CHAPTER 2

Timed interactions between the Hox expressing non-organizer mesoderm and the Spemann organizer generate positional information during vertebrate gastrulation

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Abstract
We report a novel developmental mechanism. Anterior-posterior positional information for the vertebrate trunk is generated by sequential interactions between a timer in the early non-organizer mesoderm and the organizer. The timer is characterised by temporally colinear activation of a series of \textit{Hox} genes in the early ventral and lateral mesoderm (i.e. the non-organizer mesoderm) of the Xenopus gastrula. This early \textit{Hox} gene expression is transient, unless it is stabilised by signals from the Spemann organizer. The non-organizer mesoderm and the Spemann organizer undergo timed interactions during gastrulation which lead to the formation of an anterior-posterior axis and stable \textit{Hox} gene expression. When separated from each other, neither non-organizer mesoderm nor the Spemann organizer are able to induce anterior-posterior pattern formation of the trunk. We present a model describing that convergence and extension continually bring new cells from the non-organizer mesoderm within the range of organizer signals and thereby create patterned axial structures. In doing so the age of the non-organizer mesoderm, but not the age of the organizer, defines positional values along the anterior-posterior axis. We postulate that the temporal information from the non-organizer mesoderm is linked to mesodermal \textit{Hox} expression.

Introduction
Anterior-posterior (AP) positional information in vertebrate embryos is generated during gastrulation. In zebrafish positional values of forebrain (and possibly midbrain) are specified at early gastrulation (Grinblat et al., 1998), whereas more posterior values are committed at the end of gastrulation (Woo and Fraser, 1998). In amphiabia only two different AP positional values (head and trunk) are differentiated in the Spemann organizer early in gastrulation (Spemann, 1931; Zoltewicz and Gerhart, 1997). Later on, at the end of gastrulation, different positional values can be discriminated in trunk neurectoderm and mesoderm (Mangold, 1933; Sahana and Grainger, 1992). In chicken, AP neural specification appears during gastrulation (Darnell et al., 1999; Muhr et al., 1999; Storey et al., 1992). In mouse, as in amphibians, only anterior identities are defined at the beginning of gastrulation (for review see (Beddington and Robertson, 1998)).

Nieuwkoop introduced a model for the induction and AP patterning of amphibian neurectoderm (Nieuwkoop, 1952), which is now generally accepted for vertebrates. After “activation”, which specifies all presumptive neurectoderm to an anterior neural fate, “transformation” leads to graded posteriorisation. Activation is obtained by antagonists of BMP and Wnt signals in the ectoderm. Signals involved such as Noggin, Chordin, Dickkopf-1 or Cerberus are generated in the organizer (for review see (Harland, 2000; Nieuhrs, 1999; Weinstein and Hemmati-Brivanlou, 1999; Wilson and Edlund, 2001). Transformation occurs due to posteriorising signals originating in non-organizer regions of the embryo (Bang et al., 1997; Bang et al., 1999; Gaunt et al., 1999; Gould et al., 1998; Itasaki et al., 1996; Kolm and Sive, 1997; Muhr et al., 1997; Muhr et al., 1999; Woo and Fraser, 1997). Gradients of different morphogens including FGF (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Muhr et al., 1997; Streit et al., 2000), retinoic acid (Durston et al., 1989; Ruiz I Altaba and Jessell, 1991; Sharpe, 1991), XWnt-3A (McGrew et al., 1997; Takada et al., 1994), and XWnt-8 (Erter et al., 2001; Kiecker and Nieuhrs, 2001), have been postulated to transform the anterior neurectoderm in a planar way (i.e. from posterior to anterior). In addition there is
evidence for the involvement of vertical short range signals (not from posterior to anterior, but from mesoderm to overlying ectoderm) (Chen et al., 2000; Nieuwkoop, 1952; Nieuwkoop and Koster, 1995; Poznanski and Keller, 1997).

