Int. J. Dev. Biol. 42: 417-421 (1998)

From selectors to realizators#

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ABSTRACT In 1975 Antonio García-Bellido proposed a framework for understanding the morphogenetic function of homeotic genes in terms of selector genes and realizator genes. Since then, much has been learnt of the molecular nature and expression patterns of the *Hox* selector genes. Our identification of realizator genes, and our understanding of how specific sets of realizators are activated in different segments, is still far from complete, however. Here we discuss the nature of the *Hox* target genes identified so far and the basis of the target specificity of *Hox* gene products.

KEY WORDS: *Hox target genes, Hox cofactors, morphogenesis, Drosophila*

Introduction

In his contribution to the Ciba Foundation Symposium in 1975, Antonio García-Bellido proposed that a hierarchy of three classes of genes, 'activators', 'selectors' and 'realizators', accounts for cell differentiation in developmental pathways. This provided a functional scheme for the control of morphogenetic processes and he "applied the name *selector gene* to (these) homeotic genes and *realizator gene* to the cytodifferentiation genes which convert the abstract signals into actual developmental operations" (García-Bellido, 1975). The key proposal was that, once activated in their appropriate territories by "activator genes", the homeotic loci would not act directly to specify morphological differences between metameric units; they would rather 'select' a battery of subordinate targets, the 'realizator' genes, encoding cellular proteins directly required in cell differentiation processes. This major conceptual advance significantly contributed to the definition of a theoretical framework for subsequent developmental genetic analysis.

The considerable amount of genetic and molecular data accumulated over the last twenty years has largely substantiated this view. It is now well established that a hierarchy of genetic modules, comprising maternal and the various classes of segmentation genes, is sequentially deployed to specify the temporal and spatial ordering of *Hox* gene activation. In addition, interactions occur between genes of the same class within the hierarchy, leading to the further definition of *Hox* expression domains (for reviews see McGinnis and Krumlauf, 1992; St. Johnston and Nüsslein-Volhard, 1992). Therefore, by 5-6 h of development, each metameric unit specifies its own identity by expressing a unique combination of *Hox* genes. The discovery of the homeodomain provided a key molecular clue to the function of the encoded proteins. The homeodomain, a sixty amino acid motif, mediates sequence-specific DNA-binding (Desplan *et al.*, 1988; Ekker *et al.*, 1994) and Hox proteins have been shown to act as positive or negative transcription factors (Krasnow *et al.*, 1989). It is therefore thought that each metamer will enter a specific morphogenetic pathway and develop unique shape, form and function depending on the target genes transcriptionally regulated by the set of Hox proteins expressed in it.

Although the question of target gene identity and function is not a novel problem, significant progress has been gained only recently (for review: Graba *et al.,* 1997). We shall here briefly summarize the strategies deployed to identify *Hox* target genes, next consider how their function contributes to pattern and morphogenesis, and finally discuss how the work on targets and cofactors of Hox proteins has improved our understanding of Hox complex gene function.

Target genes identification

Various strategies have been deployed in searching for Hox target genes. Each one presents real advantages but clear shortcomings as well, and none among them stands out as optimal. Classical genetics has been almost ineffective, presumably because mutations in target genes do not express simple homeotic-related phenotypes. *In vitro* studies were also unsuccessful, due to the poor DNA binding specificity displayed by homeodomain-

Abbreviations used in this paper: abd-A, abdominal-A; Abd-B, abdominal-B; Antp, Antenapedia; cnn, centrosomin; dpp, decapentaplegic; Dfd, Deformed; exd, extradenticle; lab, labial; Scr, Sex comb reduced; tsh, teashirt; Ubx, Ultrabithorax; wg, wingless.

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0214-6282/98/$10.00

containing proteins (Ekker *et al.*, 1994). The majority of putative targets have been identified on the basis of homeotic gene control of the expression patterns of previously characterized genes or enhancer trap lines. For such putative targets it is difficult to know whether the control by Hox proteins is direct or not. For one of them only, the *decapentaplegic (dpp)* gene, has definitive proof been obtained that regulation occurs directly (Capovilla *et al.*, 1994; Sun *et al.*, 1995). The subtractive hybridization procedure, developed to isolate sequences differentially transcribed upon *Hox* gene overexpression, suffers from the same drawback (Feinstein *et al.*, 1995). Another recently proposed method consists of a yeast screen of genomic DNA fragments able to activate a reporter gene in the presence of a Hox protein (Mastick *et al.*, 1995). While in principle limiting the search to positively regulated sequences, at non physiological concentrations of the Hox transregulator and in the probable absence of assistant cofactors, the method has the significant advantage that it selects directly controlled targets in an *in vivo* context. Immunoprecipitation of DNA fragments bound *in vivo* to Hox proteins and subsequent cloning of transcription units in their vicinity also appears specially designed for cloning direct effectors (Gould *et al.*, 1990a; Graba *et al.*, 1992). The two last procedures present the additional advantage of identifying Hox response elements in the targets, key tools to analyze in molecular terms the mode of action of Hox proteins.

