Shaping animal body plans in development and evolution by modulation of *Hox* expression patterns

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Summary

Most animals exhibit distinctive and diverse morphological features on their anterior-posterior body axis. However, underneath the variation in design and developmental strategies lies a shared ancient structural blueprint that is based on the expression patterns of *Hox* genes. Both the establishment and maintenance of the spatial and temporal distribution of *Hox* transcripts play an important role in determining axial pattern. The study of many animal systems, both vertebrate and invertebrate, suggests that the mechanisms used to establish *Hox* transcription are nearly as diverse as the body plans they specify. The strategies for maintenance of *Hox* expression pattern seem more conserved among different phyla, and rely on the action of Pc and trx group genes as well as auto- and cross-regulation among *Hox* genes. In mice, the sharing of regulatory elements coupled with auto- and cross-regulation could explain the conservation of the clustered arrangement of *Hox* genes. In contrast, fly *Hox* genes seem to have evolved insulators or boundary elements to avoid sharing regulatory regions. Differences in *Hox* transcription patterns can be correlated with morphological modifications in different species, and it seems likely that evolutionary variation of *Hox* cis-regulatory elements has played a major role in the emergence of novel body plans in different taxa of the animal kingdom. BioEssays 20:116–125, 1998.

INTRODUCTION

Hox genes are expressed in discrete domains of the body along the anterior-posterior axis, and are required for the proper morphological differentiation of all or part of these expression domains. Hox proteins are homeodomain transcription factors which assign different identities to body regions by the differential regulation of numerous downstream genes, many of which are regulated by multiple Hox proteins.[1] *Hox* gene organization is distinctive and enigmatic: the genes map in clusters and the order of individual genes within a cluster correlates with their spatial expression pattern on the anterior-posterior axis of the body.[2]

Although *Hox* genes were first genetically and molecularly characterized in the fruit fly *Drosophila melanogaster*, a great deal of information concerning their functions has recently been obtained from experiments in other insects, nematodes, and mice. The deep knowledge thereby obtained has allowed useful comparative studies of *Hox* patterns of expression throughout the animal kingdom; constituting one of the first systematic attempts to understand the molecular basis of the evolution of developmental patterning. The expanding efforts to uncover functional roles of *Hox* genes in animal development has recently even provided

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explanatory models for certain human birth defects. Both synpolydactyly and hand-foot-genital syndrome are apparently due to mutations in human *Hox* genes[3,4].

The Importance of *Hox* Transcriptional Regulation

Why is it important to understand the trancriptional regulation of *Hox* genes? One way to address this is to ask whether the deployment of Hox proteins in precise patterns is instructive or merely permissive for morphological pattern. Certainly the global controls that limit *Hox* gene expression to large metameric domains play an instructive role, for ectopic expression of Hox proteins outside those domains, either in flies or mice, can result in large scale homeotic transformations. In fact, many of the dominant homeotic transformations seen in the original *Drosophila* mutants are the result of altered expression of *Hox* genes.[2]

The experiments of Mann[5] and Castelli-Gair and Akam[6,7] have shown that the detailed modulation of *Hox* spatial and temporal patterns of expression within metameres also plays an instructive role. Segmental fields in the *Drosophila* embryo have groups of cells with the potential of developing Keilin's organs (the larval equivalent of legs) and spatially separate groups of cells with the potential of developing as spiracles. Neither potential is realized in abdominal segments, due to the suppressing power of Ubx and other bithorax complex proteins which are expressed in most cells of these segments. In the second and third thoracic segments, Ubx is transcriptionally activated in a intricate pattern that includes the spiracle primordia, and consequently these segments lack spiracles. In the leg primordia of the same segments, Ubx expression is absent during early stages of embryogenesis, which allows these primordia to form Keilin's organs. Thus, within a given metamere, detailed differences in the place and time where a *Hox* gene is expressed plays an important role in determining morphologically different cellular fates.

Similar results concerning the requirements for precise spatial and temporal patterns of Hox expression were recently obtained in *C. elegans*. Salser and Kenyon[8] studied the functions of the Antp homolog mab-5 in the V5 lineage of the lateral ectoderm. The entire V5 lineage expresses mab-5 periodically during development, and a spatial pattern of mab-5 protein accumulation is generated within the lineage. Salser and Kenyon activated ectopic mab-5 expression at inappropriate times and in inappropriate cells of the V5 lineage, and found that both the on and off states of mab-5 expression have important instructive value for the normal morphogenesis of posterior neural and epidermal structures of the nematode.

These insightfully detailed experiments have uncovered the fact that upstream regulation of *Hox* genes can be an important process for the generation of pattern within a *Hox* functional domain. For many of the *Hox* genes, variations in expression pattern occur in all tissues and throughout much of embryogenesis. What are the regulatory mechanisms that account for the complex temporal and spatial alterations in postestablishment *Hox* expression pattern? Considering the importance of the segment polarity signalling functions such as *wingless (wg), hedgehog (hh)*, and *decapentaplegic (dpp)* in the generation of patterning information, it seems likely that these signals will be very important in the detailed variation of *Hox* transcriptional patterns, but only a few inroads have been made into this unexplored territory[9,10].

ESTABLISHMENT OF *Hox* EXPRESSION

How it Works in *Drosophila*

In the fruit fly, *Hox* transcriptional patterns are established during syncytial and early cellular blastoderm in stripes on the anterior-posterior axis. At this time, the embryo has an asymmetric distribution of numerous transcription factors of both maternal and zygotic origin, produced by the coordinate, gap, and pair-rule genes. These proteins form an overlapping series of gradients and stripes that expose every nucleus on the anterior-posterior axis to unique combinations of transcription factors. Alterations in the expression patterns of these proteins, or loss of their functions, can result in constrictions, expansions or deletions of *Hox* expression domains[2]. Although not yet characterized to the same level of detail, the regulatory elements that establish stripes of Hox transcription are presumably similar to enhancers that direct stripes of pair-rule gene expression[11].

Enhancers that establish *Hox* expression domains have been shown to possess gap and pair rule protein binding sites that are important for their function.[12] The most convincing evidence for a direct interaction between gap proteins and *Hox* regulation come from the study of *Hab* mutations. Shimell et al.[13] discovered that the *Hab* phenotype was due to a single base substitution in the *iab-2* regulatory region of *abd-A* that resulted in the destruction of a binding site for the Krüppel gap repressor protein, leading to ectopic expression of *abd-A*. In another study, Qian and coworkers[12] identified binding sites within a *Ubx* regulatory element for the gap proteins Hunchback and Tailless, as well as for the pair-rule protein Fushi tarazu. The changes in regulatory element activity due to loss of specific binding sites resemble the changes observed in *tailless, hunchback*, or *fushi tarazu* mutant backgrounds, suggesting that the regulatory input of all these proteins is integrated by the enhancers that establish the initial pattern of *Ubx* transcription.

