Molecular Dissection of Hox Gene Induction and Maintenance in the Hindbrain

Minireview

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"Preformation is represented by DNA, not by . . . a tiny adult in every sperm . . ."
—Antonio García-Bellido (1998)

"The key to understanding how genomic regulatory networks . . . work lies in experimental analysis of cis-regulatory systems at all levels of the regulatory network."
—Maria Arnone and Eric Davidson (1997)

The analysis of developmental control mechanisms has for many years been dominated by *Entwicklungsmechanik* ("experimental embryology"), a discipline whose principal premise is to uncover biological constraints by studying the behavior of embryonic cells when placed in conditions other than their normal environment. The best experiments designed along these principles can disclose epigenetic signals and responses, such as induction, which govern changes in the direction of cell differentiation. It has been known since the 1920s that signals from a special region, the "organizer," can divert the fate of prospective skin to nervous system, and that the induced nervous system is perfectly patterned along its head-to-tail (anterior–posterior) axis. Three models were proposed to account for this. The first, advanced by Mangold (1933), suggested that distinct organizer activities are responsible for inducing the head, trunk, and tail of the embryo. In support for this, organizers from donor embryos older than gastrula stage induce a nervous system lacking anterior brain regions (Knezevic et al., 1998, and references therein). A second model (Waddington and Needham, 1936) proposes that initial signals from the organizer ("evocation") produce a nervous system without any regional character and that its pattern is conferred by later signals ("individuation"). Indeed, it is possible to generate a neural plate that expresses general neural markers, but no region-specific markers (Streit et al., 1997). Finally, Nieuwkoop and Nigtevecht (1954) suggested that the nervous system generated by initial neuralizing signals ("activation") is always anterior in character (forebrain), and that subsequent signals can alter ("transform") the fate of some of these cells, generating posterior nervous system. Consistent with this model, Cox and Hemmati-Brivanlou (1995) showed that a tail portion of the amphibian embryo can cause a head portion (mainly prospective forebrain) to express intermediate (hindbrain) neural markers. Although there is some supporting evidence for each model, these three views are apparently incompatible.

How many different signals are involved? At one extreme, Mangold's model could be interpreted to mean that there are as many distinct signals as there are regions in the nervous system. At the other, Nieuwkoop's model is consistent with just one, or very few, graded signals, which act quantitatively to impart progressively more posterior character to the nervous system as development proceeds. But embryos seem to be more resourceful than the investigators who study them, and mechanisms other than those implied by these three models can at least refine anteroposterior pattern. For example, the prechordal mesendoderm emits signals that can anteriorize the hindbrain (Dale et al., 1997; Foley et al., 1997; Pera and Kessel, 1997), and interactions between adjacent regions within the neural tube can regulate the expression of regional markers (e.g., Itasaki et al., 1991; Martinez et al., 1991). Despite this richness of ideas and experimental efforts, it seems remarkable that we still don't know how many signals are required to pattern the nervous system, from where these signals emanate, or how they produce complex patterns of expression of many different markers along the axis.

Some of the targets of these signals within the hindbrain and trunk have been discovered. The *Hox* genes are organized in clusters, such that their arrangement along the chromosome (in the 3′-to-5′ direction) reflects both their most anterior border of expression (3′ being more anterior) and the onset of their expression (3′ being earlier). We also know that these *Hox* genes can be regulated by retinoids, which control their expression in a time- and concentration-dependent way: higher doses or longer treatments induce progressively more posterior (5′) genes in the cluster (reviewed by Marshall et al., 1996). This finding, and the discovery that FGF can mimic tail/trunk mesoderm in inducing more posterior nervous system from prospective forebrain (Cox and Hemmati-Brivanlou, 1995; Muhr et al., 1997) appear to support Nieuwkoop's model.