One group of transcriptional factors that is important for trunk patterning in vertebrates is the *Hox* family of homeotic selector genes, which specify more posterior AP positional values and are clustered in functional units. Their 3' to 5' order in a *Hox* cluster matches the A to P sequence and time sequence in which they are expressed in neurectoderm and dorsolateral mesoderm along the body axis (“spatial colinearity” (Duboule and Dolle, 1989; Graham et al., 1989); “temporal colinearity” (Deschamps et al., 1999; Duboule and Morata, 1994; Gaunt and Strachan, 1996; Izpisua-Belmonte et al., 1991; Leroy and De Robertis, 1992)). Connections between temporal colinearity and spatial colinearity leading to AP patterning have been suggested (Duboule and Morata, 1994; Gaunt, 2000), but remain unclear.

Here we describe the temporally colinear activation of a series of *Hox* genes in non-organizer mesoderm of Xenopus laevis during gastrulation. This early *Hox* expression is transient, unless it is stabilised by signals from the Spemann organizer. Neither non-organizer mesoderm nor the Spemann organizer are able to induce AP pattern formation, when separated from each other. Only their cooperation results in AP patterning.

We propose a model which is based on timed interactions of the non-organizer mesoderm (characterised by the temporally colinear expression of a series of *Hox* genes) with the Spemann organizer. These interactions depend on convergence and extension. Different portions of non-organizer mesoderm interact with the Spemann organizer at different times. In doing so their age encodes positional values along the AP axis in both mesoderm and ectoderm. We postulate that this positional information coming from the non-organizer mesoderm is connected to early mesodermal *Hox* expression, hence the temporally colinear *Hox* sequence is translated into a spatial pattern.

**Results**

A temporally colinear sequence of *Hox* expression in the non-organizer mesoderm during gastrulation

We used PCR and an improved whole mount in situ hybridisation method to detect the initial expression of Xenopus *Hox* genes. Nine *Hox* genes examined were expressed in a temporally colinear sequence in non-organizer mesoderm, starting early in gastrulation (*Hoxd-1, Hoxb-4, Hoxc-6, Hoxa-7, Hoxb-9* are shown in Fig. 1A, B; *Hoxa-1, Hoxb-1, Hoxb-7, Hoxd-13* are not shown). The initial expression was localised in the non-organizer mesoderm and excluded from the Spemann organizer. Later expression spread to the presumptive neurectoderm (shown for *Hoxd-1*, Fig. 1C), giving increased expression on both sides of the organizer gap. Ectodermal expression was generally neurectodermal, with only *Hoxd-1* transiently showing non-neural ectodermal expression (not shown). Ventral expression of each *Hox* gene then faded out, leaving two strong dorsolateral patches, which later spread anteriorly (compare Fig. 6A). These findings show that gastrula non-organizer mesoderm contains a temporally colinear activated sequence of *Hox* genes indicating the presence of a timed signalling cascade.

Separation of the non-organizer mesodermal cascade and organizer functions

This signalling cascade in the non-organizer mesoderm can be separated from organizer functions by ventralisation or deleted by dorsalisation.
Fig. 1  Spatial and temporal Hox expression during gastrulation. Results for Hoxd-1, Hoxb-4, Hoxc-6, Hoxa-7, and Hoxb-9 are shown. Hoxa-1, Hoxb-1 and Hoxb-7 also fit this sequence (not shown). Hoxd-13 expression did not begin before the end of gastrulation (stage 15, not shown).

(A) WISH for five Hox genes at five different stages. Vegetal views, dorsal up.

(B) Diagram showing the onset of the temporally colinear expression of five different Hox genes as analysed with WISH (red) and PCR (blue).

(C) Localisation of Hoxd-1 expression in mesoderm and ectoderm. Embryos cut into halves across the dorsolateral blastopore lip at stages 10.5 and 11. One half analysed with Xbra, the other with Hoxd-1. The Xbra expression domain is outlined. The initial Hoxd-1 expression is located within the mesoderm. At stage 11 it is expanded into the presumptive neurectoderm (arrowheads).

Fig. 2  Hox expression in ventralised and dorsalised embryos.

(A) Hoxc-6 in embryos ventralised with UV. WISH (vegetal views of stages 10.5 to 12.5, lateral view of stage 26). Similar results were obtained for Hoxd-1, Hoxb-4, Hoxa-7, and Hoxb-9 (not shown).