How General Are the Regulatory Circuits That Establish *Hox* Expression Domains?

Given the extraordinary conservation of *Hox* expression patterns in animal development, one might expect that the

regulatory networks upstream of the *Hox* genes were equally well conserved. However, many of the cellular developmental processes that occur prior to *Hox* expression; such as oogenesis, early cell division patterns, and segmentation, are highly variable among different members of the animal kingdom. Recent experimental results indicate that this variability is apparently associated with a diverse set of strategies and mechanisms for establishing the initial boundaries of *Hox* gene expression in different animal phyla.

Drosophila is rather unique among insects in that much of its segmentation and body axis patterning occurs in a nuclear syncytium. In other insects similar patterning decisions are made in both syncytial and cellular contexts. In the locust *Schistocerca gregaria* and the beetle *Tribolium castaneum*, the posterior segments are constructed from a proliferative zone by cell division[14], a situation that shares some similarities to the developmental context in which *Hox* expression is established in other animals such as chordates. Despite the differences in developmental context, the patterns of expression of some gap and pair-rule transcripts in *Tribolium* embryos is reminiscent of patterns found in *Drosophila* embryos, and suggests some gap and pair-rule genes play a conserved role in insect embryonic patterning[15–17]. One interesting new *Tribolium* patterning mutant, called jaws, has homeotic transformations in the segments derived from the syncytium, but lacks segments derived from the proliferative zone. jaws apparently does not correspond to any of the well characterized members of the *Tribolium Hox* cluster. It is possible that this mutation may map in the beetle homologue of a *Drosophila* pair-rule or a gap gene, or it might identify a novel mechanism of early *Hox* regulation in a cellular context[18].

Outside the Insect Class, the extant data on *Hox* gene establishment controls have not yet implicated gap or pair-rule type genes as upstream regulators. Experiments in *C. elegans* indicate that the pattern of expression of worm *Hox* genes are determined in part by mechanisms independent of global position along the anterior posterior axis[19]. Normally, the *Hox* gene *mab-5* is expressed near the posterior end of the worm, a domain that includes cells of the V6 group. The two V6 cells that express this *Hox* protein are descendants of the ABarp blastomere. When other blastomeres (EMS and P2) are ablated early in embryogenesis, other cells acquire a ABarp identity and give rise to ectopic V6 cells located in novel positions on the worm anterior-posterior axis. The ectopic V6 equivalents also establish *mab-5* expression in their new positional/cellular environments. Similar results have been obtained on the establishment controls for *Hox* expression in leech development, which is apparently controlled by a mix of timing and lineage inputs that are not absolutely fixed to anterior-posterior position[20]. Although no mechanisms are yet known to explain these position-independent controls on the establishment of *Hox* expression, these observations suggest that many invertebrates use different strategies to establish *Hox* expression than have been characterized in *Drosophila*.

A few trans-acting factors have been shown to directly regulate *Hox* transcription in mammals. Two are proteins encoded by the genes *Krox20*[21] and *kreisler*[22]. *Krox20* encodes a zinc finger transcription factor expressed at high levels in rhombomeres 3 and 5 (r3/5) in the hindbrain of developing mice. *Hoxb-2*, which is expressed in a *Krox20*-dependent manner, contains three Krox20 protein binding sites in its promoter region, that are required for *Krox20*-dependent reporter transcription *in vivo*. kreisler encodes a maf/b-Zip protein and it is required for the establishment of Hoxb-3 expression in r5. The r5 pattern can be recapitulated by a 45 bp element derived from *Hoxb-3* upstream sequences which contains Kreisler protein binding sites plus an Ets-type consensus binding site. Both binding sites are required to establish the correct transcription pattern. None of the above genes have known homologues in *Drosophila* that act in the coordinate, gap, or pair-rule pathways to establish *Hox* gene expression.

As expected in a cellularized system, many cell-cell signaling molecules have been found to regulate *Hox* expression in vertebrates. Perhaps the best studied signal is retinoic acid (RA)[23,24]. Exposure of embryos to RA induces homeotic transformations as well as ectopic expression of *Hox* genes; e.g., *Hoxb-1*[25]. The response of *Hoxb-1* to RA is likely to be directly mediated by RAR proteins, as the mutation of RAR binding sites abolished the activity of a small *Hoxb-1* establishment enhancer in mouse embryos[26].

Even though the evidence regarding the mechanism of establishment controls in most model animal systems is still scanty and scattered, the extant results indicate that the developmental genetic circuitry controlling anterior-posterior patterning upstream of the *Hox* genes is not widely conserved throughout the animal kingdom.

Maintenance of Spatially Localized Expression and Function of *Hox* Genes

Since the initial experiments of Lewis[27], there has been steadily increasing evidence that *Hox* functions in *Drosophila* are required both for the initial assignment of metameric identities as well as the maintenance of such identities. When *Hox* gene expression is ablated at times ranging from hours to days after the time of initial activation, homeotic transformations or loss of metamere identity can result.[2] The persistent expression of the homeotic genes in spatial patterns apparently allows cells to retain a memory of their anterior-posterior position as they proceed through embryogenesis and find themselves in new cellular contexts. This does not mean that all late expression patterns of *Hox* proteins are conferring positional information, as there are examples where *Drosophila Hox* genes have apparently adopted novel functions unrelated to axial patterning[28].

The Polycomb-Group and Trithorax-Group Genes

The genes in the Polycomb (Pc) group maintain the boundaries of *Hox* gene expression by repressing *Hox* transcription outside their normal domains of activity. In *Pc* mutants, posterior *Hox* genes such as *abd-A* and *Abd-B* are expressed in more anterior regions of the embryo, and the phenotypic result is a partial transformation towards posterior abdominal identity.[29–31] Pc acts in multimeric complexes and each complex probably include a subset of many members of the Pc group.[32] Polytene chromatin binding studies, and tests of gene expression patterns from genes outside the *Hox* complexes, indicate that *Hox* genes are not the only transcription units that respond to the repressive effects of Pc group functions.[32] Pc response elements (PREs) map to discrete regulatory sites within bithorax complex DNA,[32,33] but due to the relatively large size of the currently defined elements[33,34] and the fact that none of the known Pc group proteins has sequence specific DNA binding functions, it is still unclear how Pc proteins are persistently tethered to specific DNA elements.

