Minireview

Temporal and spatial patterning of an organ by a single transcription factor

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Abstract

During the formation of animal organs, a single regulatory factor can control the majority of cell-fate decisions, but the mechanisms by which this occurs are poorly understood. One such regulator, the nematode transcription factor PHA-4, functions together with various *cis*-regulatory elements in target genes to regulate spatial and temporal patterning during development of the pharynx.

Animal organs are composed of multiple varied tissues, which must form coordinately in the right place and in the right sequence during development [1,2]. A process as complex as organ formation requires precision and selectivity of gene expression on a number of spatial and temporal levels. Certain genes are expressed in all of the cells that will constitute the particular organ, thus conferring an organ identity upon a field of cells, while other genes are expressed specifically in subsets of cells, thus allowing differentiation of tissue types within the organ. Both of these kinds of gene-expression program in organogenesis are coordinated and regulated temporally so that the expression patterns follow a precise sequence. It might be expected that the various levels of control would require a large number of transcriptional regulators, but an astonishing finding from more than a decade of research is that complex patterns of cell-fate determination and differentiation can be regulated by single 'selector' genes [3,4]. A selector gene encodes a gene regulator, typically a transcription factor, which autonomously regulates cell-fate decisions within cells of the nascent organ. An example is the *Caenorhabditis elegans* transcription factor PHA-4, a member of the FoxA family, which regulates formation of the foregut - or pharynx - that pumps material from the environment into the gut of the animal [3,5,6] . But how does a single transcription factor orchestrate the diversity of gene-expression patterns that emerges during organogenesis? This question has lacked experimental elucidation until now. In two microarray studies that build upon their previous work on PHA-4 [7], Susan Mango and her associates at the University of Utah have shown for the first time how a selector transcription factor functions with a combination of *cis*-regulatory elements to regulate cell-fate determination both spatially [8] and temporally [9].

Identification of *cis*-regulatory elements that function in organ patterning

To identify genes that are primarily expressed in the pharynx, Mango and colleagues [8,9] profiled transcripts from the mutant strains *par-1* and *skn-1*. Worms with *par-1* mutations produce an excess of pharyngeal cells following transformation of gut cells to a pharyngeal fate, whereas *skn-1* animals produce no pharyngeal cells owing to transformation of pharynx precursors into body muscle and epidermis (Figure 1). Comparing expression levels between *par-1* and *skn-1* animals increased the sensitivity of the analysis, as differences in specific expression levels were much larger than would be seen in a more traditional comparison, such as between wild-type and *skn-1* animals. Thus, genes that would have been excluded in a traditional comparison, such as genes that are expressed only in subsets of pharyngeal