Nature and function of *Hox* target genes: realizators versus transcriptional regulators

So far, some twenty candidate *Hox* target genes have been identified in *Drosophila*. While this small set of genes does not allow us to draw up a panoramic overview of the functions deployed downstream of the homeotic complex, it however deserves several general comments. Examination of the nature of the encoded proteins and of genetic data that are available for most of the target genes, first emphasizes that only three of them correspond to realizator genes, in the sense of the word given by García-Bellido; meaning that their products specify at a cellular level characteristics that are central in morphogenetic processes, such as the rate and orientation of mitoses, size and shape of cells, cell-cell adhesion and communication, and eventually expression of terminal differentiation products. Consistent with a direct involvement of the three gene products in basic cellular processes, the cytoskeleton-associated β3-tubulin presumably has a role in cellular morphology changes (Hinz *et al.,* 1992), the centrosomal protein Centrosomin (Cnn) in the assembly or stabilization of microtubular networks (Li and Kaufman, 1996) and the homophilic cell adhesion protein Connectin in the formation of neuromuscular connections (Gould and White, 1992; Nose *et al.*, 1992).

However, all the other identified targets encode regulatory molecules, either transcription factors or cell signaling proteins (reviewed in Graba e*t al.*, 1997; see also Isaac and Andrew, 1996 and Andrew *et al.*, 1997), and thus themselves function to select the activity of downstream genes. Therefore, the genetic cascade that controls pattern formation extends to tiers beyond the *Hox* complex genes, before finally reaching the activation of realizator genes thought to provide the basic cellular functions required for differentiation processes.

Significant in the context of the control of morphogenetic programs, is the role of signaling proteins for the connection of embryonic germ layers. Germ layers develop almost independently during early embryogenesis, but become functionally interconnected as morphogenesis proceeds under the control of Hox proteins. The best example for such a mechanism involves Dpp and Wingless (Wg). These molecules are produced in the visceral mesoderm under the control of *Ultrabithorax (Ubx)* and *abdominal-A (abd-A)*, and are secreted towards the midgut endoderm where they regulate the activity of the Hox gene *labial (lab)* and thereby control gut cell differentiation.

Nature and function of *Hox* target genes: multiple Hox control and multiple roles

Another noteworthy point is that most if not all the known targets are controlled, positively or negatively, by several *Hox* gene products. It therefore appears that Hox proteins, according to their relative abundance and differential affinity for target DNA sequences, compete for the regulation of overlapping sets of downstream genes. Competition for common targets, together with the changes in activity of individual Hox proteins upon interaction with assistant cofactors (see below), provides a fundamental clue for understanding how the specificity of action of Hox genes is achieved in the whole animal. We must relate these recent observations that Hox proteins recruit overlapping set of downstream genes to a comment of García-Bellido about the diversification of morphogenetic traits during evolution: “The appearance of new selector genes does not demand new realizator genes, but only a quantitatively different utilization of those already existing, so that, in this sense, the amount of genetic information required for evolutionary complication is kept to a minimum” (García-Bellido, 1975).

From this point of view, it is also significant to note that target genes fulfil essential functions in developmental processes other than downstream of Hox proteins. Most are expressed early in development and play critical roles before becoming subject to homeotic control. For example, the *dpp* product acts as a morphogen for the establishment of the dorsoventral polarity in early embryogenesis (St Johnston and Gelbart, 1987), *wg* is a prototypic segment polarity gene (Nüsslein-Volhard and Wieschaus, 1980), *teashirt (tsh)* defines the basal identity of trunk segments (Fasano *et al.*, 1991). Thus, Hox proteins have recruited targets from genes involved in various developmental mechanisms and have coordinated their functions in morphogenetic programs.