The trithorax (trx) group mutations are genetically defined by their ability to suppress Pc group phenotypes.[35,36] Loss of an individual trx group function (such as *trithorax* itself) does not abolish expression of homeotics but does have differential affects on the amounts of expression from different homeotics, ranging from severe to mild.[37,38] Some trx group functions like *brahma (brm*), contribute to the SWI/SNF complexes of *Drosophila*, and antagonize the repressive effect of Pc group proteins.[38] The trx group functions that act in the SWI/SNF-like complexes are presumably regulating the accessibility of activators to regulatory sequences of *Hox* genes.[39] Another trx group protein, GAGA factor/*Trl*, is a component of another chromatin modeling function called the NURF complex, which acts by promoting nucleosome mobility at heat shock promoters, and perhaps at homeotic promoters as well.[40–42]

There is abundant evidence that many members of the Pc and trx groups are widely evolutionarily conserved at both a structural and functional level.[32,43] The current evidence supports the idea that these genes apparently represent an ancient system for the stable maintenance of gene expression patterns, so ancient that a Pc homolog was recently discovered in plants.[44]

Autoregulatory and Cross-Regulatory Mechanisms

Some *Hox* genes in flies and mice seem to rely on autoactivation circuits for the maintenance of expression during development. In the case of the *Drosophila* head genes *Deformed (Dfd)*, and *labial (lab)*, this is largely mediated through the direct binding of the Dfd and Lab proteins to autoactivation enhancers for the respective genes.[45–47] Two mouse homologs of *lab* and *Dfd* (*Hoxb-1* and *Hoxb-4*) also use autoactivation to achieve persistent expression.[48,49] Autoactivation enhancers in *Hox* complexes are so well conserved that they can be switched between mouse and fly developmental contexts, and still function appropriately.[48–51] The autoactivation of *Dfd* and *lab* genes is dependent not only on the respective Dfd or Lab protein functions but also on the *extradenticle (exd*) function; embryos that lack both maternal and zygotic Exd protein do not maintain either *Dfd* or *lab* transcription, although the transcriptional regulation of many other *Hox* genes is unaffected.[47,52]

The mammalian counterparts of the *Drosophila* Exd protein are the Pbx class of proteins.[53] The importance of Exd/Pbx function for autoactivation of *Hox* transcription was discovered by analysis of a small regulatory element that maps in the 5′ flanking sequence of the mouse *Hoxb-1* gene.[48] This *Hoxb-1* element contains multiple composite binding sites that cooperatively interact with Hoxb-1 and Pbx proteins in vitro, and that direct reporter expression in rhombomere 4 of mouse embryos. All Hox response elements so far characterized require Exd protein function in order for the Hox protein to activate transcription via the element.[52] The need for Exd protein function in the case of both *lab* and *Dfd* autoactivation may be a reflection of this apparent general requirement.

Hox gene expression can also be maintained by autoregulatory circuitry that is largely indirect. Within the visceral mesoderm cells, the Ubx protein expression is maintained through autoregulatory mechanisms dependent on *dpp* and *wg* intercellular signals.[54] Recent work has indicated that the *wg* signal and *dpp* signal are integrated on nearby sequences in a *Ubx* enhancer element by *Drosophila* LEF-1 family and *Drosophila* CREB family transcription factors, respectively.[9,10] Bienz has persuasively argued[54] that such indirect autoactivation circuits, dependent on multiple inputs, are well-buffered against developmental noise, and better at stabilizing expression patterns in fields of cells than are simple direct autoregulatory circuits. The biological complexity of a regulatory circuit may thus be a virtue for the organism possessing it, if not necessarily for an investigator attempting to dissect its component parts.

Cross regulatory relationships among the *Hox* genes also play an important role in determining their transcriptional patterns. The Bithorax complex proteins Ubx, Abd-A, and Abd-B are all capable of repressing the transcription of more anterior *Hox* genes,[2] and there is evidence in one case reported by Appel and Sakonju[55] that this repression is exerted by direct interaction with *Hox* protein binding sites in the genes that are being repressed. The cofactors, if any, that might assist in Hox-mediated repression, are as yet unknown. It seems unlikely that Exd plays any role, since cross regulatory repressions are unaffected in embryos that lack all Exd function.[56] Positive cross regulation can also

assist in the normal maintenance of *Drosophila* and mouse *Hox* gene expression patterns.[49,57]

Enhancers Sharing as An Explanation for the Maintenance of Clustered Arrangements of *Hox* Genes

Part of the fascination of the *Hox* genes has been their arrangement in linear clusters in which the order of the genes reflects the order of their expression boundaries on the embryonic anterior-posterior axis. How this colinear arrangement is generated is still a matter for speculation. However, recent work has provided strong evidence to support the idea[58] that shared enhancers regulating different *Hox* promoters might provide a reason for the evolutionary cohesion of the complexes. Gerard and colleagues[59] have found that a small regulatory region, which binds RA receptor proteins in vitro, is required for setting the anterior boundary of expression of two adjacent mouse *Hox* genes, *Hoxd*-10 and *Hoxd*-11. The mutation of this element in the context of the normal *Hox-d* complex appears to be sufficient to derepress both *Hoxd-10* and *Hoxd-11* promoters in more anterior body cells. A shared enhancer element is also required for the activities of both the *Hoxb-3* and *Hoxb-4* promoters in transgenic constructs.[49] This shared enhancer contains an evolutionarily conserved sequence called CR3, which includes two Hox protein binding sites that are essential for its function. The CR3-containing element can also drive a Hox-dependent pattern of reporter expression in *Drosophila* embryos. The prediction is that mice in which CR3 is deleted will lose function of both the *Hoxb-3* and *Hoxb-4* promoters in cis, although this evidence is not available at present. Interestingly, the CR3 element in the Hoxb complex is ''auto''-regulated by the paralogous Hoxd-4 protein in mouse embryos, a phenomenon Gould and colleagues term pararegulation.[49]