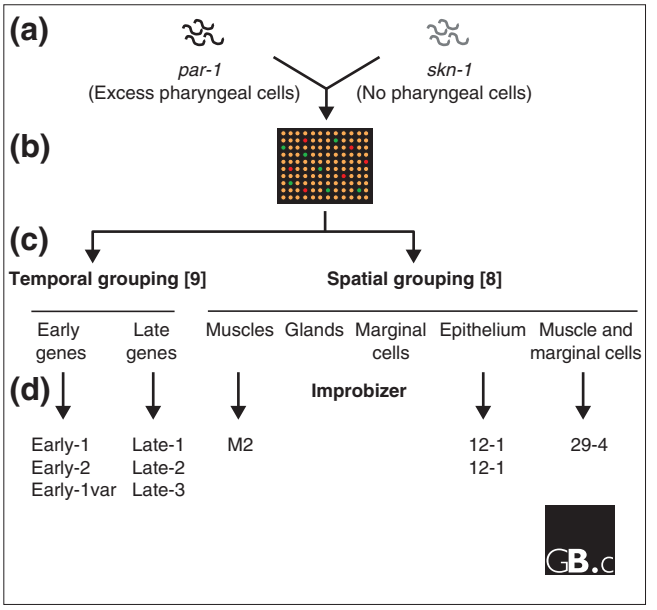

Figure 1
An outline of the experimental strategy used by Mango and colleagues [8,9] to identify regulatory motifs that specify temporal and spatial patterns of gene expression during pharyngeal development. **(a)** RNA was isolated from worms with mutations in the *par-1* or *skn-1* genes, which have excess or no pharyngeal cells, respectively. **(b)** The RNA from the two strains was compared using a whole-genome microarray. **(c)** Transcripts with high levels of expression in *par-1* worms compared with *skn-1* worms were selected and sorted into groups according to their temporal [9] or spatial [8] pattern of expression. For the temporal groupings the genes were divided into those expressed early or late in pharynx development; for the spatial groupings they were divided into those expressed in the muscles, glands, pharyngeal marginal cells or epithelium, plus those that were expressed in both the muscles and the marginal cells. **(d)** The promoters of the genes in each group were analyzed using the Improbizer algorithm to find sequence elements that were significantly enriched in each group; these were named Early-1, M2, and so on. A selection of these is shown.

cells or are expressed at very low levels, were readily detected from the *par-1* to *skn-1* comparison.

The next stage of the analysis was the identification of regulatory elements within the promoters of the identified pharyngeal genes. The pharynx-specific genes were grouped according to their temporal and spatial expression patterns and sequences in the proximal regions of the promoters of grouped genes were analyzed for overrepresented sequence elements (Figure 1). One factor that contributed to the success of this stage was the recently completed genome sequence of the related nematode *Caenorhabditis briggsae* [10,11]; conservation of sequences between the genomes of *C. elegans* and the closely related *C. briggsae* is often used to make a case for their biological relevance [10]. When Mango and colleagues [8,9] looked at pharyngeal gene promoters, they found that the proximal 500 base-pairs of promoter sequence were the most conserved between *C. elegans* and *C. briggsae* genes; they therefore decided to limit their analysis to these regions, thus increasing their chances of identifying sequences motifs of biological relevance.

Another important factor contributing to the success of this stage was the use of the Improbizer algorithm [12], which identifies sequence motifs that occur at significantly high rates within a sample pool and which has the advantage that *a priori* knowledge of the *cis*-regulatory sequence is not required. Thus, when used on a population of genes associated with a particular biological activity, Improbizer can identify novel sequences involved in gene regulation associated with that particular activity.

The criteria used for subdivision of the pharynx-specific genes into temporal and spatial classes were a critical aspect of the experimental design. In the study by Gaudet *et al.* [9], the pharynx-specific genes were subdivided into two temporal classes, depending on whether expression began during mid-embryogenesis ('early' genes) or at the start of terminal differentiation of the pharynx ('late' genes). This grouping was used to identify sequence elements that were enriched in one temporal group compared with the other. In the study by Ao *et al.* [8], the total complement of pharyngeal genes was subdivided into five groups on the basis of their spatial expression patterns. Sequence elements that were particularly enriched in the promoters of each group were identified as potential *cis* elements involved in regulation of spatial expression patterns. In both studies [8,9], the rich resources available to *C. elegans* biologists, including databases of expression patterns obtained from *in situ* hybridization studies [13], three-dimensional 'Topo' maps for identifying genes with shared expression patterns [12] and the wealth of detailed studies on embryogenesis and larval development, were crucial in creating spatial and temporal groupings of genes that were analyzed with the Improbizer algorithm.

The results of these analyses were a set of sequence motifs that were found to be overrepresented in promoters of particular subgroups of pharyngeal genes (Figure 1). But are these motifs actually used for gene regulation in the developing worm? Many microarray and bioinformatic approaches flounder when it comes to biological validation of the sequence motifs identified, but Mango and colleagues [8,9] took a multipronged approach that not only allowed them to test the identified sequences for biological relevance but also provided information about the function of each promoter element. The initial validation test was for enhancer activity of the identified motif in the context of a minimal exogenous promoter driving a reporter gene. This assay allowed the investigators to evaluate the regulatory element on three different criteria: whether the sequence was sufficient to activate expression and act as an enhancer, whether expression was primarily pharyngeal, and whether it was sufficient to confer a temporal pattern of expression. These tests not only confirmed pharyngeal expression and temporal patterns of

expression for candidate sequences, but in one case also showed that an element acted as a repressor. In the second round of validation tests, pharyngeal genes containing each candidate regulatory element were identified, and site-directed mutagenesis of the element was used to evaluate whether loss of function led to loss of the temporal pattern of expression. The native context of the identified temporal elements was further investigated by searching the promoters of the 'early' and 'late' groups of genes for conserved clustering or combinations of temporal elements. The patterns identified were also used in a bioinformatics search to find additional pharyngeal genes that had not been identified from the microarray experiments, further validating the biological relevance of the identified sequences.