(B) Diagram showing the onset of Hox expression (detected with WISH) in ventralised (dark blue) and control embryos (light blue). In ventralised embryos the temporally colinear sequence is still present.

(C) Mesodermal Hox expression (WISH) in ventralised embryos. Embryos cut through dorsolateral blastopore lips in controls and the corresponding region in ventralised embryos. Hoxd-1 at stage 11, Hoxc-6 at stage 12, Hoxa-7 at stage 12.5. The white line indicates Brachet’s cleft, separating involuted mesoderm from overlying ectoderm. Neurectodermal Hox staining in control embryos (C,
(D) *Hox* expression is absent in embryos that were dorsalised with LiCl. Dorsalised embryos (LiCl) and controls (con) at stage 12.5 (vegetal views) and stage 26 (lateral views). Results of a WISH for *Hoxd-1*, *Hoxc-6* and *Hoxb-9*. Analysis of *Hoxb-4* and *Hoxa-7* showed similar results (not shown). Arrowheads indicate the anterior *Hox* expression boundary.

Zygotic UV irradiation hyperventralises Xenopus embryos by blocking dorsal Wnt pathway activation and organizer development (Larabell et al., 1997; Scharf and Gerhart, 1983). We examined the consequences of this on early *Hox* expression. In ventralised embryos the expression of *Hox* genes expanded to fill the entire ring of mesoderm in the marginal zone and the organizer gap disappeared (shown for *Hoxc-6*, Fig. 2A). These ventralised embryos had a correctly timed early mesodermal *Hox* expression sequence (Fig. 2B), but this was transient (indistinct or absent at later stages, compare example in Fig. 2A). Ectodermal *Hox* expression was absent and normal AP *Hox* expression zones failed to form (Fig. 2C, C'). We conclude that ventralisation results in blocking ectodermal *Hox* gene expression and AP patterning, whereas the non-organizer mesodermal *Hox* sequence remains intact.

Lithium chloride treatment of early Xenopus embryos causes hyperdorsalisation via Wnt pathway activation, inducing all mesoderm to Spemann organizer (Hedgepeth et al., 1997; Kao and Elinson, 1988). Dorsalisation prevented *Hox* gene expression during gastrulation. Also in later stages *Hox* gene expression was absent or weak and the AP pattern failed to develop (Fig. 2D). Dorsalisation essentially deletes the *Hox* sequence and blocks AP patterning.

Neither ventralised nor dorsalised embryos generated a trunk with an AP pattern. We grafted early gastrula organizer tissue into ventralised embryos (Fig. 3A-F). This not only restored well-developed embryos, but also the normal sequence of *Hox* expression zones (Fig. 3D-F, compare also Fig. 3L), suggesting the presence of an interaction between the Spemann organizer and the non-organizer mesoderm.

**Timed interactions between the non-organizer mesoderm and the Spemann organizer generate AP pattern information**

We analysed whether organizer tissue explanted at different gastrula stages (representing different AP portions of the organizer) induced different parts of the trunk as it has been suggested for head and trunk (Spemann, 1931; Zoltewicz and Gerhart, 1997). Surprisingly the restored trunk portions in ventralised embryos did not considerably differ (Fig. 3G-N). Likewise experiments with organizer transplants of different sizes (1/4 of an organizer to 1 organizer) did not result in different portions of restored AP pattern (not shown). Therefore we tested whether timed interactions between the non-organizer mesoderm and the Spemann organizer specify the AP pattern. We recombined ventralised embryos with organizer tissue in different timed protocols and analysed the restored axial pattern, using a series of AP markers, including *Hox* genes.

