The hex of Hox*

The Antennapedia (Antp) and Ultrabithorax (Ubx) mutants of Drosophila melanogaster are surely the two most celebrated mutants in biology. Antp causes flies to develop legs where their antennae should be, and Ubx causes them to develop another pair of wings in place of the relatively inconspicuous balancing organs called halteres (Figure 1). Such transformation of one body part into another is called homeosis (a process described by W. Bateson as far back as 1894). Mutations that cause a propensity for such transformations to be genetically inherited are called homeotic mutations and define the homeotic genes. A major scientific triumph of Drosophilists (in particular, Edward B. Lewis (1918–2004)) was to demonstrate that homeotic genes are arranged in multi-gene complexes (the Antennapedia and bithorax complexes, ANT-C and BX-C) that are arrayed in a proximal to distal sequence in the chromosome, and that the more distal genes in a complex are expressed only in the more posterior segments of the developing embryo; the so-called spatial 'colinearity principle'. Homeotic genes code for transcription factors that contain an ~60 amino acid residue DNA-binding domain, the homeodomain, which is encoded by an ~180 bp evolutionarily conserved DNA sequence called the homeobox. Hox genes are the subset of homeobox-containing genes in the Hox clusters, and they underlie development in pretty much all animal phyla tested. In the chordates, Hox gene expression shows not only spatial but also temporal colinearity, and is thought to represent the ancestral state. Ironically, Drosophila Hox gene expression is now recognized to be a derived state, that too one caught on its way to evolutionary degeneration. A paralogous cluster of homeobox genes defines the paraHox complex. Other homeobox genes are found dispersed in the genomes of plants, animals and fungi. Recently, about 120 scientists from around the world as-

sembled at CCMB, Hyderabad to share the magic of current Hox research. This report highlights some results presented at the workshop. Unfortunately, my limitations as a ‘note-taker’ prevented me from doing justice, such as it is, to the 42 posters that were so enthusiastically presented at the meeting.

Drosophila Hox genes

Why are Hox genes clustered?

The D. melanogaster ANT-C and BX-C are located in chromosome 3R. The ANT-C contains five genes: labial (lab), proบางคนศ (pb), Deformed (Dfd), Sex combs reduced (Scr) and Antennapedia (Antp). The BX-C contains three genes Ubx, Abd-A and Abd-B. The expression of these genes is driven in individual segments by large, autonomous, cis-regulatory domains. The ANT-C also contains a few other Hox-related genes such as ftz, bcd, zen1 and zen2. In contrast to the two complexes of D. melanogaster, all the Hox genes are contained in a single cluster (HOM-C) in Tribolium and several other insect species. Susan Celniker (Lawrence Berkeley National Laboratory, USA) scanned the non-coding sequences of the Hox complexes in eight different Drosophila species whose genome sequencing is underway to identify conserved cis-regulatory modules bound by upstream regulators of the complex and for conserved microRNAs. These species showed a number of different ways in which HOM-C could be ‘broken’ (e.g. in D. virilis the

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Figure 1. Effects of mutating the Hox gene Ultrabithorax (Ubx): a. A normal fly (Drosophila melanogaster). Note the pair of wings, and in the immediately posterior segment, a pair of halteres. b. Expression of the gene CG32062 in larval tissues (imaginal discs) that develop into the adult wing and haltere. Note differential expression of this gene in the wing (left) and haltere (right) discs. c. Halteres are completely transformed into wings in a fly that carries a 'loss-of-function' mutation in Ubx. d. CG32062 expression in haltere disc is now indistinguishable from that in the wing disc. e. Wings are completely transformed into halteres in a fly that carries a 'gain-of-function' mutation in Ubx. f. CG32062 expression in wing disc now resembles that in the haltere disc. Imaginal discs are most evident in (f) (Figure taken from PhD dissertation of Ruchi Bajpai.)
Hom cluster is broken between Ubx and abd-A). Nevertheless the cis-regulatory elements for binding of Hox gene regulators and genes for microRNAs were found to be conserved. Peter Holland (University of Oxford, UK) suggested that Hox gene clustering is more pronounced in species with slow development such as vertebrates and amphibians. Clustering might be crucial for sequential gene expression which underlies temporal colinearity of Hox gene expression. On the other hand in fast developing species like Drosophila, temporal colinearity of Hox gene expression is not as crucial, although spatial colinearity is retained. The Parahox gene cluster (Parahox = Gsx, Xlox, Cdx) is conserved in mammals, amphibians and amphioxus, but shows evidence of breakage and dispersal in Drosophila, worms, ascidians and teleost fish. Holland suggested that the reason that Hox genes are clustered in teleosts and Drosophila but the Parahox genes are not, may be because Hox genes share enhancers and selection maintains the interdigitation of their regulatory elements, but enhancers are not shared by the Parahox genes.

That loss of Hox gene clustering occurs along with loss of temporal colinearity was proposed also by Daniel Chatroux (University of Bergen, Norway) to explain degeneration of the Hox cluster in the urochordate tunicate, Oikopleura dioica. This organism has a compact genome with nine dispersed Hox genes, and two unlinked Parahox genes. It has lost a number of Hox genes, but has also amplified other Hox genes. The degeneration of the tunicate Hox/Parahox clusters happened alongside the anatomical regression of these animals.