A model for combinatorial transcriptional control driving temporal patterning

The validation assays allowed Gaudet *et al.* [9] to address the core question of their study: how the PHA-4 binding sites and the temporal elements work together to regulate the timing of gene expression during pharyngeal organogenesis. Using synthetic promoters with various combinations of PHA-4 sites and the temporal *cis*-regulatory elements they had identified, Gaudet *et al.* [9] established a model of how transcriptional regulation drives temporal patterning (Figure 2). The essence of this model is that, although no one element is sufficient to drive expression, PHA-4 sites act combinatorially with 'early' or 'late' elements to drive gene expression at specific times. Gaudet and Mango [7] had previously shown that for many genes the binding affinity of PHA-4 for its promoter element could determine the timing of expression: genes with high-affinity binding sites were expressed earlier in development and genes with low-affinity binding sites were expressed later in development. These two modes of transcriptional regulation, differences in PHA-4 binding-site affinity and combinatorial activation of expression, together seem to account for the temporal expression patterns of the majority of pharyngeal genes. The work by Ao *et al.* [8] implicates a similar, albeit less complex, combinatorial system in spatial specification of gene expression during pharyngeal morphogenesis. For example, the M2 motif (see Figure 1) appears to confer muscle-cell identity upon cells whose pharyngeal identity has already been specified by PHA-4 activity.

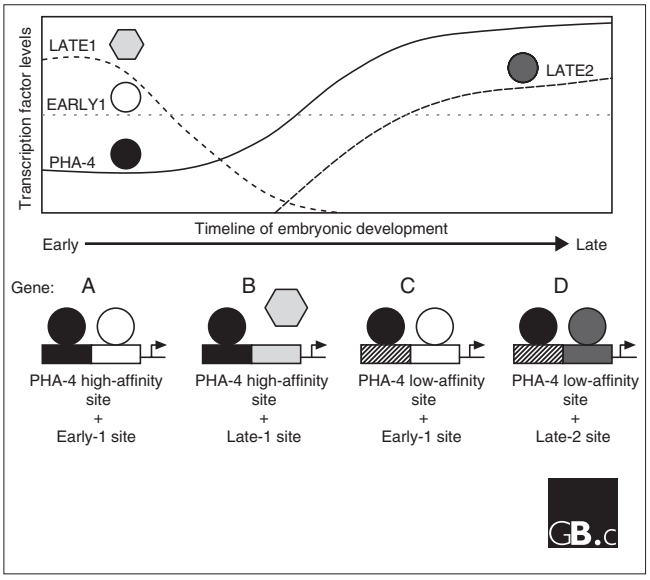

Figure 2
A model for the temporal control of pharyngeal gene expression as proposed by Gaudet *et al.* [9]. The temporal expression patterns of four transcription factors are shown at the top, and the promoters of four genes (A-D) that are expressed at different times during pharyngeal development are shown below. EARLY1, LATE1 and LATE2 are the putative transcription factors assumed to bind to the promoter elements Early-1, Late-1 and Late-2 identified by Gaudet *et al.* [9] and shown in Figure 1; the factors themselves have not been identified. Varying combinations of PHA-4-binding sites and temporal *cis*-regulatory elements drive expression of genes A-D at different times during pharyngeal development. In this model neither the PHA-4-binding site nor any of the temporal elements alone is sufficient for gene activation. Early expression of gene A is driven by recruitment of PHA-4 (black circle) to a high-affinity site (black box) along with recruitment of the putative EARLY1 factor (white circle) to an Early-1 site (white box). As PHA-4 is present at low levels early in development, only a gene carrying a high-affinity PHA-4 site can efficiently recruit PHA-4 for activation. As PHA-4 levels increase over the course of development, however, genes such as C that carry a low-affinity PHA-4 site (hatched black and white boxes) can also be activated. The onset of expression of gene C is primarily controlled by the affinity of PHA-4 for its site rather than by the Early-1 site or the EARLY1 factor, which may be expressed at stable levels throughout development. Expression of gene B is derepressed when the putative repressor LATE1 (light gray hexagon) falls to low enough levels to vacate the Late-1 site (light gray box). The timing of expression of a gene carrying a Late-1 site could be further retarded if the Late-1 site was paired with a low-affinity PHA-4-binding site. Transcription of gene D is activated late in development when the putative factor LATE2 (dark gray circle) rises to high enough levels to be recruited to the Late-2 site (dark gray box). The timing of expression of gene D could be advanced by pairing the Late-2 site with a high-affinity PHA-4-binding site.