Boundary Elements

In contrast to the mouse *Hox* gene complexes, the *Drosophila Hox* genes are split into two separate gene complexes (ANT-C and BX-C), and individual transcription units and intergenic regions within these complexes occupy much more DNA.[2] It seems plausible that this different general arrangement is due to an increased autonomy on the part of individual *Hox* genes in *Drosophila*, with fewer (if any) shared regulatory elements. Even the individual enhancers that regulate the patterns and amounts of expression for particular Hox genes normally function with a high degree of autonomy. The evidence for this relies on deletion mutations of *Hox* regulatory sequence that have been shown to result in abnormal levels of *Hox* expression due to the fusion of two distant enhancer elements. One of the best characterized examples resides in the Fab-7 region, originally defined by a deletion mutation that fused the iab-6 and iab-7 enhancers.[60] The *Fab-7* deletion results in parasegment 11 adopting levels of Abd-B transcription that are normally only seen in parasegment 12. The deleted DNA has been proposed to contain an insulator element that segregates two independent cis-regulatory domains.[60] Two recent papers provide strong support for this hypothesis, showing that Fab-7 region DNA can block the function of a wide variety of enhancers, but only when the Fab-7 element is interposed between an enhancer and promoter.[61,62] Genetic results indicate that such boundary elements may be common in the BX-C, separating parasegment specific enhancers that could potentially act on the same, and/or on different *Hox* promoters in BX-C DNA.[63,64]

CHANGES IN *Hox* EXPRESSION PATTERNS AND EVOLUTIONARY DIVERSIFICATION

Subtle Changes of Expression Within *Hox* Domains

The mechanisms by which Hox genes might contribute to evolutionary diversification has long been a subject of speculation.[29] Recent studies have provided strong correlative evidence that subtleties in *Hox* expression pattern within their anterior-posterior domains account for some of the variation in morphological pattern between insect groups. For example, caterpillars, the larvae of butterflies and moths, differ from other insects in possessing appendages in abdominal segments, known as prolegs. In flies and beetles, the *Hox* genes *Ubx, abd-A*, and *Abd-B* suppress the appearance of legs in the abdomen.[65] Warren and coworkers[66] found that butterfly Ubx and Abd-A proteins, although present early throughout the abdomen, no longer accumulate in the few cells that give rise to the prolegs, which are marked by *Distal-less* expression. Since functional tests cannot yet be performed in butterfly larvae, it is unclear whether the changes in Hox expression are required for proleg formation or are just a by-product. Nevertheless, this work supports the hypothesis that the acquisition of novel segmental morphology in an insect group has arisen by localized modification of *Hox* expression.

Another example of a correlation between a minor variation in *Hox* expression pattern and morphological change is reported by Rogers et al.,[67] who analyzed the expression patterns of the *Hox* gene *Sex combs reduced (Scr*) in the insect orders Diptera, Thysanura, Othoptera, and Hemiptera. In *Drosophila, Scr* is expressed in most cells of labial and T1 segments, and is required for their identity in embryonic and imaginal development.[68] *Scr* has a conserved pattern of expression in the labial segment, but a variable expression pattern in the first thoracic segment among the species tested. Thysanurans seem to lack an *Scr* expression domain which is conserved among the other insects tested. This expression domain may be determining the production of specialized patches of comb-type bristles

similar to *Drosophila* sex combs. It is possible that these cells began expressing *Scr* in the pterygote lineage, which branched from thysanurans early in insect evolution and includes Diptera, Orthoptera, Hemiptera, and other winged orders. Nonetheless, it is also possible that *Scr* expression was lost in those cells in the thysanurans, for the ancestral condition remains unknown. *Scr* is expressed in many epidermal cells of the T1 segment in flies, beetles, and butterflies,[65] suggesting that cells within this segment evolved *Scr* expression before the radiation of holometabolous insects.

However, the danger of inferring a causal relationship between variations in *Hox* expression pattern and morphological variation is also pointed out by Rogers and coworkers.[67] In flies and beetles, the loss of *Scr* function results in the ectopic development of wing primordia from dorsal-anterior cells near the base of the prothoracic leg. One group of cells that seems to consistently express *Scr* in the animals tested from the Dipteran, Thysanuran, Othopteran, and Hemipteran orders is located in this prothoracic dorsal regions. Rogers and coworkers suggest that *Scr* expression in this position mediates the repression of wing formation in T1 segments.[67] However, they note that this patch must predate the appearance of wings since it is present in the apterygote (wingless) lineage of Thysanurans. So it appears that this previously existing patch of *Scr* expression pattern was co-opted to prevent wing development and had other functions or no function in primitive insects or their ancestors.

Global Changes in *Hox* Pattern of Expression

In contrast to the subtle variations within *Hox* expression domains, there is no such variation in the overall boundaries of *Hox* expression in different insects. This is consistent with the conserved pattern of insect tagmosis.[65] What happens when comparisons are made not within a class but between classes, such as Insecta and Branchiopoda? Branchiopod crustaceans (e.g., *Artemia*) differ significantly in design from insects: they possess a thorax with eleven segments all harboring legs (as opposed to only three in insects) and many postgenital ''abdominal'' segments. Averoff and Akam[69] has shown that *Antp, Ubx*, and *abd-A* are expressed in most of the thoracic segments of Artemia whereas they have overlapping but distinct domains in insects. Averoff and Akam[69] have proposed that the crustacean thorax is homologous to the insect trunk, and that the Hox expression patterns in *Artemia* reflect a primitive condition. In this animal, neither *Ubx* nor *abd-A* seem to suppress leg formation, and since they do so in *Drosophila* (and presumably in other insects), a key evolutionary event in the lineage leading to insects must have been the restriction of *Ubx* and *abd-A* expression to more posterior regions.

Judging by the structural criteria used in arthropod taxonomy, it seems that the evolution of different classes within this phylum may have been concurrent with, and perhaps based on, large scale changes in *Hox* domains of expression. The body plans of each arthropod subphylum (Chelicerata, Uniramia, Crustacea, Trilobita) and many classes within them are characterized by specific segmental variations such as which segments bear appendages and what kind, and the precise pattern of segment fusions (tagmosis).[70] These are modifications that are consistent with a very large number of *Hox*-dependent morphological structures being redeployed on the anterior-posterior axis, i.e., they are similar to homeotic transformations, which in *Drosophila* are known to be due to either large scale changes in *Hox* expression, or loss-of-function mutations—the equivalent of a change in *Hox* gene number. Since changes in the number of *Hox* genes appear (so far) to be associated with even greater phylogenetic distances, the morphological diversity among the arthropod classes may be dependent on a spectacularly successful evolutionary experiment with *Hox* domains of expression.

The Chordata also present a case in which changes in *Hox* expression seems to correlate with dramatic alterations in the domain boundaries of *Hox* expression in different higher taxa. Birds and mammals (different subclasses within Chordata) differ in the number of vertebra allocated to different regions (cervical, lumbar, sacral, etc.). In chickens and mice, the boundaries of expression of some *Hox* genes are associated with the transitions from one vertebral type to another rather than to a particular numerical position in the vertebral field, as if specific *Hox* genes were locked into specifying certain type of vertebrae.[71] Since the expression of Hox proteins in different somites seems to depend on discrete regulatory elements, such broad changes in *Hox* domain size might involve alterations in upstream transcriptional regulation.