**Ageing the non-organizer mesoderm (Fig. 4A)**. We combined identically aged Spemann organizers (stage 10) with progressively older ventralised embryos containing exclusively non-organizer mesoderm. Early organizer transplantation induced an almost complete axis (Fig. 4A). Organizers transplanted progressively later induced tail, but gave progressively larger anterior deletions (Fig. 4A). We concluded that the AP positional value in the Xenopus trunk is defined by the developmental age of the non-organizer mesoderm, at which it interacts with the Spemann organizer.
Ageing the Spemann organizer (Fig. 4B). We combined progressively older organizer tissue with identically aged ventralised embryos. This was achieved by explanting organizer tissue at the beginning of gastrulation and cultivating it for different times. It has been shown before that these explants still develop and differentiate (Slack and Forman, 1980; Wilson and Keller, 1991). Organizer explants of different ages (0 to 6 hours after the beginning of gastrulation) were implanted into stage 10 ventralised embryos. Spemann organizers aged for different times generated similar portions of the AP axis. The AP pattern was not progressively deleted (Fig. 4B). An identical observation was made with organiser transplants taken directly from different developmental stages (compare Fig. 3G-N). After more than 6 hours cultivation (corresponding to the end of gastrulation) the Spemann organizer lost the ability to rescue axis formation (not shown). We conclude that different trunk AP levels are generated by timed interactions between the non-organizer mesoderm and Spemann organizer. The non-organizer mesoderm provides information required for AP patterning of the trunk. The Spemann organizer stabilizes this information, its age only determines whether the mechanism still functions.

Timed application of Noggin protein (Fig. 4C). The organizer function involves antagonism to BMP signalling (De Robertis and Sasai, 1996). BMP antagonism permits Hox expression in ectoderm (i.e. nascent neurectoderm) (Lamb and Harland, 1995; Smith et al., 1993).
Fig. 4 Timed interactions between the Spemann organizer and the non-organizer mesoderm (NOM).

(A) Ageing the non-organizer mesoderm (isolated in ventralised embryos). A ventralised embryo with no implant (UV), an untreated control embryo (con), and recombinations of organizer mesoderm from stage 10 (0h SO) with ventralised embryos of different ages after the beginning of gastrulation (0h, 2h, 4h, 6h NOM). Embryos are positioned with their head up and dorsal to the right. They were analysed with WISH using axial markers, including En-2 (midbrain-hindbrain border), Krox-20 (hindbrain), Hoxb-4 (posterior hindbrain), Hoxc-6 and Hoxa-7 (anterior spinal cord), Hoxd-13 (posterior spinal cord). Expression of Krox-20 (arrowed) and Hoxd-13 illustrate the results. Pictograms indicate restored part of axis (based on conclusion from all markers).

(B) Ageing the Spemann organizer. A ventralised embryo without implant (UV), an untreated control (con), and recombinations of stage 10 ventralised embryos (0h NOM) with organizer tissue (SO) aged for 0h, 2h, 4h, 6h after beginning of gastrulation. Embryos orientated and WISH analysed as in (A). Krox-20 expression (arrowheads) and Hoxd-13 illustrate the results. Pictograms indicate restored part of axis. The age of the organizer implant does not affect the restored axial values.

(C) Timed restoration of organizer functions by Noggin protein (nog) injection. Ventralised embryos were injected with Noggin protein into the blastocoel (schematic drawing) at different blastula and gastrula stages. Embryos were analysed as above. Left panel stained for En-2/Krox-20/Hoxc-6/Xbra, right panel for Krox-20/Hoxd-13. Embryos are orientated as in (A), arrows point to Krox-20 expression. Top, noninjected ventralised embryos (UV). Rows 2-5 show ventralised embryos injected with Noggin at the indicated stages. Bottom, control embryos (con).

Early treated embryos restore head (grey color in the corresponding pictograms) and anterior trunk (Krox-20 expression, blue colors in pictograms). Later treated embryos show progressively less head (grey) and more trunk (anterior trunk marked by Krox-20 and blue colors in pictograms, posterior trunk marked by Hox genes and Xbra, yellow and red colors in pictograms). Very late on, there is an extensive zone of Hoxd-13 expression (posterior trunk) and anterior trunk markers (eg. Krox-20) have reached the anterior end of the embryo.
It also dorsalis mesoderm (De Robertis and Sasai, 1996; Lemaire and Kodjabachian, 1996; Smith et al., 1993). Consequently, the ectopic expression of noggin in ventralised early Xenopus embryos can rescue axis formation (Smith et al., 1993).

We injected Noggin protein into the blastocoel of different blastula and gastrula stages. Timed application of Noggin protein should convert ectoderm and mesoderm to a dorsal state at defined times. This might capture particular AP identities, depending on the stage at which Noggin is applied.