Regulatory domains in BX-C

Expression of Abd-B in fly segments A5, A6, A7 and A8 is regulated by the specific cis-regulatory domains iab-5, iab-6, iab-7 and iab-8 respectively. The proximal-distal order of the cis-domains corresponds to the anterior-posterior order of the segments they specify (colinearity principle). In A4 or more anterior segments, all the cis-domains are silenced by PcG proteins binding to Polycomb Response Elements (PREs) found in each regulatory domain. In A5, only iab-5 is thought to be active; in A6, both iab-5 and iab-6 are active but only iab-6 drives the expression of Abd-B. iab-7 controls the expression of Abd-B in A7. Loss of function of iab-7 causes activation of iab-6 in A7, as seen by reduced levels of Abd-B in A7 leading to transformation of A7 (or more strictly, PS12) to A6. Domain boundaries play a fundamental role in maintaining the functional autonomy of the sequentially activated domains of the homeotic genes.

The level of Abd-B protein increases in a step-wise fashion from A5 to A8, suggesting that the distal enhancers (like those in the iab-7 domain) are stronger than the more proximal enhancers (like those of iab-5). However, when the individual enhancers are tested in transgenic systems, they all appear to drive the expression of the reporter genes at comparable high levels. To explain this, it has been suggested that intervening boundaries reduce the activity of the cis-regulatory regions. Henrik Gyurkovics (Biological Research Centre, Hungary) reported the phenotypes of flies containing combinations of different BX-C deficiencies, a BX-C duplication and a PcG mutation. The PcG mutant pC40 gives a strong posterior transformation of A7 to A8. This phenotype is suppressed by Dp(C5)/+ strongly enhanced by Df(P9)+ and less strongly enhanced by Df(iab-7). Dp(P5) is a tandem duplication of BX-C, Df(P9) is a deficiency of BX-C and Df(iab-7) is a deficiency of only the iab-7 element. These findings indicate that the cis-regulatory regions of Abd-B can be hyperactivated and that the PREs on the two homologues can interact both in trans and cis. Francois Karch (University of Geneva, Switzerland) took advantage of P-element insert near the Fab-7 boundary to replace the Fab-7 boundary by Fab-8 via gene conversion. This replacement, however, resulted in no phenotypic change. He also described the effect of targeting Escherichia coli dam methyltransferase, as a Gal-4-Dam fusion construct, to the UAS-Fab-7 boundary. Dam methyltransferase methylates the adenines in GATC. Recruitment of Dam methyltransferase to Fab-7 in this way resulted in GATC methylation at Fab-7. This methylation decayed 5 kb away from the Fab-7 target. However, methylation was also seen at the Abd-B promoter (65 kb away). This implied that Fab-7 and the Abd-B promoter are brought into close proximity with each other. Methylation of the Abd-B promoter was greater in head tissue where Abd-B is silenced than in the abdomen, which includes tissue in which Abd-B is active. Robert A. Drewell (University of Nevada, USA) addressed the question of how the iab-5 enhancer interacts with the Abd-B promoter which is 55 kb distal to iab-5 and has at least two intervening insulators, but not with the abd-A promoter which is only 48 kb proximal. A 254-hp element, the promoter tethering element (PTE), from the Abd-B promoter was shown to be required for iab-5 to drive the Abd-B promoter in transgenic embryos. In competition assays of abd-A-lacZ versus Abd-B-cat, this element directed iab-5 to the more distant Abd-B promoter. In contrast, the iab-2 enhancer drives expression from the closest promoter, which is ordinarily that of abd-A. It would now be of interest to determine which iab-5 element(s) are required for interaction with the PTE. Jumin Zhou (Wistar Institute, USA) showed that the Drosophila CTCF homologue interacts with the Fab-8 boundary both in vitro and in vivo and is required for enhancer blocking by Fab-8 in transgenic flies. He identified a novel DNA element, the Promoter Targeting Sequence (PTS) in iab-8 that targets an enhancer to a specific promoter in a strain-specific fashion. PTS also mediates interference between two enhancers, i.e. the activity of one enhancer (e.g. H1) inhibits that of another (e.g. H6) for the same promoter. Enhancer-interference might provide the basis of enhancer selection, whereby Abd-B interacts mainly with one regulatory domain in a specific abdominal segment. Vivek S. Chopra (CCMB, Hyderabad) screened for enhancers of different mutant alleles of iab-7. He found that trithoraxgleich (trg) mutations showed strong A7 to A6 homeotic transformation when combined with iab-7 hypomorphic alleles; resembling a complete loss of function of iab-7. This suggested that trg has an iab-7 activation function. The trg mutation was originally identified based on its recessive germ cell migration phenotype and classified as a trxG member. The trg locus has been mapped to a ~300 kb region in 91A3.

Paul Scheld (Princeton University, USA) evaluated different DNA segments of Fab-7 for enhancer-blocking activity when inserted between the βz enhancer and a lacZ reporter construct in transgenic flies. Gel shift assays with nuclear extracts from early or late embryos and comparison of the enhancer-blocking elements with their mutated derivatives revealed that different nuclear factors are present in the early and late developmental stages that bind different DNA elements of Fab-7 to generate the observed
insulator activity. Thus chromatin domains appear to be composed of developmentally restricted sub-elements, thereby imparting plasticity to domain function.