A Hierarchical Model for the Evolutionary Impact of Changes in the *Hox* Pathways

Developmental pathways such as the ones defined by the *Hox* genes offer a framework to categorize genetic changes by their impact on morphological design. Figure 1 diagrams different levels at which *Hox* expression or function could be modified by mutations in regulatory elements or *Hox* gene number in the course of evolution, hierarchically ordered according to the depth of their regulatory and morphological consequences. The importance of regulatory element variation in morphological evolution is likely to be very general. For example, Li and Noll[72] have constructed hybrid genes to demonstrate the importance of cis-regulatory elements in the evolutionary diversification of the *prd* homeodomain class of developmental regulators.

In a *Hox* pathway, the most superficial changes would occur in single *Hox* response elements for downstream genes (level 4 in Fig. 1). These are likely to be the most

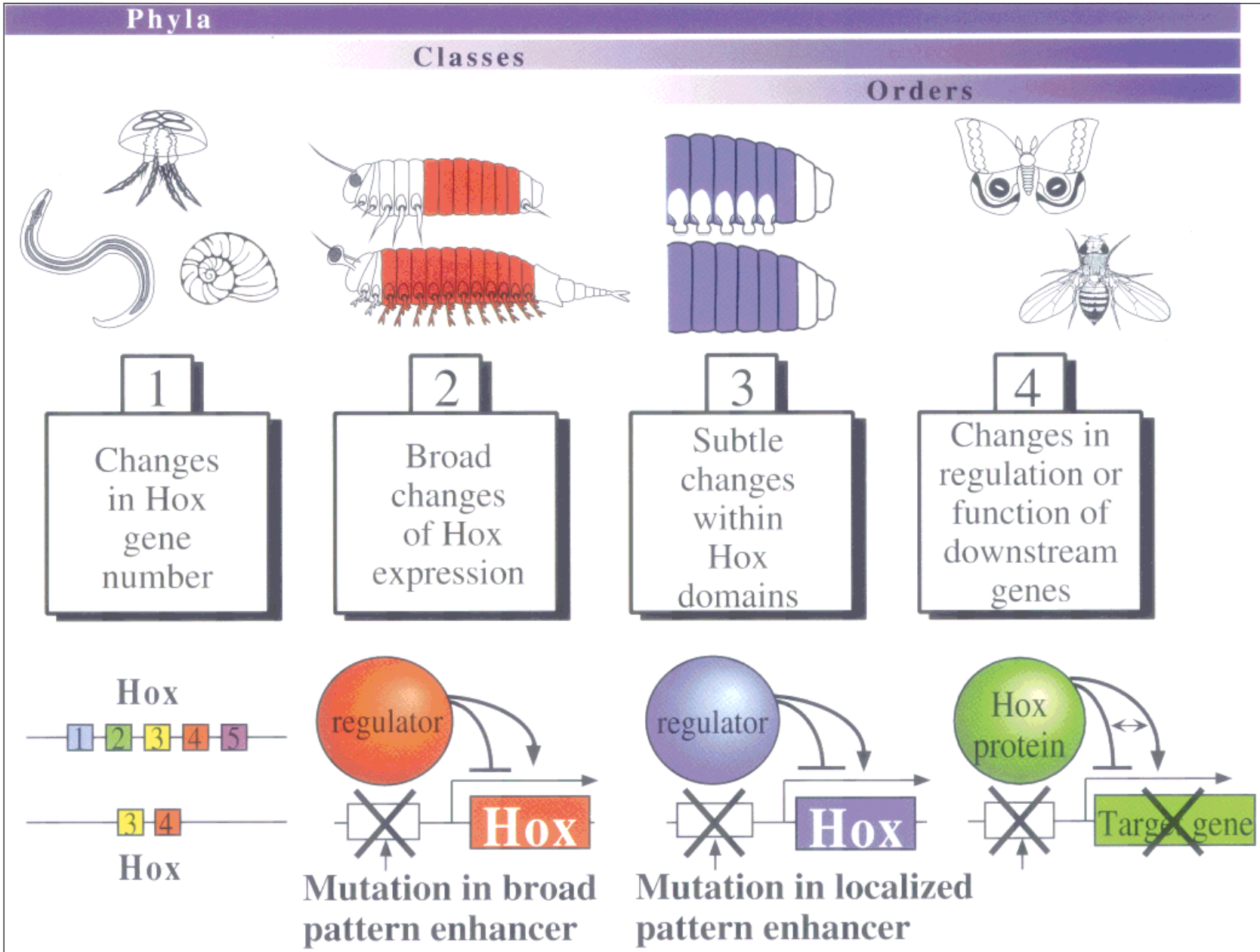

Figure 1. A hierarchy of evolutionary changes in Hox pathways arranged by their presumed morphological impact. The numbered boxes list some of the changes that Hox pathways can undergo during evolution. The diagrams below the boxes indicate likely molecular mechanisms mediating such changes. Above the boxes, diagrams show the modifications of body plan associated with the corresponding changes in Hox pathways. The graded bars at the top of the figure suggest that a rough correlation exists between levels at which Hox pathways vary, and animal taxonomic levels.

pervasive changes in evolutionary variation of *Hox* pathways, presumably involved in subtle differences between most or all animals species,[e.g.,73] and even as polymorphisms in populations within a species. Of course, the impact of any single change would depend on the hierarchical level of the gene whose expression is controlled, which could range from very high (in autoactivation circuits, the *Hox* genes themselves), to rather low (if the downstream gene itself had no subsequent regulatory function).[e.g., 74]

We imagine that the next level of regulatory element variation in *Hox* pathways would result in subtle changes in the details of a Hox transcription pattern within a preexisting large domain of expression (level 3 in Fig. 1). For example, changes in *abd-A* and *Ubx* expression in the cells of the butterfly larval abdomen correlate with the formation of prolegs.[66] Mechanistically, this could be caused by the mutation of binding sites which abolish trx-like or autoactivation function in just a few cells, or by the failure to establish expression in those cells. Many *Hox* downstream gene expression patterns would presumably change in these few cells. At least two autoregulatory elements are known to consist of independent modules that supply Hox expression in overlapping spatial and temporal patterns and different tissue types,[75–77] which might facilitate evolutionary experimentation. In another example, *Scr* seems to have acquired new domains of expression in the evolution of different insect lineages.[67] It is possible that such variation in *Hox* pattern accounts for some of the changes in anatomical organization that characterize the different orders (Fig. 1). It will be interesting to determine whether even more subtle changes in *Hox* pattern can be correlated with minor morphological differences among insect species.