Spatial and temporal patterning pathways may use similar mechanisms

How universal is the model of combinatorial transcription control proposed by Gaudet *et al.* [9]? Certainly, no *Drosophila* biologist working on pattern formation would be surprised by the findings of Mango and colleagues, and the model describing the transcriptional control of temporal patterning is striking in the resemblance that it bears to the classical models of anterior-posterior patterning in the *Drosophila* embryo [14]. The most obvious similarity is that in both nematode pharynx development and fly anterior-posterior patterning, gene expression, either at a particular time or at a particular point in space, is specified by a unique combination of regulatory molecules and *cis*-regulatory elements. These unique combinations are generated by the same mechanisms in both systems; for example, there is graded expression of regulatory molecules across axes, such

as the increasing levels of PHA-4 from early to late in *C. elegans* embryogenesis and the increasing levels of Hunchback protein along the posterior-anterior axis of the *Drosophila* embryo. Furthermore, in both systems the varying affinity of a transcription factor for its binding site creates a finer gradation of responses, as described for PHA-4 sites in pharyngeal genes (Figure 2) and as in the case of Hunchback binding sites along the promoter of its target genes, such as that encoding the transcription factor Even-skipped [15].

Temporal patterning of the developing pharynx is also similar to temporal patterning of another *C. elegans* organ, the epidermis or hypodermis. The 'heterochronic' pathway is a dedicated genetic pathway that regulates the timing of cell-fate determination in the hypodermis during post-embryonic development in *C. elegans* [1]. As with the pharyngeal pathway, temporally graded levels of key heterochronic molecules, many of which are transcription factors, specify the timing of cell-fate decisions. However, unlike the pharyngeal pathway elucidated so far, two of the heterochronic regulatory genes, *lin-4* and *let-7,* code for microRNAs that act post-transcriptionally to downregulate protein expression [16-18]. It may be that temporal patterning of the pharynx also involves undiscovered microRNA regulators; for example, PHA-4 expression is regulated by the *let-7* miRNA [19]. Mango and colleagues [7-9] limited their search for regulatory sequences to promoter regions but, as pointed out by the authors, it is also possible that expression is temporally regulated through sequence elements in the introns and 3′ untranslated regions (UTRs) of pharyngeal genes, perhaps through microRNA rather than protein regulators. One possibility is that microRNAs may themselves behave like selector factors. The *lin-4* and *let-7* microRNAs are both expressed in a temporally graded manner during larval development and appear to have a large number of regulatory targets, much like the selector transcription factor PHA-4 [18,20]. MicroRNAs may use similar strategies of acting synergistically with temporally regulated factors, in combination with differential affinities for their 3′ UTR binding sites, to control the timing of cell-fate decisions [20].

The work by Gaudet *et al*. [9] elucidates some of the transcriptional strategies used to control the timing of gene expression during *C. elegans* pharyngeal development. Similar strategies may be used in other developmental pathways, such as the heterochronic pathway in the hypodermis. The principles of the temporal control of development are being elucidated primarily in *C. elegans,* but the striking similarities between the mechanisms of temporal and spatial patterning [1] and the strong conservation of the *let-7* microRNA and *pha-4* across animal phyla [5,6,20,21] suggest that what is learnt in the lowly worm may well be applicable to higher species, such as humans.

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