One well studied Drosophila insulator is the 320 bp element found in the 5' UTR of the gypsy transposable element. It contains 12 binding sites for the Su(Hw) Zinc-finger DNA binding protein. Staining of polytene chromosomes with anti-Su(Hw) antiserum reveals hundreds of Su(Hw) binding sites that presumably represent endogenous insulators. However, only one of them (between the genes yellow and Achte-scute) was shown to actually exhibit enhancer blocking activity. Rob White (University of Cambridge, UK) reported that staining of interphase nuclei shows that Su(Hw) binding sites are gathered into 20–40 speckles that are associated with the nuclear periphery or around the nucleolus. He performed ChIP analysis with anti-GFP antiserum in flies transgenic for Su(Hw)-GFP fusion protein. He identified 105 endogenous Su(Hw) binding sites in a 3 Mb tiled region of Drosophila 2L and used their sequences to derive an extended consensus sequence for Su(Hw) binding. This consensus differed somewhat from the 12 Su(Hw) binding sites in gypsy and did not show clustering as in gypsy.

Although engrailed (en) is not a Hox gene, it encodes a homeodomain protein that plays an important role in development. Judith Kassis (NIH, USA) showed that multiple enhancers upstream of the en promoter are bracketed by a 181 bp sequence called the pairing sensitive element (PSE) that acts as a PRE involved in pairing specific silencing (PSS) in transgenic assays. It was proposed that pairing between the PSEs facilitates the interaction between the enhancers and the en promoter to regulate the control of transcription. Kassis also undertook the task to find all the DNA-binding proteins (e.g., Spl/KIF family of Zn-finger proteins) and target sites in this PRE.

Epigenetic control

In Drosophila, Polycomb group (PcG) proteins form distinct chromatin complexes that maintain the repressed states of Hox genes during development. The PRC1 complex contains the proteins PC (polycomb), PH (polyhomeotic), PSC (posterior sex combs) and dRING 1 (Sce) plus zeste and several other general transcription factors. The ESC-E(Z) (or PRC2) complex contains ESC (extra sex combs) and E(Z) (enhancer of zeste), SU(Z)12 (suppressor 12 of zeste) and the histone-binding protein NURF55. E(Z) is a SET domain protein with histone H3 lysine 27 methyltransferase activity (H3K27); the chromodomain of PC binds to H3K27 tails. Additionally, Pho (pleiohomeotic) and Pho (pho-like) homologues of human YY1 protein are sequence-specific DNA-binding proteins that interact with and recruit subunits of the ESC-E(Z) complex to the PREs in DNA. Juerg Mueller (EMBL, Germany) purified two distinct Pho protein complexes from Drosophila embryos. Neither of these complexes contains PRC1 or ESC-E(Z) components. One complex, called Pho repressive complex (PhoRC), contains Pho together with dSmHMT, a novel protein and binds to the lab-7 PREs in embryos. Ubx is misexpressed in dSmHMT mutant clones of the wing disc, thus showing that dSmHMT is a PcG gene required for Hox silencing. MBT repeats of dSmHMT bind to Pho as well as to lysine mono- and di-methylated tails of histones H3 and H4. ChIP analysis of wing and haltere discs revealed that Pho and dSmHMT are bound to PREs in both the ON and OFF states. This implies that ON and OFF do not differ necessarily in the binding to PcG and trxG proteins but that differential enzymatic activities of the bound HMTases can bring distinct histone methylation patterns in these two states. Peter Verrijzer (Erasmus University Medical Center, Netherlands) identified another small conserved sequence element, the PcG binding element (PBE) close to Pho-binding sites in the bxd PRE that is required for PC-induced silencing in vivo. Scanning Force Microscopy revealed that cooperative binding of Pho-PCC to the bithoraxoid PRE-PBE sequences wraps the DNA into a nucleosome-like structure called the silencerosome. Protein ubiquitylation also has a role in PcG-based silencing. Ubiquitin-specific protease 7 (USP7) is found associated with guanosine monophosphate synthetase (GMPS). USP7 mutant males display a ‘wings held-out’ phenotype that was shown to be enhanced by heterozygosity for gmps mutants. USP7–GMPS complex removes ubiquitin moiety from histone H2B as well as some other chromatin-bound proteins. The GAGA-factor which binds the Ubx promoter is encoded by Trithorax-like (Trl). Rakesh Mishra (CCMB) made yeast two-hybrid screens to identify partners of GAGA. These included lola-like and dmTAF3. Mutations in lola-like reduce PRE-mediated silencing of Ubx, whereas dmTAF3; Trl double mutants enhance the Ubx phenotype. Thus GAGA can recruit both activating and repressive complexes to its target promoter by interacting with different partners. Rick Jones (Southern Methodist University, USA) reported that ChIP assays of wild type and PcG mutant wing discs reveal the presence of multiple PcG complexes at the bxd PRE located ~25 kb upstream of the Ubx promoter. A model for hierarchical recruitment protein complex was proposed in which Pho and Pho interact with and recruit subunits of the ESC-E(Z) complex to the bxd PRE. Histone methylation by E(Z), as well as direct Pho–PC interaction, then recruits the PRC1 subunits and thereby silences transcription from the Ubx promoter. Interestingly, RNA pol II is present at the silenced Ubx promoter in wing discs, together with Kismet, a trxG protein involved in chromating remodelling. Thus PcG may repress via a block in transcription initiation or elongation. Jeff Simon (University of Minnesota, USA) reconstituted the ESC–E(Z) complex from baculovirus-expressed recombiant proteins and showed that the histone methyltransferase (HMTase) activity of the reconstituted complex has primary specificity for H3K27. Although E(Z) is the HMTase catalytic subunit, the noncatalytic partners SU(Z)12 and ESC are necessary to enhance this activity by 100x. Missense mutations in the CXC domain of E(Z), in surface loops of ESC, and in the conserved VRFs domain of SU(Z)12 disrupted HMTase activity, but the mutant proteins retained the ability to assemble into the complex. Since SU(Z)12 can bind mononucleosomes in vitro, it is conceivable that the noncatalytic partners of E(Z) also chaperone the complex to the nucleosome. E(Z) and ESC are conserved between Drosophila and Caenorhabditis elegans, but in C. elegans another mononucleosome binding protein called MES-3 substitutes for SU(Z)12. ESC-like (ESCL), shares ~60% homology with ESC and can substitute for ESC in the postembryonic complex. Reduced escl gene dosage enhances escl loss of function phenotypes. Double knockdown of ESC and ESCL by RNAi caused Ubx to be derepressed in wing-disc derived cells. Phosphorylation of H3 serine 28 antagonized methylation on K27. Phosphorylated H3S28 is enriched at polytene puffs, consistent with the idea that it is an activating modification.
Valerio Orlando (Dulbecco Telethon Institute, Italy) showed that mutations in the RNAi machinery enhanced mutant PcG homeotic phenotypes and that the Dicer and Argonaute proteins are also localized in the nucleus. Small RNAs were found derived from PcG repressed intergenic segments, suggesting the involvement of RNAi pathway and ncRNA (non-coding RNA) in epigenetic silencing of BX-C by PcG proteins. Giacomo Cavalli (CNRS, France) showed that homozygosity for a Fab-7-miniwhite transgene inserted near the scalloped (sd) locus caused sd to be silenced. But this silencing was alleviated if Fab-7 was deleted from the BX-C. 3D-FISH/immunostaining revealed that in a significant fraction of embryonic nuclei, the Hox and sd genes co-localize in nuclear bodies enriched in PC protein. The co-localization is seen only in tissues in which the Hox genes are silenced. This long-range silencing required both PcG as well as components of the RNAi machinery that are co-localized with PcG bodies. However, comparably definedtrxG bodies contain both active and inactive genes; thus co-regulated genes undergo coordinated nuclear compartmentation. Such contacts might impose constraints on chromosome evolution.