The next hierarchical level of *Hox* pathway variation in evolution is probably conferred by mutations that result in a wholesale redeployment of the anterior-posterior domain of a Hox pattern (level 2 in Fig. 1). This might involve expansion or contractions of *Hox* expression throughout one or many metameres. In these cases, many *Hox* downstream genes would have altered expression patterns in many cells. Mechanistically, broad alterations in *Hox* patterns of transcription (e.g., the restriction of Ubx and abd-A to abdominal segments in insects as compared to *Artemia*[78] could have been generated by the gain of gap repressor binding sites in *Hox* cis-regulatory elements. From this point of view, the lesions in Krüppel protein binding sites in *Hab* mutant chromosomes[13] can be thought of as mimicking an ancestral condition. The shifts in *Hox* boundaries seen in comparisons of bird and mammal patterns of expression,[71] could similarly be due to the acquisition or loss of binding sites for upstream factors like *Krox20* or *kreisler*, or by changes in the expression patterns for such regulators.[21,22] Such mutations could have driven deep reorganizations of the body plan, and may be more frequently associated with differences at the taxonomic level of classes.

The deepest level at which *Hox* pathways change in evolution is likely to be at the level of alterations in *Hox* gene number (level 4 in Fig. 1). Gene duplication is postulated to permit the establishment of wholly new developmental strategies,[79] and thereby generate the dramatic differences in form that distinguish phyla or subphyla. There are examples in recent evolutionary history of individual Hox type genes that apparently have rapidly diverged to acquire nonhomeotic functions after being duplicated.[reviewed in 80] This evolutionary divergence is probably partly due to rapid coding evolution, as well as novel spatial and temporal patterns of expression.

It has been argued[70,81] that the most profound innovations in animal design occurred during the Cambrian period, and that fundamental architectural novelty of the type that distinguishes the higher taxa has not arisen since. Apparently, dramatic innovation is no longer tolerated at fundamental branch points of developmental pathways controlling metazoan body plans. There are likely to be many reasons why such branch points are resistant to change, but among them is that as more and more downstream genes and cells are incorporated into a developmental genetic pathway, the less flexible that pathway becomes, since changes of expression of some of the downstream genes leads to disruptive consequences. In Figure 1 we have attempted to hierarchically order some of the evolutionary modifications in *Hox* developmental pathways according to their presumed impact on morphology, and suggested that this order could roughly correlate with taxonomic levels, and also with the number of cells and downstream genes affected.

A fascinating problem in biology is to understand the developmental and molecular mechanisms behind the patterns of macroevolution. The comparative study of *Hox* gene expression patterns have already proven a very useful tool to define plausible and realistic connections between patterning genes and evolutionary changes. There appears to be a great need for more comparative expression studies, but also for the beginnings of a comparative study of *Hox* cis-regulatory modules and downstream elements whose variation probably underlies much morphological change in animal evolution. As a prelude, genuinely detailed knowledge of the structure and function of the component parts of such elements from model organisms will be required. An exciting prospect for the future will be to reconstruct the history of some crucial regulatory elements, how they have changed at various branch points of evolution and how that has led to changes in form and design in the animal kingdom.

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REFERENCES

1 Botas, J. (1993) Control of morphogenesis and differentiation by HOM/Hox genes. *Curr Opin Cell Biol* 5:1015–1022.

2 McGinnis, W. and Krumlauf, R. (1992) Homeobox genes and axial patterning. *Cell* 68:283–302.

3 Muragaki, Y., Mundlos, S., Upton, J. and Olsen, B.R. (1996) Altered growth and branching patterns in synpolydactyly caused by mutations in HOXD13. *Science* 272:548–551.

4 Mortlock, D.P., Post, L.C. and Innis J.W. (1996) The molecular basis of hypodactyly (Hd): A deletion in *Hoxa13* leads to arrest of digital arch formation. *Nature Gene* 13:284–289.

5 Mann, R.S. (1994) *engrailed*-mediated repression of *Ultrabithorax* is necessary for the parasegment 6 identity in *Drosophila. Development* 120: 3205–3212.

6 Castelli-Gair, J., Greig, S., Micklem, G. and Akam, M. (1994) Dissecting the temporal requirements for homeotic gene function. *Development* 120:1983–1995.

7 Castelli-Gair, J. and Akam M. (1995) How the Hox gene *Ultrabithorax* specifies two different segments: The significance of spatial and temporal regulation within metameres. *Development* 121:2973–2982.

8 Salser, S. and Kenyon C. (1996) A *C. elegans* Hox gene switches on, off, on and off again to regulate proliferation, differentiation and morphogenesis. *Development* 122:1651–1661.

9 Eresh, S., Riese, J., Jackson, D.B., Bohmann, D. and Bienz, M. (1997) A CREB-binding site as a target for decapentaplegic signalling during *Drosophila* endoderm induction. *EMBO J* 16:2014–2022.

10 Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S.C., Grosschedl, R. and Bienz, M. (1997) LEF-1, a nuclear factor coordinating signalling inputs from wingless and decapentaplegic. *Cell* 88:777–787.

11 Small, S., Kraut, R., Hoey, T., Warrior, R. and Levine M. (1991) Transcriptional regulation of a pair-rule stripe in *Drosophila. Genes Dev* 5:827–839.

12 Qian, S., Capovilla, M. and Pirrotta, V. (1993) Molecular mechanisms of pattern formation by the BRE enhancer of the Ubx gene. *EMBO J* 12:3865–3877.

13 Shimell, M., Simon, J., Bender, W. and O'Connor, M. (1994) Enhancer point mutation results in a homeotic transformation in *Drosophila. Science* 264:968–971.

14 Patel, N. (1994) The evolution of arthropod segmentation: Insights from comparisons of gene expression patterns. *Development* suppl: 201–207.

15 Sommer, R.J. and Tautz, D. (1993) Involvement of an orthologue of the *Drosophila* pair rule gene hairy in segment formation of the short germ band embryo of *Tribolium* (Coleoptera). *Nature* 361:448–450.

16 Wolff, C., Sommer, R., Schroder, R., Glaser, G. and Tautz, D. (1995) Conserved and divergent expression aspects of the *Drosophila* segmentation gene *hunchback* in the short germ band embryo of the flour beetle *Tribolium. Development* 121:4227–4236.