Actually, very little is known of precisely how target genes work together to control proper organogenesis. To date, the single documented case of a mechanistic integration of Hox target gene functions is the control of morphogenesis in the central part of the midgut. Three targets, *dpp*, *wg*, and *cnn* are essential for this process. In the visceral mesoderm, Cnn is thought to participate in the reorganization of the microtubular network of cells involved in central constriction formation (Heuer *et al.,* 1995; Li and Kaufmann, 1996); *dpp* and *wg* are required first for a feed-back regulatory loop that maintains their own expression and *Ubx* transcription in the appropriate territories (Hursh *et al.,* 1993; Thüringer *et al.*, 1993; Xiang *et al.,* 1996), and second for the production of the transcription factor *Tsh*. In the endoderm, signaling by *Dpp* and *Wg* is necessary for a nuclear translocation of *Extradenticle* (*Exd*) and for a differential activation of *lab*, which in turn, and probably through an interaction with *Exd*, drives the expression of target genes that commit cells to distinct differentiation pathways (Hoppler and

Bienz 1995; Mann and Abu-Shaar, 1996). This is the unique example to date of how Hox-dependent coordination between cell signaling, transcription regulation and dynamics of cellular morphology results in the induction of a morphogenetic process. How the functions of other targets are integrated in morphogenetic control networks is far less understood, which illustrates that work on the identification and functional characterization of *Hox* target genes is just in its infancy.

Target gene regulation

The patterning information in the overlapping domains of Hox "selector" gene expression is translated into segment-specific morphology through the differential activities of target genes in different segments. How are these differential activities achieved? The regulation of some target genes has been studied and leads us to a few generalizations: individual target genes tend to be regulated by several Hox "selector" genes; target gene expression tends to be highly tissue-specific and the same target gene can show different responses to homeotic genes in different tissues or at different stages in development; the endogenous regulatory sequences controlling target gene expression tend to be long, typically several kilobases in length.

Understanding the molecular basis of this complex regulation involves first the identification of the DNA sequences within these large regulatory regions that are directly involved in the homeotic response (i.e., Hox response elements). This search for *in vivo* binding sites has been hampered by the poor sequence specificity exhibited by Hox proteins *in vitro*. This lack of sequence specificity *in vitro* contrasts with the specific effects of Hox proteins *in vivo* and led to the hunt for cofactors that could interact with specific members of the *Hox* family to enhance the sequence selectivity of their binding. The paradigm was provided by yeast mating type products (reviewed in Johnson, 1995). The yeast homeodomain products a1 and α2 bind poorly to DNA on their own but specificity is enhanced through protein-protein interactions. The a1-α2 heterodimer recognizes a target sequence that consists of two monomer binding sites separated by a fixed number of base pairs. A separate set of target sites are recognized by α2-α2 homodimers with two monomer binding sites with a different spacing. The constraint on the spacing is dependent on an additional interaction with a MCM1 dimer which stabilizes the configuration of the α2 -α2 dimer and determines the spacing of the DNA-binding surfaces. Also, protein-protein interactions may lead to conformational changes which affect DNA binding. Through these protein-protein interactions, complexes exhibiting high specificity of DNA binding can be formed from subunits which individually show little sequence specificity.

Hox cofactors and target specificity

Recent studies have provided strong evidence that this yeast paradigm will also hold for the Hox genes of *Drosophila* and for *Hox* genes in general. The best studied cofactor, *Exd*, fits well with the yeast model (reviewed in Mann and Chan, 1996). *Exd* (and its vertebrate homologs, the *PBX* genes) encodes a homeodomain product with considerable sequence similarity to yeast a1. It shows cooperative binding with *Hox* gene products on some target genes and specific *Hox/Pbx* heterodimers show individual target sequence specificity. The *in vitro* effects have been strongly supported by *in vivo* experiments where short sequences, that cooperatively bind *Exd* and a Hox protein, function as *in vivo* target sites exhibiting specific *Hox* gene control. Analysis of the Repeat 3 sequence of the mouse *Hox B1* regulatory sequences led to a 20bp sequence that cooperatively binds *Hox B1/Pbx* or the homologous *Drosophila* heterodimer, *Lab/Exd*. This sequence is capable of driving reporter gene expression in *Drosophila* in a pattern which closely overlaps the expression pattern of the *lab*. This experiment provided the first evidence that a short sequence, comparable in size to the yeast haploid specific gene operator, was capable of acting as a *Hox* response element *in vivo*. Its small size implies that its function depends on only a few binding proteins and raises hopes that *Hox* target gene specificity can be understood by the investigation of the interactions between a few proteins at a specific target site. This expectation is strengthened by the observation that a small change in the target site sequence dramatically alters the response: changing two base pairs switched the regulation from a *Lab* response to control by *Deformed* (*Dfd*; Chan *et al*., 1997).