**Hox protein cofactors**

The cofactors extradenticle (EXD) and Homothorax (HTH) increase the DNA-binding specificity of Hox proteins by interacting with the short motif called hexapeptide (HX) or YPWM found in nearly all Hox proteins. Although recruitment of cofactors to abdA HX increased its DNA-binding specificity, Yacine Graba (CNRS) showed that the HX motif was dispensable for EXD recruitment, thus suggesting that EXD also interacts with other abdA domains. Also the ‘quality’ of the DNA binding site of the Hox protein was important for selecting the mode of EXD recruitment. Mutation of the abdA HX motif led to activation of dpp rather than its repression. The dpp enhancer has nine abdA-binding sites, of which three are required for activation and four for repression. The wild type and HX mutant abdA proteins had the same ability to footprint the dpp enhancer. This suggested that an intramolecular regulatory circuit controls the abdA activation/repression switch.

Antp is required for specification of the entire second thoracic segment (no Antp-l- mitotic recombinant cells can be induced in the adult wing or notum). Frederic Prince (Biozentrum, Switzerland) showed that ey-Gal 4; UAS-Antp; UAS-N causes transformation of the eye to wing and leg. Both transformations were suppressed by overexpression of exd. Ectopic wing formation required the YPWM motif, which is situated amino terminally to the homeodomain, and did not occur if the motif was mutated. But the mutation did not affect the formation of ectopic leg structures. UAS-Bip2 (Bip2 = dTAF3) enhances the eye-to-wing transformation, but not if YPWM is mutated to AAAA. Bip2 was shown to genetically interact with the Antp\[^{13}\] (Antennapedia–Cephalothorax) allele. Thus BIP2 also is a cofactor that interacts with Antp via the YPWM motif.

