unaffected by the graft rotation, causing the final appearance of the grafted wing to resemble that of the unoperated wing, with black scales at the newly proximal end and white scales at the newly distal end. Alternatively, if the incision sites surrounding the graft do not permit a morphogen to enter from the proximal bands, the graft would be expected to remain white and free of black scales. However, if morphogenic signaling is not responsible, the effect should be as if the graft had been rotated after all pattern formations were complete. This latter result was obtained, with black and white scales visible in their original locations relative to the autograft and at opposite locations relative to the wing (Fig. 6B). Because the extreme proximal and distal ends of the graft formed small regions of now-misplaced color, in regions sharply delineated by the graft boundaries, we concluded that for this pattern element the fate of these cells was not changed by surgery. Similar results were also obtained from surgeries of more proximal forewing regions (data not shown). These data suggest that heparin has the ability to alter wing pattern in this region without disrupting normal or abnormal morphogen signalling.

Discussion

We have found that several sulfated polysaccharides mimicked or antagonized cold shock, and were able to cause the physical displacement of band patterns without affecting morphogens directly. To explain these results, we propose that polysaccharides alter the level of a cold shock hormone produced outside the wing, that modifies the activity of an intracellular regulatory factor that is expressed in a gradient along the proximodistal axis. The postulated regulatory factor serves to determine the coloration of bands in the proximal and distal regions of the wing and to control the suppression of eyespots in areas repatterned as wing margin bands.

Heparin and dextran sulfate may affect a regulatory factor whose activity varies along the proximodistal axis

Because heparin treatment can alter wing pattern in the proximal forewing without the involvement of long-range signalling, it must affect the interpretation of regulatory information at the local level. This information could be in the form of either cell-autonomous regulatory molecules, or molecules that act over a distance that is too small to be noticeable in a surgically altered wing. The simplest case is that polysaccharide treatment affects a regulatory factor whose expression level or activity state varies according to the proximodistal position of each cell and determines which color a scale cell adopts (Fig. 7). If its levels reach one extreme at the wing margin and proximal forewing bands, then changes in the overall level of the factor result in the expansion or contraction of band patterns centered on these motifs. Based on the observation that the width of several bands is generally proportional to their displacement from the wing margin, it is possible that polysaccharides merely multiply the effective concentration or activity of the factor by a constant, resulting in the proportional expansion of a linear gradient of factor level (see Fig. S3).

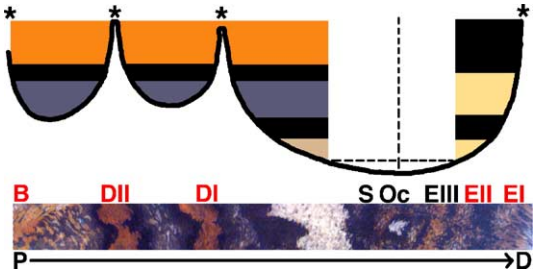

Fig. 7. Model of wing repatterning with cold shock and antagonists. The level of an intracellular regulatory factor (plotted curve) varies along the proximodistal axis of the wing (left to right). Threshold levels of this factor (above plotted curve) determine wing color at each position. The slope of the curve thus determines the width of each colored band. Pattern changes occur as polysaccharides increase or decrease of the level of regulatory factor throughout the wing, causing patterns to shift toward or away from the putative peaks of factor activity (asterisks). Below, a portion of the dorsal forewing is labeled with the color motifs of the Schwanitsch nymphalid ground plan (B, DII, DI, Oc, EIII, EII, EI). Motif S denotes the heparin insensitive small triangular region between the white band and the eyespots in the anterior forewing. The motifs shifted proportionally by both heparin and dextran sulfate are shown in red. One or more selector genes may distinguish the color patterns of distal and proximal domains (left and right of the vertical dotted line), which are demarcated by the eyespots. Elements below a minimum threshold of regulatory factor (horizontal dotted line) are patterned by independent mechanisms, but are overridden by the other elements in heparin-treated pupae.