Injecting Noggin protein into the blastocoel of a ventralised blastula (stage 9) fixed an anterior part of the AP axis. Stripes of the hindbrain marker Krox-20 were expressed in the middle of the embryo (Fig. 4C). The anterior marker otx-2 was expressed in an extensive domain in front of them (not shown). Progressively later Noggin protein injection (at stage 10, 10.5, 11.5) led to the rescue of progressively more posterior parts of the hindbrain and trunk and to the loss of anterior parts (see pictograms in Fig. 4C).

Fig. 5 Neurectodermal Hox expression requires signals from organizer mesoderm and non-organizer mesoderm.

(A) Wrap assay. Spemann organizer tissue (SO) and/or non-organizer mesoderm (NOM) are wrapped in two (ectodermal) animal caps (AC).

(B) Wrap assays (fixed around the end of gastrulation) were dissected and analysed for ectodermal Hoxd-1 expression using WISH. Tissue localisation is indicated in the corresponding schematic drawings. Hoxd-1 expression: blue stipples. Only combined Spemann organizer (SO) and non-organizer mesodermal tissue (NOM) induce Hoxd-1 expression (arrowheads) in ectodermal animal caps (AC).

(C-D) Ectodermal lineage tracing in Wraps containing non-organizer and organizer mesoderm. A Wrap after in situ hybridisation for Hoxd-1 (arrowheads in C) and the corresponding fluorescence staining. In the magnified sectors the arrowheads indicate that the tissue borders in the Wraps correspond to the borders between mesodermal implant and fluorescence labeled ectoderm. The main portions of Hoxd-1 staining are ectodermal.

These results are in accordance with the idea that progressively later influence of the Spemann organizer on non-organizer mesoderm leads to the formation of more posterior portions of the AP axis.

An alternative explanation is based on recent findings describing that a BMP-4 function is necessary for initial activation of Hox genes during gastrulation (Wacker et al., in press). The later Noggin is injected the later BMP-4 is inhibited. Consequently more genes of the Hox sequence are initiated resulting in an increased portion of the restored trunk. However
since the timed appearance of Hox genes is not sufficient to form an AP pattern (compare nontreated UV ventralised embryos), another Noggin induced function must be involved in a time dependent way.

**The role of Hox genes in the interaction between non-organizer mesoderm and Spemann organizer**

To test whether the establishment of stable Hox expression in neurectoderm requires both non-organizer mesoderm and organizer signals, we wrapped pieces of tissue from the Spemann organizer, non-organizer mesoderm, or both, in ectodermal animal caps (Fig. 5A). Neither organizer nor non-organizer mesoderm alone induced Hox expression in the surrounding ectodermal tissue. Hox expression occurred only in triple recombinates (organizer + non-organizer mesoderm + ectoderm) (Fig. 5B). To verify that Hox expression was ectodermal we used rhodamine dextrane labeled animal caps in a Wrap assay containing both organizer and non-organizer mesoderm. This experiment shows that the main part of the induced Hox expression was ectodermal (Fig. 5C,D). We conclude that neurectodermal Hox expression requires signals from both the Spemann organizer and from the non-organizer mesoderm.

We are currently analysing whether the Hox proteins themselves are involved in the signalling of positional information to the neurectoderm or whether mesodermal and neurectodermal Hox gene expression are regulated by the same signals.

**Discussion**

We have demonstrated the presence of a time mechanism in the non-organizer mesoderm during gastrulation, which is characterised by the appearance of a temporally colinear sequence of Hox genes. This sequence can be isolated by ventralisation. Its interaction with the Spemann organizer is crucial for the formation of an AP axial pattern and for the activation of Hox gene expression in the neurectoderm. We postulate that Hox genes are not only responding to AP patterning signals. Rather they are required for the AP patterning signals themselves.

