Developmental Biology 388 (2014) 205–215

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Evolution of Developmental Control Mechanisms

The bristle patterning genes *hairy* and *extramacrochaetae* regulate the development of structures required for flight in Diptera ☆

Marta Costa [a], Manuel Calleja [b], Claudio R. Alonso [c,*], Pat Simpson [a,*]

[a] Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3 EJ, UK
[b] Centro de Biología Molecular Severo Ochoa, C/ Nicolás Cabrera, 1, Universidad Autónoma, 28049 Madrid, Spain
[c] John Maynard Smith Building, School of Life Sciences University of Sussex, Brighton BN1 9QG, UK

ARTICLE INFO

Article history:
Received 10 July 2013
Received in revised form
18 December 2013
Accepted 21 December 2013
Available online 30 December 2013

Keywords:
Drosophila
Calliphora vicina
scute
Sensory organs
Flight motor

ABSTRACT

The distribution of sensory bristles on the thorax of Diptera (true flies) provides a useful model for the study of the evolution of spatial patterns. Large bristles called macrochaetes are arranged into species-specific stereotypical patterns determined via spatially discrete expression of the proneural genes *achaete–scute (ac–sc)*. In *Drosophila ac-sc* expression is regulated by transcriptional activation at sites where bristle precursors develop and by repression outside of these sites. Three genes, *extramacrochaetae (emc), hairy (h)* and *stripe (sr)*, involved in repression have been documented. Here we demonstrate that in *Drosophila,* the repressor genes *emc* and *h,* like *sr,* play an essential role in the development of structures forming part of the flight apparatus. In addition we find that, in *Calliphora vicina* a species diverged from *D. melanogaster* by about 100 Myr, spatial expression of *emc, h* and *sr* is conserved at the location of development of those structures. Based on these findings we argue, first, that the role *emc, h* and *sr* in development of the flight apparatus preceded their activities for macrochaete patterning; second, that species-specific variation in activation and repression of *ac-sc* expression is evolving in parallel to establish a unique distribution of macrochaetes in each species.

Introduction

It is becoming clear that the evolution of developmental patterns is associated with changes in the networks of genes underlying the specification, differentiation and distribution of pattern elements. However, the specific molecular regulatory mechanisms involved and the way in which developmental networks evolve are only beginning to be explored. One mechanism for innovation is the co-option of pre-existing regulatory genes and/or networks for new roles. This has been documented in several cases, including the evolution of segmentation, heart development, butterfly wing spots, dorsal appendages of dipteran eggs and the neural crest (Keys et al., 1999; Meulemans and Bronner-Fraser, 2005; Olson, 2006; Chipman, 2009; Vreede et al., 2013). Co-option involves the rewiring of an existing gene network allowing it to affect the behavior of new cellular processes. This could occur through changes in a small number of components, such as changes in the expression domains of regulatory proteins, modification of their regulatory capacity, variation in *cis*-regulatory element composition at gene targets or changes in protein interaction domains in target proteins (Averof and Akam, 1995; Averof and Patel, 1997; Sucena and Stern, 2000; Alonso et al., 2001; Ronshaugen et al., 2002; Gompel et al., 2005; Erwin and Davidson, 2009). However identification of the molecular changes remains challenging because innovations are generally infrequent (Kopp, 2011) and their genetic analysis requires tractable experimental systems in which a morphological difference can be clearly attributed to a specific genetic alteration (Stern, 2000). The distribution of sensory bristles on the thorax of Diptera provides a useful model in which to address these questions (Simpson et al., 1999). Here we explore the possibility that an ancestral gene network has been recruited during the evolution of bristle patterns.