These experiments show that subtle changes in DNA sequence can switch the target site regulation. However, they also present a picture of individual target sequences being regulated by individual *Hox* genes: i.e., a *lab*-specific and a *Dfd*-specific target. It is not clear that this gives an accurate impression of *in vivo* binding specificity. To register in this response element assay a *Hox* gene product must not only bind but must also activate. A *Dfd* specific response could be produced from a target that binds several other *Hox* products as well as Dfd protein but upon which only Dfd is capable of forming a complex with activation activity. Other *Hox* gene products may bind but fail to activate or may even repress. The analysis presented does not directly address this issue by, for example, looking at the effect of ubiquitous *Ubx* expression on the activity of the "*Dfd-specific*" target. However, it is striking that ubiquitous expression of *Dfd* from a *hs-Dfd* construct led to strong expression of *Dfd* in head segments but little expression in trunk segments where *Dfd* may have to compete with other *Hox* gene products. This would be consistent with this element being bound not only by *Dfd* but also by *Sex comb reduced (Scr)*, *Antenapedia* (*Antp*), *Ubx* and *Abd-A*. In the published figure there is some support for enhanced expression in posterior segments indicating that Abdominal-B (Abd-B) may not bind this element. Regulation of a *Hox* response element by this same set of *Hox* genes was shown in the case of the *Hox-B4 CR3* element although it was not definitively demonstrated that all these *Hox* genes were acting on a single binding site (Gould, *et al*., 1997).

Across the Hox gene complexes there are three main groups of homeobox sequences: *lab, Dfd-abd-A* (i.e., *Dfd, Scr, Antp, Ubx, abd-A*), and *Abd-B*. These groupings also show some relationship to the morphological segmental types regulated by these genes divided into head, (gnathal plus trunk) and genital. These morphological types may roughly correspond to the regulation of three sets of target genes. The interaction of *Hox/Exd* dimers on subtly different target sequences provides an explanation for the regulation of specific targets by the *Lab* and *Dfd-AbdA* groups. However, we still require mechanisms for specificity within the Dfd-AbdA group and for specific binding by *Abd-B* which lacks the YPWM motif that appears critical for interaction with Exd. Analysis of the regulatory regions of target genes indicates that many respond to regulation by at least several members of the *Dfd-AbdA* group

although the resolution of these analyses has not been sufficient to conclude that all these genes compete for the same binding site. Competition for the same target sites would, however, provide an explanation for the phenotypic suppression/posterior dominance phenomenon whereby more posterior genes within this group have a dominant effect over more anterior genes (Gonzalez-Reyes and Morata, 1990). The differences in activity between the gene products within this group may not lie so much in individual binding site preferences but rather their relative hierarchy of effects on the same target sequences. We do not yet know whether this dominance scale reflects binding affinity or, as suggested by Pinsonneault *et al.* (1997), a functional hierarchy.

Regulation of Exd availability

The function of Exd as a cofactor enhancing the binding specificities of *Lab* and *Dfd-AbdA* group *Hox* gene products suggests a widespread if not ubiquitous availability of the Exd protein. Exd is produced as a maternal product giving uniform expression followed by patterned zygotic expression. Uniform Exd was initially thought to be sufficient for its function as additional maternal *exd* or *hs-exd* was able to replace the zygotic requirement. However, recently it was observed that the availability of Exd is regulated by its nucleo-cytoplasmic localization (Mann and Abu-Shaar, 1996; Aspland and White, 1997). Initially it is cytoplasmic but after gastrulation it enters the nucleus in a defined spatial and temporal pattern. When *exd* is expressed uniformly from a hs-construct or maternally, its availability in the embryo is still patterned by regulation of its nucleo-cytoplasmic location. Interestingly the zygotic *exd* expression follows a similar distribution to the pattern of the nuclear accumulation of Exd suggesting that it may autoregulate. The pattern of nuclear Exd has only been shown to be developmentally relevant in the imaginal discs. Normally, peripherally (proximally) Exd is nuclear and centrally (distally), it is cytoplasmic. Expression of high levels of Exd in the central region using the UAS-GAL4 system overrides the nucleo-cytoplasmic regulation and leads to distal nuclear Exd and phenotypic consequences. In the embryo the prominent features of the pattern from mid-extended germ-band onwards include: 1) in the epidermis: nuclear accumulation in anterior segments is followed by a markedly cytoplasmic zone in ventral parasegment 2. The anterior border of parasegment 3 provides a sharp anterior border to a zone of high level nuclear accumulation in ventral parasegments 3-5 which then tails off posteriorly; 2) there is a centrally located zone of nuclear accumulation in the endoderm and 3) in the late embryonic CNS there is a heterogeneous pattern of nuclear accumulation which is predominantly thoracic.