**Role of the Spemann organizer**

Functions of the Spemann organizer include formation of dorsal mesoderm (prospective notochord and prechordal mesoderm), dorsalisation of non-organizer mesoderm (e.g. prospective somites), induction of neurectoderm (by antagonizing BMP and Wnt signals) and initiation of gastrulation movements (for review see (Harland and Gerhart, 1997)). The organizer does not directly create an AP pattern (except for the aforementioned separation between head and trunk organizer). In our experiments neither its size nor its age have a direct effect on the positional values of the surrounding tissue. However, its functions affect AP patterning in different indirect ways. First it is involved in restricting the initial Hox expression to the non-organizer mesoderm (Wacker et al., in press). Second it is the source for neural activation signals, which is a prerequisite for neural transformation and thus for AP patterning. Third it initiates morphogenetic movements, namely convergence and extension, which are postulated to be a crucial component of AP patterning (see our model below). And finally it sequentially stabilises positional identities from the non-organizer mesoderm as indicated in our transplantation experiments.

**Neural transformation**

Recent insights into the determination of regional differences within the trunk are based on the “activation and transformation model of neural patterning” (Nieuwkoop, 1952).
Organizer signals (BMP and Wnt antagonists) induce anterior neurectoderm (“activation”). Posteriorising signals then transform anterior neurectoderm into more posterior neurectoderm (“transformation”). As stated in the introduction gradients of different morphogens have been postulated to mediate transformation acting in a planar way. However evidence has been presented that vertical signalling is necessary for AP patterning as well (Chen et al., 2000; Nieuwkoop, 1952; Nieuwkoop and Koster, 1995; Poznanski and Keller, 1997). The need for vertical signalling in AP patterning has also been shown in chick (Muhr et al., 1999). Here we present evidence that these vertical transforming signals originate from the non-organizer mesoderm. This is in accordance with former observations describing a role of paraxial mesoderm (descending from the non-organizer mesoderm) in transformation (see introduction). Recent observations in zebrafish demonstrating that heterotopic transplantations of non-organizer mesoderm result in formation of posterior trunk portions (Agathon et al., 2003) point to the importance of non-organizer mesoderm, as do experiments demonstrating in Xenopus that the entire vegetal half (including all non-organizer mesoderm), but not the organizer is able to transform neural tissue (Fujii et al., 2002).

The proposed mechanisms of vertical and graded planar signalling could interact in different ways, thereby accounting for evidence for both vertical and planar signals during axial patterning. Planar gradients could create a diffuse or incomplete posteriorisation without detailed positional values. Vertical signals could then refine and focus the pattern. Experiments supporting this mechanism have been published for ectodermal Hoxb-1 expression (Poznanski and Keller, 1997). The opposite mechanism is also conceivable. Vertical signals could place landmarks which are then used to set up gradients (i.e. define positions for morphogen sources or sinks).

Some proposed morphogens however are also involved in dorsoventral patterning and could thereby act indirectly by affecting the size or characteristics of the Hox expressing non-organizer mesodermal domain (e.g. XWnt-8 (Hoppler and Moon, 1998), FGF and BMP-4 (Wacker et al, in press)). Some are known regulators of morphogenetic movements (e.g. the Wnt-pathway (Kühl et al., 2001), the FGF-pathway (Conlon and Smith, 1999; Griffin et al., 1995)) and could affect the AP pattern by changing the timing of organizer/non-organizer mesoderm interactions, which we have demonstrated to be crucial for AP pattern formation. Some of these pathways are also known regulators of Hox genes (retinoic acid (Godsave et al., 1998), Wnt-8 (Kiecker and Niehrs, 2001), FGF (Pownall et al., 1996)).