Eyespots were only observed to be affected by polysaccharides when the wing margin bands are displaced in close proximity to the eyespots (Fig. 1). This result suggests that effects on eyespot patterning are the consequence of shifts of marginal bands (or a factor that positions them), rather than due to the direct action of heparin on the eyespot inductive mechanism. There appears to be an antagonism between the mechanisms that promote formation of the wing margin elements and the eyespots: hindwing cauteries create eyespot-like patterns that are truncated at the most proximal marginal band (Nijhout, 1980); when eyespot size is expanded by drugs such as lithium, the eyespots are truncated in the same way (Serfas and Carroll, in preparation); and wing imaginal disks from which the outer edge has been surgically removed early in the fifth instar form a new wing margin that overrides the eyespots (Nijhout and Grunert, 1988). Because eyespots or cauteries were sometimes partially truncated to form open semicircles in each of these cases, it appears that the recipient tissue becomes incapable of responding to the eyespot inductive signal as it takes on a distal wing margin positional identity. Heparin, by expanding the region of tissue with this positional identity, eliminates eyespots secondarily to its effects on proximodistal fates.

Polysaccharides probably affect a heparin-binding protein at a site outside the wing

The polysaccharides examined are large (~500 kDa), highly charged compounds which either do not occur in animals (dextran sulfate, fucoidan) or are normally restricted to extracellular spaces (heparin). In either case, it is unlikely that these compounds cross the cell membrane, so their target is likely a receptor or extracellular protein. Because heparin has an irregular sulfation pattern with many potential binding sites, molar comparisons to other inhibitors are misleading, but by weight, it is at least 40-fold more effective than molsin or sodium tungstate, and the dosage that is effective is comparable to that for authentic heparin-binding proteins in vertebrates. Among the polysaccharides tested, a clear structure–activity relationship favors key molecular features of heparin. The most widely diverged polysaccharide structures bind in an antagonistic or inhibitory manner. Accordingly, we conclude that a target protein specifically recognizes an endogenous polysaccharide closely related to heparin.

Hemolymph from a cold-shocked pupa can induce a mild cold shock response in some recipients, suggesting that a cold shock hormone remains present in the hemolymph (Otaki, 1998). Polysaccharides, molsin, and tungstate could act by changing the amount of hormone released, binding to the hormone, or blocking its reception. We favor the first model. We observed that alterations from these drugs or cold shock are almost perfectly symmetrical, with far greater similarity between wings from the same animal than between wings of different animals from the same experimental group (see Fig. S2). This behavior differs from that of drugs that reduce eyespot size, most of which affect the wing on the injected side twice as severely as the uninjected counterpart (Serfas and Carroll, in preparation). Even when heparin was injected into one forewing and dextran sulfate was injected into the other at three successive hourly time points, no asymmetry was seen (data not shown). This result suggests that these drugs likely influence the secretion of cold shock hormone by a structure located near the body midline, rather than act on receptor function within the wing.

The known drugs with heparin-like effects may work by related mechanisms. The crude extract “molsin” may contain polysaccharides. However, if present, these must remain conjugated to proteins, because “molsin”, unlike heparin, is inactivated by phenol:chloroform extraction or brief 80°C incubation (data not shown). Although the effects of “molsin” have been ascribed to the release of aromatic amino acids by aspergillopeptidase A that serve as pigment precursors (Umebachi and Osanai, 2003), we have not observed any patterning effect from treatments that inhibit pigment production (Serfas and Carroll, in preparation). Tungstate and molybdate (Otaki, 1998) have the same charge and structure as sulfate and can compete with sulfate as a substrate or inhibitor for sulfate transporters (Markovich, 2001). In addition, phosphotungstate and silicotungstate are effective anticoagulants that directly compete with heparin for antithrombin III binding (Tajima et al., 1989). Although it has been suggested that tungstate alters wing pattern by acting as a tyrosine phosphatase inhibitor (Otaki, 1998), we have observed no polysaccharide-like effects from tests of the tyrosine phosphatase inhibitor phenylarsine oxide or a dozen tyrosine kinase inhibitors (Serfas and Carroll, in preparation). Furthermore, tungstate and dextran sulfate compete to generate a weak effect (Fig. 3M), which suggests that tungstate acts on a target accessible to dextran sulfate in the extracellular space, rather than an intracellular phosphatase.