Many species of the sub-order Nematocera, the most ancient lineage of Diptera, display a uniform covering of randomly positioned but equally spaced bristles of similar size, a distribution thought to represent the ancestral state (McAlpine, 1981). Flies of the Cyclorrapha, a more recently derived lineage, also display uniformly spaced small bristles, microchaetes, but bear in addition large bristles, called macrochaetes, that are an evolutionary novelty of the Cyclorrapha. Macrochaetes are found in stereotypical, species-specific arrangements on the mesonotum (Simpson et al., 1999; Simpson and Marcellini, 2006). Expression of proneural genes

* Corresponding authors.
E-mail addresses: c.alonso@sussex.ac.uk (C.R. Alonso), p.simpson@zoo.cam.ac.uk (P. Simpson).

0012-1606/$ - see front matter © 2014 The Authors. Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2013.12.032

of the *achaete–scute (ac–sc)* family (Bertrand et al., 2002) regulates development of bristle precursors and the evolution of bristle patterns correlates with evolution of the temporal and spatial expression patterns of these genes (Simpson and Marcellini, 2006). Ubiquitous proneural gene expression can account for the pattern of uniformly spaced microchaetes (Pistillo et al., 2002; Wülbeck and Simpson, 2002). In contrast, macrochaetes arise from patterned *ac-sc* expression such that discrete domains of expression prefigure the sites at which macrochaete precursors will develop (Cubas et al., 1991; Skeath and Carroll, 1991; Wülbeck and Simpson, 2000; Pistillo et al., 2002). The evolution of macrochaetes is therefore associated with the acquisition of a spatially restricted pattern of *ac-sc* expression that furthermore evolves between species.

Investigation into the genetic regulation of *ac-sc* activity in *Drosophila melanogaster* has uncovered two gene networks that are partially redundant. On the one hand the transcription factors encoded by *pannier* (*pnr*) and the genes of the *Iroquois complex* (*Iro-C*) activate transcription in the proneural clusters (Gomez-Skarmeta et al., 1996; Garcia-Garcia et al., 1999). Activation requires numerous *cis*-acting regulatory elements scattered throughout the *ac-sc* complex *(AS-C)* that appear to have evolved along with duplication events at the *AS-C* in the lineage leading to the Cyclorrapha (Gomez-Skarmeta et al., 1995; Skaer et al., 2002; Negre and Simpson, 2009). In parallel to the activators, a second set of factors antagonizes *ac-sc* function by preventing accumulation of *ac-sc* products resulting from basal promoter activity at sites outside the positions of the proneural clusters (Garrell and Modolell, 1990; Van Doren et al., 1991; Van Doren et al., 1994; Usui et al., 2008). Three antagonists have been studied, the products of the genes *stripe* (*sr*), *extramacrochaetae* (*emc*) and *hairy* (*h*). They are expressed in partially overlapping discrete spatial domains and are sufficient to correctly position bristle precursors under experimental conditions of uniform Sc expression (Rodriguez et al., 1990; Cubas and Modolell, 1992; Brand et al., 1993; Dominguez and Campuzano, 1993; Fernandes et al., 1996; Usui et al., 2008). None of these factors act via the *cis*-regulatory sequences of the *AS-C* that are the targets for Pnr and the Iro-C transcription factors (Usui et al., 2008). Thus patterning of bristles by *sr*, *emc* and *h* acts independently from patterning by activation of *ac-sc*.