17 Brown, S. and Denell, R. (1996) Segmentation and dorsoventral patterning in *Tribolium. Cell Dev Biol* 7:553–560.

18 Sulston, I. and Anderson, K. (1996) Genetic analysis of embryonic patterning mechanisms in the beetle *Tribolium castaneum. Cell Dev Biol* 7:561–571.

19 Cowing, D. and Kenyon, C. (1996) Correct *Hox* gene expression established independently of position in *Caenorhabditis elegans. Nature* 382:353–356.

20 Nardelli-Haefliger, D., Bruce, A.E.E. and Shankland, M. (1994) An axial domain of HOM/Hox gene expression is formed by morphogenetic alignment of independently specified cell lineages in the leech *Helobdella. Development* 120:1839–1849.

21 Sham, M.H. (1993) The zinc finger gene *Krox20* regulates *HoxB2 (Hox2.8)* during hindbrain segmentation. *Cell* 72:183–196.

22 Manzanares, M., Cordes, S., Kwan, C.T., Sham, M.H., Barsh, G.S. and Krumlauf, R. (1997) Segmental regulation of *Hoxb-3* by kreisler. *Nature* 387:191–195.

23 Boncinelli, E., Simeone, A., Acampora, D. and Mavilio, F. (1991) Hox gene activation by retinoic acid. *Trends Gene* 7:329–334.

24 Krumlauf, R. (1994) Hox genes in vertebrate development. *Cell* 78:191–201.

25 Marshall, H., Nonchev, S., Sham, M.A., Muchamore, I., Lumsden, A. and Krumlauf R. (1992) Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity. *Nature* 360:737–741.

26 Marshall, H., Studer, M., Popperl, H., Aparicio, S., Kurolwa, A., Brenner, S. and Krumlauf, R. (1994) A conserved retinoic acid response element required for early expression of the homeobox gene *Hoxb-1. Nature* 370:567–571.

27 Lewis, E.B. (1964) Genetic control and regulation of developmental pathways. In *The Chromosomes in Development*, (ed. M. Loke) Academic Press, New York.

28 Hoppler, S. and Bienz, M. (1994) Specification of a single cell type by a *Drosophila* homeotic gene. *Cell* 76:689–702.

29 Lewis, E.B. (1978) A gene complex controlling segmentation in *Drosophila. Nature* 276:565–570.

30 Struhl, G. and Akam, M. (1985) Altered distributions of Ultrabithorax transcripts in extra sex combs mutant embryos of *Drosophila. EMBO J* 4:3259–3264.

31 Wedeen, C., Harding, K. and Levine, M. (1986) Spatial regulation of *Antennapedia* and *bithorax* gene expression by the *Polycomb* locus in *Drosophila. Cell* 44:739–748.

32 Simon, J. (1995) Locking in stable states of gene expression: Transcriptional control during *Drosophila* development. *Curr Opin Cell Biol* 7:376–385.

33 Chan, C.S., Rastelli, L. and Pirrotta, V. (1994) A *Polycomb* response element in the *Ubx* gene that determines an epigenetically inherited state of repression. *EMBO J* 13:2553–2564.

34 Chang, Y.L., King, B., O'Conner, M., Mazo, A. and Huang, D.H. (1995) Functional reconstruction of *trans* regulation of the *Ultrabithorax* promoter by the products of two antagonistic genes, *trithorax* and *Polycomb. Mol Cell Biol* 15:6601–6612.

35 Shearn, A. (1989) The *ash-1, ash-2*, and *trx* genes of *Drosophila melanogaster* are functionally related. *Genetics* 121:517–525.

36 Kennison, J.A. (1993) Transcriptional activation of *Drosophila* homeotic genes from distant regulatory elements. *Trends Gene* 9:75–79.

37 Breen, T.R. and Harte P.J. (1993) trithorax regulates multiple homeotic genes in the bithorax and Antennapedia complexes and exerts different tissue-specific, parasegment-specific and promoter-specific effects on each. *Development* 117:119–134.

38 Tamkun, J.W., Deuring, R., Scott, M.P., Kissinger, M., Pattatucci, A.M., Kaufman, T.C., and Kennison J.A. (1992) *brahma*: A regulator of *Drosophila* homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. *Cell* 68:561–572.

39 Pazin, M.J. and Kadonaga J.T. (1997) SWI2/SNF2 and related proteins: ATP-driven motors that disrupt Protein-DNA interactions? *Cell* 88:737–740.

40 Biggin, M.D. and Tijan R. (1988) Transcription factors that activate the *Ultrabithorax* promoter in developmentally staged extracts. *Cell* 53:699–711.

41 Farkas, G., Gausz, J., Galloni, M., Reuters, G., Gyurkovics, H. and Karch F. (1994) The *Trithorax-like* gene encodes the *Drosophila* GAGA factor. *Nature* 371:806–808.

42 Tsukiyama, T. and Wu C. (1995) Purification and properties of an ATP-dependent nucleosome remodeling factor. *Cell* 83:1011–1020.