A recent paper (Gould et al., 1998) provides a novel approach to the study of neural patterning, combining the ideas of *Entwicklungsmechanik* with a molecular dissection of the enhancer elements regulating the expression of one of the *Hox* genes, *Hoxb4*, in the hindbrain. The authors use the finding that two separate elements direct expression of *Hoxb4*. An early neural enhancer (early NE) becomes active at 8.25 days and accounts for the fuzzy anterior boundary of endogenous *Hoxb4* expression that appears before rhombomeres become distinct (Figure 1). By 9.5 days, when a second element (late NE) is activated, the anterior border of endogenous *Hoxb4* expression becomes fixed at its normal boundary, between rhombomeres 6 and 7 (r6/r7). Gould et al. (1998) use transgenic mouse lines expressing a reporter gene driven by either the early or the late NE to investigate the signals that control *Hoxb4* expression.

Do the signals that establish the early and late boundaries of expression reside within the neural tube itself, or do they emanate from neighboring structures? Surprisingly, Gould et al. (1998) obtain a different answer for each enhancer. In hindbrain explants cultured alone, expression driven by the late NE develops even if the explants are dissected from young (8.25 day) embryos. Therefore, signals that drive the late NE are autonomous
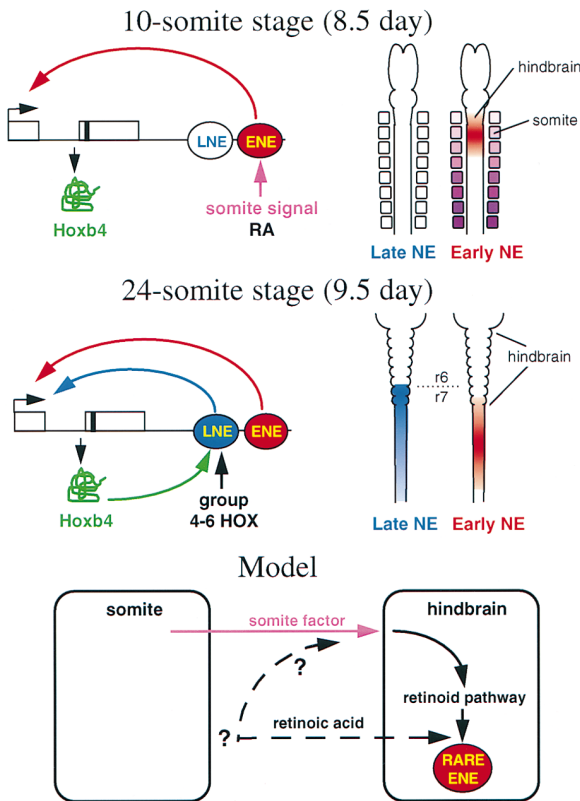
Figure 1. A Model for the Regulation of *Hoxb4* Expression in the Hindbrain

At an early stage, before hindbrain subdivisions (rhombomeres) become visible, an early neural enhancer (ENE; red) directs expression of *Hoxb4* in the hindbrain, without sharp boundaries. The ENE is activated by graded signals from the neighboring somites (pink/purple). About one day later, a second neural enhancer (LNE; blue) is activated by Hoxb4 protein itself (green) and by the products of *Hox* genes in the paralogous groups 4–6 (Gould et al., 1997). The boundary of expression driven by the LNE becomes fixed at the border between rhombomeres 6 and 7 (r6/r7). By this time, expression driven by the ENE has receded more posteriorly. A model for the control of expression of the ENE is presented, which requires the retinoid pathway, and which activates a retinoic acid response element (RARE) contained within the ENE. Exogenously added retinoic acid (which may also be produced by somites) mimics the somite-derived signal. Modified from Gould et al. (1998), with kind permission of Dr. R. Krumlauf.

to the neural tube or have already been received by the 10-somite stage and only require maturation. Indeed, it had already been shown that Hoxb4 protein itself activates this enhancer, providing positive feedback regulation (Gould et al., 1997). By contrast, strong and stable expression from the early NE is seen only when explants are cut from 24-somite stage embryos. Therefore, some character of the hindbrain must change, under the influence of neighboring structures, at some time before the 24-somite stage.