Bristle patterns are subject to constraints imposed by structures on the thorax that are important for flight. For instance no bristles of any sort are positioned over the ridges, sutures and wing processes that are part of the flight motor (McAlpine, 1981). In addition macrochaetes, but not microchaetes, are excluded from the sites of attachment of flight muscles (Usui et al., 2004). Interestingly, the expression domains of *sr, emc* and *h* correlate with the regions from which these structures arise. So are all three genes required for the development of these structures? The flight motor of the Diptera is a highly conserved feature that was probably present in an early ancestor of this insect order long before macrochaetes appeared. If *sr*, *emc* and *h* play a role in specifying parts of the flight motor this would be likely to precede that for macrochaete patterning. It is indeed well documented that *sr* plays an important role in the development of tendons (Volk, 1999; Ghazi et al., 2003). Furthermore some of the sutures on the notum fail to form when the activity of *emc* is impaired (de Celis et al., 1995). Here we show that, in *D. melanogaster*, both *emc* and *h* are required for development of thoracic sutures, wing hinge sclerites, scutellum and scutellar lever arm. We also show that the expression of *sr, emc* and *h* in regions that give rise to the flight apparatus is conserved in *Calliphora vicina*. This is in contrast to the spatial expression of *emc* on the dorsal scutum where, like that of *ac-sc*, expression evolves in a dynamic fashion between the two species and correlates with changes in macrochaete patterns. We therefore suggest that functions of the genes related to flight are ancient and that their roles in bristle patterning might have been co-opted relatively recently in the lineage leading to the Cyclorrapha. Patterning of bristles by *emc*, *h* and *sr* would not require the evolution of any new features at the *AS-C* itself, whereas patterning through transcriptional activation is associated with gene duplication and the acquisition of numerous *cis*-regulatory elements (Skaer et al., 2002; Simpson and Marcellini, 2006; Negre and Simpson, 2009). Thus we also argue that the two mechanisms might have evolved sequentially.

Materials and methods

Fly rearing

Drosophila melanogaster flies were kept at 25 °C and fed on standard food. *Calliphora vicina* flies were kept at room temperature and fed on sucrose. Larvae were kept at room temperature and fed on minced meat.

Gene cloning

Fragments of the genes *hairy* and *extramacrochaetae* were isolated from genomic DNA extracts from *Calliphora vicina* using degenerate PCR primers. Hairy and Emc sequences from several Dipteran species were aligned using CLUSTALW software and degenerate primer pairs were designed based on these alignments. The degenerate primers used for *hairy* were the following: Forward h_F1 5′ GARAARACNGTNAARCA YYTICA 3′; h_F2 5′ CARGYNGCNGA YCCIAARRT 3′; Reverse h_R1 5′ CCRTTIGGNARYTTNGTNGG 3′; h_R2 5′ CCANGGYCTCCANGGYTGNTCYTC 3′; h_R3 5′ACIAGISWNAGNGGYTGYTG3′.

The primers were designed for nested PCR, with h_F1 and h_R2 being the outer ones. The degenerate primers used to isolate *emc* were the following: Forward emc_F1 5′A TGAARDSNHTNACNGCIGTITG 3′; emc_F2 5′ GGNGARAAYGCNGARATIMARATGTA 3′; Reverse emc_R1 5′GTRTTNGGNSWYTGICKRTC 3′; emc_R2 5′ TGNCKRTCNVYNAGIGG 3′.

In this case emc_F1 and emc_R1 were the outer ones. The gene fragments obtained were cloned into pGEM-T Easy Vector (Promega) and sequenced. The identity of the fragments was verified by using BLAST with default values for algorithm parameters. In order to test for any species cross-contamination of the gene fragments obtained, specific PCR primers were designed and tested on new genomic DNA samples. Following isolation of gene fragments, the SMART™ RACE cDNA Amplification Kit (Clontech) was used to obtain the complete coding region and the manufacturer′s protocol was followed.

RNA in situ hybridization

Digoxigenin-labelled (Roche) and/or fluorescein-labelled RNA (Roche) probes were made following standard protocols. The orthologous fragments of *hairy* and *emc* obtained by degenerate PCR primers were used as a transcription template. For *C. vicina scute* a fragment isolated by (Pistillo et al., 2002) was used. In *D. melanogaster* there are two isoforms of *sr, srA* and *srB* (Frommer et al., 1996). An orthologue of *srB* was isolated in *C. vicina* by (Richardson and Simpson, 2006). For *stripeB*, the template was a fragment of the first exon cloned from genomic DNA using the following specific primers: forward- 5′ ACATGCCTGTTTAAGACCAC 3′; reverse- 5′ TGTATTCAAATCTCCCTGCT 3′. For *D. melanogaster*, the 5′UTR plus the first exon of *hairy* and *emc* was used as transcription template. These fragments were isolated from genomic DNA using specific primers.