Implications for models of wing pattern organization and evolution

Interspecific comparisons of butterfly patterns have inspired proposals of a “nymphalid ground plan” composed of several conserved symmetry centers (reviewed in Nijhout, 1991). Of these elements, the proximal wing motifs B, DII, and DI and the wing margin bands EI/EII were each expanded by heparin and contracted by dextran sulfate. The observed alterations are consistent with a simple model in which a regulatory factor reaches peak levels at B, DII, and DI, but all similarly-colored bands composing these motifs may be determined by the same thresholds of factor activity. Although the proximal and distal domains of the wing clearly have distinct color schemes, they respond to similar concentrations of polysaccharides prior to a similar critical period 14–17 h after pupation. It is possible that a gradient of a single regulatory factor in each wing region may influence the positioning of most of the conserved motifs in the butterfly wing, but additional diversity of coloration is regulated by a difference of interpretation of this gradient between the proximal and distal domains of the wing. An early proposal for the evolution of elaborate wing patterns in higher Lepidoptera by Süffert and Henke (reviewed in Nijhout, 1991) suggested that a single band pattern underwent mirror-image

duplications along the proximodistal axis. Our observations suggest that this could have occurred by the induction of novel peaks of a regulatory factor at several positions along this axis.

Acknowledgments

We thank Jane E. Selegue and Victoria A. Kassner for their excellent technical assistance in all aspects of the project from its inception. We thank Catherine L. Smith, Jamie Carroll, John Schlitz, Ashley Lange, Kathryn L. Clark, Andrea L. Hummitzsch, Megan Swaab, Rachel Leisso, Jessica Luckason, Sujata Patel, Rianna La Fave, Lauren Hansen, Marie Bechler, Cristi Frazier, Hannah Burrack, Chika McTier, Jessica Jacobson, Margaret Ritter, and Richa Misra who have conscientiously maintained the *Precis* colony in good condition for the past five years. We thank Brad Hersh and John Yoder for helpful comments on the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.09.014.

References

Beldade, P., Brakefield, P.M., 2002. The genetics and evo devo of butterfly wing patterns. Nat. Rev., Genet. 3, 442–452.

Belenkaya, T.Y., Han, C., Yan, D., Opoka, R.J., Khodoun, M., Liu, H., Lin, X., 2004. *Drosophila* Dpp morphogen movement is independent of dynamin-mediated endocytosis but regulated by the glypican members of heparan sulfate proteoglycans. Cell 119, 231–244.

Brakefield, P.M., Gates, J., Keys, D., Kesbeke, F., Wijngaarden, P.J., Monteiro, A., French, V., Carroll, S.B., 1996. Development, plasticity and evolution of butterfly eyespot patterns. Nature 384, 236–242.

Brunetti, C.R., Selegue, J.E., Monteiro, A., French, V., Brakefield, P.M., Carroll, S.B., 2001. The generation and diversification of butterfly eyespot color patterns. Curr. Biol. 11, 1578–1585.

Carroll, S.B., Gates, J., Keys, D.N., Paddock, S.W., Panganiban, G.E., Selegue, J.E., Williams, J.A., 1994. Pattern formation and eyespot determination in butterfly wings. Science 265, 109–114.

French, V., Brakefield, P.M., 1995. Eyespot development on butterfly wings: the focal signal. Dev. Biol. 168, 112–123.

Galant, R., Skeath, J.B., Paddock, S., Lewis, D.L., Carroll, S.B., 1998. Expression pattern of a butterfly achaete-scute homolog reveals the homology of butterfly wing scales and insect sensory bristles. Curr. Biol. 8, 807–813.

Goldschmidt, R.B., 1938. Physiological Genetics. McGraw-Hill, New York.

Greco, V., Hannus, M., Eaton, S., 2001. Argosomes: a potential vehicle for the spread of morphogens through epithelia. Cell 106, 633–645.

Green, J.B., New, H.V., Smith, J.C., 1992. Responses of embryonic Xenopus cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm. Cell 71, 731–739.

Jiggins, C.D., Mavarez, J., Beltran, M., McMillan, W.O., Johnston, J.S., Bermingham, E., 2004. A genetic linkage map of the mimetic butterfly, *Heliconius melpomene*. Genetics (doi:10.1534/genetics.104.034686).

Joron, M., 1998. Diversity in mimicry: paradox or paradigm? TREE 13, 483–489.