The EXD and HTH co-factors are not present in the wing and halter discs, so UBX must either be acting alone or with other co-factors. Bradley Hersh (University of Wisconsin-Madison, USA) analysed the regulatory regions of the genes sal (spalt) and knot, two direct targets of UBX that are expressed in the wing, but are repressed by UBX in the haltere. The 1.4 kb region upstream of the sal promoter has sites for binding of UBX as well as putative binding sites for Mad (mothers against Dpp), a transcriptional effector of the Dpp pathway. Loss of function clones for Mad in the haltere disc cause derepression of sal; thus Mad collaborates with UBX to repress sal in the haltere. The knot regulatory element has nine Ubx-binding sites in two redundant blocks. In the more conserved block, only UBX binding appears to be required for repression of knot. But the other divergent block has additional sequence not bound by UBX, but is required for full repression. Thus the divergent region appears to require a new co-factor for full repression. In other words, UBX collaborates with multiple co-factors to regulate target genes in the haltere.

**Drosophila proboscipedia (pb) mutants display homeotic transformation of adult labial mouthparts to distal prothoracic legs. The labial-to-leg transformation requires hedgehog activity. Thus Pb protein appears to antagonize hedgehog. David Cribs (CNRS, France) showed that Pb also down regulates wg and dpp in labial discs, apparently by inhibiting upregulation of wg and dpp by the transcription factor cubitus interuptus Ci. His group has analysed the functional relationship between Pb and Ci, and are testing whether other Hox proteins are involved in similar interaction to regulate morphogen signalling.

**Downstream targets**

During postembryonic development of the Drosophila CNS, each thoracic neuroblast produces ~ 100 progeny, whereas their abdominal counterparts produce only about six. This difference is due to an abdominal-specific burst of abd-A within neuroblasts that prevents precursor divisions by activating H99 cell-death genes. More neuroblasts are produced in abd-A clones in the abdomen. Alex Gould (MRC-NIMR, UK) reported that the transcription factor grainhead (grh) is required together with abd-A to trigger neuroblast apoptosis. grh appears to make neuroblasts competent to undergo Abd-A induced apoptosis in a cell-type specific and temporal pattern. An FRT-mosaic screen resulted in the identification of several mutations that produce an abd-A-like neuroblast over-proliferation phenotype. Among the genes identified was Dskl, a novel anti-proliferative kinase that upregulates abd-A levels thereby promoting apoptosis of abdominal neuroblasts.

Cyclin E plays a critical role in the specification of neuroblasts and thus in the differentiation of neurons and glia. Christian Berger (University of Mainz, Germany) reported that cyclin E appears to be a Hox gene target. Ectopic expression of abd-A and Abd-B was found to repress cyclin E. The cis-regulatory regions of D. melanogaster and D. pseudoobscura cyclin E genes appeared to have a CNS-specific enhancer with possible binding sites for Abd-A and Abd-B. Berger conducted gel-shift and supershift experiments which showed that cyeE is indeed a target of these Hox proteins. Bioinformatic analysis revealed that the D. melanogaster gene dally (division abnormally delayed) has 11 clustered Ubx-binding consensus sequences of which five are conserved in D. pseudoobscura, suggesting a possible functional significance. Dally is a glycoprotein required for diffusion of wingless and Dpp signalling.

Ubx down regulates activities of the signalling centres such as A/P and D/V organizers, to specify haltere fate. It does this by altering the relationship between morphogen signalling components in the wing and haltere discs. In the haltere Ubx restricts both the amount and diffusion of Dpp morphogen, thereby limiting
signal transduction and cell proliferation. Kalpana Makhijani (CCMB) used a transgenic reporter construct to show that dally, which is expressed in both anterior and posterior compartments of the wing disc, is not expressed in the posterior compartment of the haltere disc. Moreover, dally was derepressed in loss-of-function mitotic clones of Ubx in the haltere. She also reported a preliminary ChIP analysis which suggested that UBX binds to sites in the dally promoter. Further analyses of Dpp signalling revealed that it is down regulated in the haltere disc both due to reduced levels of Dpp and due to its inability to diffuse into the posterior compartment, probably due to the absence of Dally.

Richard Mann (Columbia University, USA) reported that Ubx alters the way in which Dpp signalling controls transcription of thickveins (tkv) and dally, molecules that modulate the way Dpp spreads and forms a gradient. There are fewer tkv receptors in the wing disc cells than in haltere disc cells; this results in a greater sequestration of Dpp in the haltere and greater diffusion in the wing. Misexpression of tkv in the wing reduced the wing size by 30%. Dpp also represses Masker of tkv (Mtr) in the haltere, but not in the wing. Additionaly, Ubx appears to contribute to tkv derepression in the haltere by making Mtv an activator of Tkv rather than a repressor. L. S. Shashidhara (CCMB) reported that over-expression of Dpp (A/P signalling), vestigial (D/V signalling) or activated form of EGFR can override the effects of Ubx and induce haltere to wing transformation. He drew parallels between down regulation of Dpp by Ubx and down regulation of Bmp2 and Bmp7 by Hox genes in mouse. Shashidhara also described efforts to identify new targets of Ubx via enhancer trap analysis, EP screens and microarray analysis (Figure 1). That Ubx reduces both dpp expression and activity in the haltere disc, perhaps by controlling expression of dally and dally-like, two proteins that help in dpp diffusion was also reported by E. Sanchez-Herrero (University Autonoma Madrid, Spain). In addition, he derived Ubx-Gal 4 lines by gene conversion of Ubx-lacZ lines and used them in combination with UAS-Ubx or UAS-Hox in an Ubx background to test whether different Ubx isoforms or distinct Hox genes can substitute for the endogenous Ubx gene in haltere development. A partial transformation of haltere to wing was observed if only isoform IVa was expressed in the haltere disc at a level similar to that in Ubx heterozygotes. Interestingly, isoform IVa does not suppress sal. All the other isoforms, as well as Ser, abd-A Dfd and Abd-B could substitute for Ubx in the formation of halteres. But Anp, lab and pb were not as efficient.