The regulated subcellular distribution indicates that *Hox* genes do not operate on a "level playing field" of Exd cofactor availability but what is the relevance of this pattern of Exd nuclear accumulation? It is interesting that both in the epidermis and in the imaginal discs the pattern of nuclear Exd is strikingly related to the pattern of expression of the *tsh* gene. *tsh* is regulated by Exd and Tsh also acts as a cofactor to *Hox* genes (Dezulueta, *et al.*, 1994; Rauskolb and Wieschaus, 1994). However the relationships between *exd*, *tsh* and the homeotic regulation of target genes are still obscure and this is an area that merits closer examination. The idea that *Hox* genes do not act on a "level playing field" appears to conflict with previous studies such as the analysis of the mutant phenotypes of *Pc*-group genes. In such mutants, *Hox* genes are released from their normal positional controls and become expressed along the entire antero-posterior axis of the embryo. Removing both maternal and zygotic expression of the *Pc*-group gene, *esc*, leads to a remarkably consistent Abdominal-8-like phenotype along the length of the embryo. As *esc* specifically affects Hox gene expression, it indicates that the *Hox* genes are able to impose a particular segmental morphology on any part of the antero-posterior axis. However, whilst the final pattern of Hox gene expression in an *esc* null is rather uniform, the emergence of the pattern is complex. In *esc*$^-$ embryos the emergence of ectopic Ubx expression, anterior to the normal domain of parasegments 5-13, shows strong expression in the epidermis in a ventral patch in parasegment 2 which appears similar in extent to the zone of cytoplasmic Exd (Gould *et al.*, 1990b). This is an intriguing parallel between the regulation of *Hox* gene expression and the nuclear accumulation of Exd and suggests that the subcellular localization of Exd may be affecting the balance between activation and repression in the homeotic auto/cross-regulatory network. The relevance of the epidermal pattern or the highly heterogenous pattern of Exd expression and nucleo-cytoplasmic localization in the central nervous system to the differential regulation of sets of downstream target genes is, however, still obscure.

We conclude by drawing up a short list of targets for future research: 1) identify further cofactors, in particular cofactors for *Abd-B*; 2) unravel the pathway linking *exd*, *tsh* and *Hox* genes to the common control of sets of target genes; 3) determine whether phenotypic suppression is represented in molecular terms in the affinity of binding interactions or in functional activities on target genes and, 4) most endogenous target gene regulatory sequences that respond to *Hox* gene control are several kilobases in length, yet *Hox* response elements can be constructed from small sequences of a few tens of base pairs. What are the extra features endowed by the large endogenous regulatory elements?

Mechanistic conservation and phylogenetic diversity

The tremendous rise of molecular studies in developmental biology during the past few years led to the surprising conclusion of a large conservation during evolution of genetic circuits that control basic developmental steps. The high degree of structural, and to some extent, functional conservation of the homeotic complex revealed that different species use the same master regulatory molecules to define the body plan organization and to induce morphogenetic processes. Not only have *Hox* genes been conserved during the course of evolution, but so have the mechanisms controlling their expression and modulating their function: genes of the *Pc-G* and *trx-G* groups assure the stable maintenance of their expression domains and the Exd/Pbx family of proteins assists Hox proteins to achieve specificity. An open question is whether or not the conservation of this genetic network also includes *Hox* target genes. In this respect, a number of the targets identified in *Drosophila* have vertebrate orthologs. Most of them however belong to gene families that have become highly diversified, and nothing is yet known about their possible regulation by vertebrate *Hox* genes. Despite the overall conservation of the genetic program which includes *Pc* and *trx* groups of genes, the homeotic complex, cofactors and possibly targets, the morphogenetic mechanisms that are initiated by Hox proteins in metazoans result in the development of very different structures. Thus, a crucial point for understanding the genetic basis of diversity is to

correlate the modifications that have been introduced in this common program to the diversification of forms and functions. Recent data from comparative developmental genetics and palaeontology clearly suggest that diversity finds its primary origin upstream of the *Hox* complex genes, by redesigning their expression domains and dynamics (Burke *et al.*, 1995; Ervin *et al.*, 1997; Shubin *et al.*, 1997). One can infer at least two additional but non exclusive ways towards morphogenetic diversification. First, at the same level of hierarchy as the homeotic complex, the recruitment or functional modification of cofactors might have allowed Hox proteins to select distinct combinations of targets and therefore a diversification of morphogenetic processes. Second, downstream of the homeotic complex, an increased complexity of downstream target sets presumably appeared to account for the evolution of pre-existing structures or the emergence of new ones. Future work devoted to the identification and characterization of new *Hox* cofactors and targets, both in *Drosophila* and in other animal models is needed to answer these questions.

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