Kent, N., Leduc, L., Crane, J., Farine, D., Hodges, S., Reid, G.J., Van Aerde, J., 2000. Prevention and treatment of venous thromboembolism (Vte) in obstetrics. J. SOGC 22, 736–749.

Keys, D.N., Lewis, D.L., Selegue, J.E., Pearson, B.J., Goodrich, L.V., Johnson, R.L., Gates, J., Scott, M.P., Carroll, S.B., 1999. Recruitment of a hedgehog regulatory circuit in butterfly eyespot evolution. Science 283, 532–534.

Koch, P.B., Brakefield, P.M., Kesbeke, F., 1996. Ecdysteroids control eyespot size and color pattern in the polyphenic *Bicyclus anynana* (Lepidoptera: Satyridae). J. Insect. Physiol. 42, 223–240.

Koch, P.B., Merk, R., Reinhardt, R., Weber, P., 2003. Localization of ecdysone receptor protein during colour pattern formation in wings of the butterfly *Precis coenia* (Lepidoptera: Nymphalidae) and co-expression with Distal-less protein. Dev. Genes Evol. 212, 571–584.

Lewis, D.L., DeCamillis, M.A., Brunetti, C.R., Halder, G., Kassner, V.A., Selegue, J.E., Higgs, S., Carroll, S.B., 1999. Ectopic gene expression and homeotic transformations in arthropods using recombinant Sindbis viruses. Curr. Biol. 9, 1279–1287.

Mallet, J., 1999. Evolution of diversity in warning color and mimicry: polymorphisms, shifting balance, and speciation. Annu. Rev. Ecol. Syst. 30, 201–233.

Marcus, J.M., Ramos, D.M., Monteiro, A., 2004. Germline transformation of the butterfly *Bicyclus anynana*. Proc. R. Soc. Lond., B Biol. Sci. 271 (Suppl. 5), S263–S265.

Markovich, D., 2001. Physiological roles and regulation of mammalian sulfate transporters. Physiol. Rev. 81, 1499–1533.

McMillan, W.O., Monteiro, A., Kapan, D.D., 2002. Development and evolution on the wing. Trends Ecol. Evol. 17, 125–133.

Monteiro, A., Prijs, J., Bax, M., Hakkaart, T., Brakefield, P.M., 2003. Mutants highlight the modular control of butterfly eyespot patterns. Evol. Dev. 5, 180–187.

Nijhout, H.F., 1980. Ontogeny of the color pattern on the wings of *Precis coenia* (Lepidoptera: Nymphalidae). Dev. Biol. 80, 275–288.

Nijhout, H.F., 1984. Colour pattern modification by coldshock in Lepidoptera. J. Embryol. Exp. Morphol. 81, 287–305.

Nijhout, H.F., 1991. The Development and Evolution of Butterfly Wing Patterns. Smithsonian Institution Press, Washington.

Nijhout, H.F., 1997. Ommochrome pigmentation of the linea and rosa seasonal forms of precis coenia (Lepidoptera: Nymphalidae). Arch. Insect Biochem. Physiol. 36, 215–222.

Nijhout, H.F., 2003. Development and evolution of adaptive polyphenisms. Evol. Dev. 5, 9–18.

Nijhout, H.F., Grunert, L.W., 1988. Color pattern regulation after surgery on the wing disks of *Precis coenia* (Lepidoptera: Nymphalidae). Development 102, 377–385.

Otaki, J.M., 1998. Color-pattern modifications of butterfly wings induced by transfusion and oxyanions. J. Insect. Physiol. 44, 1181–1190.

Otaki, J.M., Yamamoto, H., 2004. Species-specific color-pattern modifications of butterfly wings. Dev. Growth Differ. 46, 1–14.

Panganiban, G., Nagy, L., Carroll, S.B., 1994. The role of the Distal-less gene in the development and evolution of insect limbs. Curr. Biol. 4, 671–675.

Tajima, Y., Amagai, H., Okamura, N., 1989. Phosphotungstate shows a heparin-like anticoagulant effect but inhibits heparin. Thromb. Res. 55, 329–339.

Umebachi, Y., Osanai, M., 2003. Perturbation of the wing color pattern of a Swallowtail Butterfly, *Papilio xuthus*, induced by acid carboxypeptidase. Zool. Sci. 20, 325–331.