The spineless-Aristapedia (ss_Aristapedia) mutant transforms the arista into a foreleg. The ss gene is expressed in the third and more distal segments of the antenna (A3, etc.) and has homology to the mammalian dioxin receptor. Ian Duncan (Washington University, St. Louis, USA) showed that a 521 bp fragment from the 5′ region of ss drives reporter expression in the third antennal segment and more distally, reproducing the normal ss expression. Early expression of the reporter was activated by both Dil and Hth. Hth is known to require for antennal identity specification and hth mutations transform antenna to leg. In turn, Ss is required for Dil expression. Ectopic expression of Antp repressed the ss enhancer independently of hth, possibly by competition with Dil. Five invariant subdomains in the 521 bp fragment were found to contain probable Hth, Exd and Dil binding sites. To search for possible Ss target genes, Duncan screened the genome for clusters of binding sites for Hth, Exd, Dil and Ss. The best candidate target gene found was darr, which requires Ss for expression and is expressed in the same pattern as ss in the antenna. A 258 bp darr fragment could drive expression in the distal antenna. Five other potential Ss targets identified encode detoxification-type enzymes (two cyp P-450s + three short chain dehydrogenases) that may be involved in odorant clearance. These findings provide a link for the homology of Ss with the mammalian dioxin receptor and the general detoxification response in mammals.

**Hox and the trachea, heart and neuromuscular movement**

The striking tracheal phenotype seen in the absence of Hox genes was described by Lewis in his 1978 review. Markus Affolter (Biozentrum) presented a detailed analysis of live embryo imaging, which suggested that of Hox proteins and their cofactors Sal, Hth and Exd act as micro-managers during tracheal morphogenesis. For example, Hth and Exd mutants showed a defect in expression of Branchless (Bnl) and defective elongation of the dorsal trunk and abd-A is required in the fusion cells, possibly to regulate E-cadherin.

Turning to the heart, the larval cardiac tube is made up of 104 distinct myocytes that differentiate into the anterior aorta, posterior aorta and the heart according to their position in the A/P axis. Antp, Ubx, abdA and abdB are expressed in non-overlapping domains of the cardiac tube. In the absence of Hox function, the whole tube adopts the identity of the anterior aorta that does not beat. Conversely, ectopic expression of Antp, Ubx and AbdA transforms the anterior aorta into the posterior cardiac tube that beats, and represses the formation of associated lymph glands. Michel Semeriva (University of Marseille, France) reported the identification of three genes that behave as potential Hox differential targets; they are activated by abdA in the heart and repressed by Ubx in the posterior aorta. Mutation in one of these genes, Okl reduces the frequency of the heart-beat, whereas overexpression prevents the beat by causing the slow depolarization phase to disappear. The adult heart is remodelled from segments A1–A5 of the larval cardiac tube (i.e. corresponding to the posterior aorta), abdA is successively required in the myocytes for the acquisition of both the larval and adult differentiated states. Specificity of abdA is switched at metamorphosis to induce a novel genetic programme. Ecdysone controls this remodeling by impring on both the regulation of Ubx expression and the modification of abdA function. K. Vijayaraghavan (NCBS, Bangalore) examined the effects of Hox mutations and misexpressions (e.g. loss of Dfd, ectopic expression of Antp) on embryonic movements and larval crawling and found that particular Hox gene functions specify the characteristic movements associated with peristaltic crawling. He also presented preliminary results of a screen to identify neural components that control larval crawling behaviour. In this screen Gal4 enhancer trap lines are used to drive UAS-tetanus toxin expression and the consequence of selective cell killing on locomotory behaviour is evaluated.