43 Muller, J., Gaunt, S. and Lawrence P. (1995) Function of the Polycomb protein is conserved in mice and flies. *Development* 121:2847–2852.
44 Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E.M. and Coupland G. (1997) A Polycomb-group gene regulates homeotic gene expression in *Arabidopsis. Nature* 386:44–51.
45 Regulski, M., Dessain, S., McGinnis, N. and McGinnis W. (1991) High-affinity binding sites for the *Deformed* protein are required for the function of an autoregulatory enhancer of the *Deformed* gene. *Genes Dev* 5:278–286.
46 Chouinard, S. and Kaufman T.C. (1991) Control of expression of the homeotic labial (lab) locus of Drosophila melanogaster: evidence for both positive and negative autogenous regulation. *Development* 113:1267–1280.
47 Chan, S.K., Popperl, H., Krumlauf, R. and Mann, R.S. (1996) An extradenticle-induced conformational change in a HOX protein overcomes an inhibitory function of the conserved hexapeptide motif. *EMBO J* 15:2476–2487.
48 Popperl, H., Bienz, M., Studer, M., Chan, S., Aparicio, S., Brenner, S., Mann, R.S. and Krumlauf, R. (1995) Segmental expression of *Hoxb-1* is controlled by a highly conserved autoregulatory loop dependent upon *exd/pbx. Cell* 81:1031–1042.
49 Gould, A., Morrison, A., Sproat, G., White, R.A.H. and Krumlauf, R. (1997) Positive cross-regulation and enhancer sharing: two mechanisms for specifying overlapping *Hox* expression patterns. *Genes Dev* 11:900–913.
50 Malicki, J., Cianetti, J., Peschle, C.L. and McGinnis, W. (1992) A human HOX4B regulatory element provides head-specific expression in *Drosophila* embryos. *Nature* 358:345–347.
51 Awgulewitsch, A. and Jacobs, D. (1992) The *Deformed* autoregulatory element functions in a conserved manner in transgenic mice. *Nature* 358:341–344.
52 Pinsonneault, J., Florence, B., Vaessin, H. and McGinnis, W. (1997) A model for *extradenticle* function as a switch that changes Hox proteins from repressors to activators. *EMBO J* 16:2032–2042.
53 van Dijk, M. and Murre, C. (1994) extradenticle raises the DNA binding specificity of homeotic selector gene products. *Cell* 78:617–624.
54 Bienz, M. (1994) Homeotic genes and positional signalling in the *Drosophila* viscera. *Trends Gene* 10:22–26.
55 Appel, B. and Sakonju, S. (1993) Cell-type-specific mechanisms of transcriptional repression by the homeotic gene products UBX and ABD-A in *Drosophila* embryos. *EMBO J* 12:1099–1109.
56 Peifer, M. and Wieschaus, E. (1990) Mutations in the *Drosophila* gene *extradenticle* affect the way specific homeo domain proteins regulate segmental identity. *Genes Dev* 4:1209–1223.
57 Reuter, R. and Scott, M.P. (1990) Expression and function of the homeotic genes *Antennapedia* and *Sex combs reduced* in the embryonic midgut of *Drosophila. Development* 109:289–303.
58 Celniker, S.E., Sharma, S., Keelan, D.J. and Lewis, E.B. (1990) The molecular genetics of the bithorax complex of Drosophila: cis-regulation of the Abdominal-B domain. *EMBO J* 9:4277–4286.
59 Gerard, M., Chen, J.Y., Gronemeyer, H., Chambon, P., Duboule, D. and Zakany, J. (1996) In vivo targeted mutagenesis of a regulatory element required for positioning the *Hoxd-11* and *Hoxd-10* expression boundaries. *Genes Dev* 10:2326–2334.
60 Gyurkovics, H., Gausz, J., Kummer, J. and Karch, F. (1990) A new homeotic mutation in the *Drosophila* bithorax complex removes a boundary separating two domains of regulation. *EMBO J* 9:2579–2585.
61 Zhou, J., Barolo, S., Szymanski, P. and Levine, M. (1996) The Fab-7 element of the bithorax complex attenuates enhancer-promoter interactions in the *Drosophila* embryo. *Genes Dev* 10:3195–3201.
62 Hagstrom, K., Muller, M. and Schedl, P. (1996) *Fab-7* functions as a chromatin domain boundary to ensure proper segment specification by the *Drosophila* bithorax complex. *Genes Dev* 10:302–3215.
63 Karch, F., Weiffenbach, B., Bender, W., Peifer, M., Duncan, I., Celneken, S., Crosby, M. and Lewis, E.B. (1985) The abdominal region of the Bithorax complex. *Cell* 43:81–96.
64 Galloni, M., Gyurkovics, H., Schedl, P. and Karch, F. (1993) The bluetail transposon: evidence for independent *cis*-regulatory domains and domain boundaries in the bithorax complex. *EMBO J* 12:1087–1097.
65 Denell, R.E., Brown, S.J. and Beeman, R.W. (1996) Evolution of the organization and function of insect homeotic complexes. *Cell Dev Biol* 7:527–538.
66 Warren, R.W., Nagy, l., Selegue, J., Gates, J. and S. Carroll (1994) Evolution of homeotic gene regulation and function in flies and butterflies. *Nature* 372:458–461.
67 Rogers, B.T., Peterson, M.D. and Kaufman, T.C. (1997) Evolution of the insect body plan as revealed by the *Sex combs reduced* expression pattern. *Development* 124:149–1557.
68 Kaufman, T.C., Seeger, M.A. and Olsen, G. (1990) Molecular and genetic organization of the *Antennapedia* gene complex of *Drosophila melanogaster*. in *Advances in Genetics. Genetic Regulatory Hierarchies in Development.* 27:309–362.
69 Averof, M. and Akam, M. (1995) Hox genes and the diversification of insect and crustacean body plans. *Nature* 376:420–423.
70 Gould, S.J. (1991): The disparity of the Burgess Shale arthropod fauna and the limits of cladistic analysis: why we must strive to quantify morphospace. *Paleobiology* 17:411–423.
71 Burke, A.C., Nelson, C.E., Morgand, B.A. and Tabin, C. (1995) *Hox* genes and the evolution of vertebrate axial morphology. *Development* 121:333–346.
72 Li, X. and Noll, M. (1994) Evolution of distinct developmental functions of three Drosophila genes by acquisition of different cis-regulatory regions. *Nature* 367:83–87.
73 Carroll, S., Weatherbee, S., Langeland, J. (1995) Homeotic genes and the regulation and evolution of insect wing number. *Nature* 375:58–61.
74 Hinz, U., Wolk, A. and Renkawitz-Pohl, R. (1992): *Ultrabithorax* is a regulator of b3 tubulin expression in the *Drosophila* visceral mesoderm. *Development* 116:543–554.
75 Thuringer, F., Cohen, S.M. and Bienz, M. (1993) Dissection of an indirect autoregulatory response of a homeotic *Drosophila* gene. *EMBO J* 12:2419–2430.
76 Zeng, C., Pinsonneault, J., Gellon, G., McGinnis, N. and McGinnis, W. (1994) Deformed protein binding sites and cofactor binding sites are required for the function of a small segment-specific regulatory element in *Drosophila* embryos. *EMBO J* 13 No. 10:2362–2377.
77 Lou, L., Bergson, C. and McGinnis, W. (1995) *Deformed* expression in the *Drosophila* central nervous system is controlled by an autoactivated intronic enhancer. *Nucl Acids Res* 23:3481–3487.
78 Averof, M. and Akam, M. (1993) HOM/Hox genes of Artemia: implications for the origin of insect and crustacean body plans. *Curr Biol* 3:73–78.
79 Holland, P.W., Garcia-Fernandez, J., Williams, N.A. and Sidow, A. (1994) Gene duplications and the origins of vertebrate development. *Development* Suppl:125–133.
80 Averof, M., Dawes, R. and Ferrier, D. (1996): Diversification of arthropod *Hox* genes as a paradigm for the evolution of gene functions. *Cell Dev Biol* 7:539–551.
81 Whittington, H.B. (1985) *The Burgess Shale*. Yale University Press, New Haven, CT.