From where do these signals emanate? The early NE does drive expression in long-term culture of hindbrain explants cut from embryos as young as the 6–7 somite stage, provided that neighboring somite tissue is included, suggesting that somite-derived signals can activate this enhancer (see also Itasaki et al., 1996). Can these signals specify regional identity? The early NE can be induced by somites in more anterior hindbrain, which does not normally express *Hoxb4*, showing that the somite signal is "instructive." However, more posterior somites can also activate the early NE in its normal territory, and they are even more potent than somites normally adjacent to this region. These experiments suggest that somites from different levels of the axis do not emit distinct instructive signals, but rather that there is a graded activity within the somite mesoderm (increasing towards the tail of the embryo), which is required for expression from the early NE enhancer (Figure 1). These properties are reminiscent of the "transforming principle" suggested by Nieuwkoop.

What is the molecular nature of the somite-derived signals? Retinoids can posteriorize the neural tube by ectopic activation of *Hox* genes (see above and Marshall et al., 1996). Gould et al. (1998) provide evidence implicating retinoid synthesis in the somite-derived signal or the responses to it (Figure 1). First, retinoic acid can induce the early NE ectopically in vivo when applied to a more anterior region of the hindbrain, and second, disulphiram (an inhibitor of retinoid synthesis) abolishes the induction of the early NE by somites in culture. Retinoids can also act directly on the neural tube: the early NE contains a retinoic acid response element (RARE); point mutations in this element abolish all sites of expression, and replacement of this RARE by that of a more anteriorly expressed *Hox* gene (*Hoxb1*) shifts the expression boundary anteriorly. Using an heroic approach, they show that retinoic acid signaling is required in vivo for correct early expression of *Hoxb4*: early electroporation of a dominant-negative retinoid receptor into the neural tube of intact chick embryos abolishes *Hoxb4* expression, but late electroporation (at a time when the late NE should be active) does not. These experiments clearly implicate retinoid signaling as necessary for the induction, but not for maintenance, of *Hoxb4* expression in the neural tube both in vivo and in vitro. The authors argue that the somite signal may not itself be retinoic acid, because filters that should allow retinoic acid to pass through diminish induction of the early NE by somites in culture, opening the possibility that the signal is a protein that activates the retinoid pathway within the responding neural tube. However, these experiments still leave open the possibility that the somite signal may be a retinoid, if this requires a protein chaperone secreted by the somite, or if the retinoid must be released very close to the neural tube by cell processes that cannot pass through the filter.

What do we learn from these experiments? At first glance, they do appear to provide considerable direct support for Nieuwkoop's "activation/transformation" model of nervous system patterning. Somites produce a signal, increasing in strength toward the tail of the embryo, and which is required for expression of *Hoxb4* driven by the early NE both in its normal domain and in more anterior regions of the hindbrain. But this paper also tells us that there is much more complexity in the system of signals and responses that pattern the nervous system than the three classical models lead us to expect. It provides a clear experimental demonstration, using a combination of transgenic mouse lines with experimental embryology in chick and mouse embryos,

that separate mechanisms exist to induce and maintain the expression of one specific gene that specifies positional address within the hindbrain. However, many of the important questions remain. For example, do the explants that do not express *Hoxb4* have a more anterior character, none at all, or do they encode conflicting positional information? What mechanisms pattern the regions of the brain anterior to the hindbrain, where *Hox* genes are not expressed? If signals are graded, what mechanisms generate the sharp boundaries between rhombomeres and adjacent domains of gene expression? There are only 13 paralogous *Hox* gene groups, but many more functionally distinct regions within the length of the nervous system; where is the remaining information encoded, and what are the signals for this? As this paper demonstrates, a combination of classical experimental embryology with the dissection of enhancer elements represents a promising new avenue that may help finally to bridge the 300-year-old gap between genetics ("preformation") and epigenesis.

Selected Reading

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