**Beyond Drosophila**

**Vertebrates**

Derived teleost fishes possess up to seven Hox clusters, whereas basal ray-finned fishes (e.g. bichir) and outgroup taxa...
such as horn sharks and lobe-finned fishes have only four *Hox* clusters. The line leading to the derived teleosts is believed to have undergone a whole genome duplication (WGD). Chi-Hua Chu (Rutgers University, USA) analysed the conserved noncoding sequences (aka phylogenetic footprint clusters, PFCs) in the extra *Hox* clusters of the teleosts, specifically zebrafish and puffer fish. She found lineage-specific patterns of element gain/loss as well as point mutations in cis-regulatory elements that are otherwise conserved in horn sharks and humans. The derived teleosts showed conservation of fewer PFCs as well as differential retention of PFCs. Other basal teleosts like mooneye and paddlefish show a partial duplication of *HoxC*, but this appears to represent another duplication event which occurred independently of the WGD of the derived teleosts. Thus evolution of the *Hox* clusters in ray-finned fishes was already quite dynamic prior to the WGD, leaving open the question of whether the WGD was responsible for the great diversity of teleost body plans. Victoria Prince (University of Chicago, USA) estimated that the WGD occurred more than 300 million years ago (MYA), but that the species radiation (>23,000 species today) occurred only about 100 MYA. These figures argue against a radiation triggering role for the WGD. Prince also described the specification by *hoxb1a* of rhombomere r4 in the developing Zebrafish hindbrain; the mouse *Hoxb1* orthologue has a similar function. Knockdown of *Hoxb1a* by microinjection of antisense morpholino compands blocked migration of r4-specific facial neurons and they resembled the nonmigratory r2-specific trigeminal neurons. A microarray analysis was done to compare mRNAs in dissected normal r4 cells, normal r2 cells and in morpholino treated *hoxb1a-deficient* r4 cells. Among the genes that were up-regulated by >1.5x by *Hoxb1a* was *prickle 1-like*, which had a discrete, punctate expression pattern in the r4-derived migrating facial neurons. Prince is now testing the effects of morpholino inhibitors of *prickle 1-like* on these neurons.

In mouse, *Hoxa2* controls the patterning of the second brancial arch neural crest derivatives and is expressed in rhomboeres r2-r5 of the developing mouse hindbrain. Stefan Tumpel (Stowers Institute for Medical Sciences, USA) described the three independent enhancer modules that drive *Hoxa2* expression. One enhancer drives expression in r3 and r5 and has Krox20 binding sites. The r4 enhancer is within the intron and has binding sites for *Hoxb1* and the r2 enhancer is within the coding sequence. The pufferfish Fugu has two *Hoxa2* paralogues that are differentially expressed in the hindbrain but showed only subtle sequence alterations, possibly due to the constraints of having the enhancers within the coding sequences.

Oliver Pourquie (Stowers Institute for Medical Sciences) presented an elegant use of two-colour fate mapping to study *Hoxb* expression during somitogenesis in the developing chick embryo. Donor embryos were labelled with Dil as well as electroporated with a Hoxb-GFP construct. Cells from these embryos were then grafted onto age-matched recipients. The Dil label tracked the donor cells and their descendants and the GFP marked the activation of the *Hox* gene. The *Hoxb* genes were activated in a temporally colinear fashion in the epiblast cells fated to give rise to paraxial mesoderm and control their ingress into the streak. The results showed that the stem cells from the anterior primitive streak gave rise to the medial somite compartment, whereas cells from the posterior epiblast populated the lateral somite compartment. Pourquie also described additional experiments in which *Hoxb9-GFP* and *Hoxb4-RFP* were successively electroporated into foci to show that the polyclone of b4 expressing cells is anterior to those expressing b9. Finally, the quantity of *Hox* gene product (e.g. expressed as expressed into two promoters, HSV-TK or CAGGS) had no effect on cell fate.

Francois Spitz (University of Geneva, Switzerland) gave insights into the regulation of posterior *HoxD* genes of mouse that came through the study of a 3 Mb inversion engineered to have one break between *d10* and *d11* genes. Inversion homozygotes were viable, but showed severe defects in vertebral column development. *Hox* genes have also been recruited to give positional cues in subsequent processes such as limb or gut morphogenesis, hair growth, hematopoesis, etc. Long distance regulation of *HoxD* gene expression in digits is under the control of a Global Control Region (GCR), that is located well outside the *Hox* complex, whereas *Hox* expression for setting up the embryonic A-P axis is subject to regulation from within the complex. They also engineered a 7cm inversion that breaks the *HoxD* cluster into two pieces. This split results in the partition of expression domains in limbs, gut and genitalia between the two sub-clusters, indicating that the GCRs are localized to either side of the cluster. Wendy Biekmore (MRC Human Genetics Unit) described two-colour 3D-FISH studies which revealed *Hox* gene reorganization during retinoic acid-induced mouse ES cell differentiation. At day 0, the *Hoxb1l* locus (b1), stained in red, was located within the chromosome 11 territory that was defined by chromosome painting in green. At day 4, b1 was extruded outside the chromosome territory and by day 10 it was drawn back into the chromosome 11 territory. In contrast *Hoxb9*, only 300 kb away from b1, was unextruded at day 4. The sequential extrusion of *Hoxb* genes outside of the chromosome territory reflected temporal colinearity of expression during mouse embryonic development. Similar decondensation and relocalization was seen for *Hoxd1* on chromosome 2, suggesting that the higher-order chromatin remodelling mechanisms that regulate colinear expression are conserved between the different *Hox* clusters. However, the *b1* and *d1* genes did not show evidence for colocalization. Additional regulatory elements (Global Control Region, GCR) that regulate *S* *Hoxd* gene expression during limb development remained throughout just within the chromosome 2 territory in an apparently constitutively decondensed chromatin structure.

C. S. Shashikanth (Pennsylvania State University, USA) described *lacZ* reporter gene studies in transgenic mice to test mutations in a 200-bp enhancer that drives mouse *Hoxc8* expression in the neural tube and mesoderm of the posterior region of the developing embryo. These studies identified nine elements for six different kinds of transcription factors. *Hoxc8* enhancers from teleost, whale, coelacanth and chicken also could drive reporter gene expression in mouse embryos, but that the anterior boundary of reporter gene expression was affected. The goal of such studies is to identify the transcription factors responsible for setting up the anterior boundary of reporter gene expression. B. De Kumar (CCMB) used a GFP reporter to demonstrate that conserved non-coding sequences located in a 7 kb region upstream of the vertebrate *HoxD* complex act to repress GFP expression in various mammalian cell lines. These results indicate that the conserved sequences act as negative regulatory elements. He also generated 12 stable transgenic ES lines and
found that GFP expression follows dynamic spatial and temporal patterns in them wherein GFP and Oct4 expression appears to become mutually exclusive. He also presented evidence suggesting that transcripts are derived from these conserved sequences, which suggests the involvement of RNA-mediated mechanisms. De Kumar presented preliminary expression pattern of these transcripts in embryoid bodies. Takashi Kondo (RIKEN, Japan) investigated higher order structure formation at the Hoxb9 promoter via transfection assays in mouse cell culture, footprinting and gel shift assays of nuclear extracts. He found that the jcp1 and jcp2 proteins bind to the 289 bp b9 promoter, but not to smaller segments of 231 or 211 bp. Thus jcp1 is a candidate higher order structure protein. siRNA-induced down regulation of jcp1 caused b9 promoter activity to increase by >3x.

**Inter cellular signalling by homeodomain proteins**

The role of homeoproteins in non-autonomous signalling was described by Alain Prochiantz (CNRS). Homeoproteins, such as mouse En-3, are packaged in the nucleus into spingolipid vesicles that are secreted out of the cell and can be internalized by other cells. The internalization and secretion signals make use of conserved domains. Prochiantz showed that an external gradient of En-2 strongly repelled growth cones of Xenopus axons from the temporal retina and, conversely, attracted axons from the nasal retina. Thus En2 appears to influence retinotectal targeting. This reponse depended on local protein synthesis and correlated with the activation of transcription initiation factors eIF4E and eIF4E-BP1. Likewise, neuronal transfer of Otx2 accelerated the postnatal maturation of parvalbumine-positive GABAergic interneurons in the neocortex in the ‘critical period’. Thus En2 and Otx-2 play roles in neuronal plasticity.

**Invertebrates**

*Nasonia vitripennis*, a parasitic wasp (hymenopteran), has a long germ band mode of development like *Drosophila*, but one that has been independently evolved. *Drosophila* embryo patterning is regulated by a gradient of bicoid protein. But the bicoid protein is a novelty that evolved after the evolutionary separation of the two insects. Claude Desplan (New York University) reported that Nasonia uses a similar gradient of the orthodenticle (otd) protein to regulate its embryonic patterning. Maternal otd mRNA is found at both poles of the Nasonia egg and plays two roles to pattern the embryo: it differentially activates anterior genes (e.g. giant, hunchback) at the anterior of the egg in a manner reminiscent of bicoid in *Drosophila* and it also activates posterior genes (e.g. caudal) much earlier than in short germband insects, like the locust Schistocerca, where their expression is delayed to the growth zone. Interestingly, the bicoid binding sites in the 5' regulatory regions of the *Drosophila* gap genes are replaced in Nasonia by binding sites for Otd.

Development in the crustacean amphipod *Parhyale hawaiensis* takes ten days. Nipam Patel (University of Chicago) cloned all its Hox gene orthologues, analysed their expression patterns and found that their expression in the stem cells (teleoblasts) occurs in a temporally and spatially colinear pattern. The Hox genes are expressed early, often while a parasegment is defined by only a single row of cells. Methods to manipulate gene expression have only begun to be developed in this system. An award-winning poster by Anastasios Pavlopoulos (University of Cambridge, UK) described the efficient transformation of Parhyale with vectors based on the Minos transposable element. Transformation of Parhyale embryos was used to demonstrate that heat-shock induced misexpression of Ubx affected the morphology of maxillipeds, which are ordinarily the feeding appendages, into locomotory appendages which are normally developed on only the more posterior trunk segments.

The centipede *Strigmania maritima* lays eggs of about 1 mm in diameter and during embryogenesis produces about 40 segments. Michael Akam (University of Cambridge, UK) reported that the genes *enlarged*, *delta* and *oddskipped* are expressed in a dynamic pattern of concentric rings reflecting travelling waves of gene activity, akin to the segmentation oscillator that underlies vertebrate somatogenesis. Subsequently, expression of Notch shows up with the intercalation of intercalary stripes. The expression patterns of the Hox genes *Antp*, *Ubx*, *abd-A* and *Abd-B*, suggests that they are regulated by interactions with the segmentation oscillator. Both the sense and anti-sense strands of Ubx are expressed in a mutually exclusive complementary pattern, suggesting a mechanism of promoter competition and that the control of Hox transcript accumulation may also involve antisense mechanisms.

Fittingly, a final session of three talks by Gius Morata (UAM, Spain), Ian Duncan and K. VijayRaghavan recounted more personal views of Ed and Pam Lewis and their contributions to the genetic, developmental and molecular studies on Hox genes, in particular, and to fostering camaraderie among scientists and nature lovers, in general.

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