A temporally colinear sequence of Hox expression is generated in the nonorganiser mesoderm (compare to Fig. 1, Fig. 6A). The Hox expression in this sequence is intrinsically transient and by itself does not generate a spatial pattern (e.g. in ventralised embryos).
Fig. 6 The time space translator model.
(A) False color representation of expression of three Hox genes during gastrulation. WISH on sibling embryos for Hoxd-1 (purple), Hoxc-6 (green), Hoxb-9 (red). Digital images were analysed and selected areas labeled with respective false colour and combined in one image. Six gastrula stages (10.5, 11, 11.5, 12, 12.5, and 13) are shown in a lateral view, anterior up, and dorsal to the right. Anterior levels of the Hox expression at the end of gastrulation are arrowed.
(B) The time space translator model. Expression of new Hox genes (different colours) is initiated in non-organizer mesoderm (NOM) at different times. Non-organizer mesodermal tissue moves toward the Spemann organizer by convergence and then extends anteriorly (arrow). When mesoderm adjacent to the Spemann organizer involutes (IM), the current Hox code is transferred to overlying neurectoderm (NE). While the early Hox sequence in the non-organizer mesoderm (solid outlined black box) is running, new cells from this region are continuously moved into the range of Spemann organizer (dashed black box) and their Hox code is then stabilised by an organizer signal. Thus the temporal Hox sequence is converted into a spatial AP pattern by continuous morphogenetic movement and stabilisation of timed information by the organizer in both involuted mesoderm (IM) and overlying neurectoderm (NE).
(C) Dorsal views. In non-organizer mesodermal cells the Hox sequence is running (solid black outline). From this domain cells are continuously moved into influence of Spemann organizer (dashed black box) by convergence and extension (arrows). The AP pattern arises in an anterior to posterior, early to late sequence by a mechanism involving progressive dorsally directed movement of mesodermal Hox expressing cells and initiation of neurectodermal Hox zones by combined signals from the non-organizer mesoderm and the organizer.
(D) Schematic diagrams depicting locations of Spemann organizer, blastopore and initial Hox expression domain in Xenopus and orthologous structures in the zebrafish (Alexandre et al., 1996), the chick (Gaunt and Strachan, 1996) and the mouse (Deschamps et al., 1999) at the beginning of gastrulation. Zebrafish and Xenopus are shown in vegetal views, chick and mouse are shown in dorsal views defined by the range of organizer signals (Fig. 6B, C). Meanwhile, convergence and extension (Keller et al., 1985; Keller and Danilchik, 1988) continuously exchange the cell population in this domain. The AP pattern arises in an anterior to posterior, early to late sequence by a mechanism involving progressive dorsally directed movement of mesodermal Hox expressing cells and initiation of neurectodermal Hox zones by combined signals from the non-organizer mesoderm and the organizer.
A model for AP patterning

We propose a new model for AP patterning of the vertebrate trunk. Defined AP zones arise via sequential interactions between the non-organizer mesoderm and the Spemann organizer, which stabilises the current \textit{Hox} expression in a domain. The \textit{Hox} status/AP identity of each pattern zone is defined only by the age of the non-organiser mesoderm at which this interaction takes place. The age of the organizer is not important. Interaction between non-organizer mesoderm and the organizer translates the temporal sequence found in the non-organizer mesoderm into a stable spatial AP sequence, which is vertically transferred to the activated neur ectoderm. We call this model a time space translator.

Nature and universality of the mechanism

AP patterning appears as a result of neural transformation, resulting from vertical and from planar signals. The transformed axial domains are characterised by \textit{Hox} gene expression zones in a spatially colinear way. In all vertebrates examined, these \textit{Hox} genes are initially expressed at the opposite side of the blastopore, or equivalent (primitive streak in chick and mouse), from the organising centre (Fig. 6D) (Alexandre et al., 1996; Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1996). The initial expression of different \textit{Hox} genes always appears in the same region, which is called the “\textit{Hox} induction field” (Deschamps et al., 1999) or “opening zone” (Gaunt, 2000). However, since the \textit{Hox} genes are expressed at different times, and gastrulation movements continuously bring new cells into this domain, different \textit{Hox} genes are expected to be expressed in different subpopulations of cells (Deschamps et al., 1999). These \textit{Hox} expression domains then progress in an anterior direction along the AP axis until they reach their final AP position. In chicken and mouse this progression does not depend on cell migration (Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1994).

A role of lateral/paraxial mesoderm in transformation has been indicated in Xenopus and in zebrafish (Bang et al., 1997; Bang et al., 1999; Barnett et al., 1998; Erter et al., 2001; Kolm and Sive, 1997). This function can be mimicked by intermediate doses of activin (inducing non-organizer mesoderm (Green et al., 1997)) and depends on Wnt-8 (Bang et al., 1999; Erter et al., 2001). In Chick and mouse interactions of paraxial mesoderm and neural tissue result in transformation as well. They define the final borders of the \textit{Hox} expression domains (Gould et al., 1998; Grapin-Botton et al., 1997; Itasaki et al., 1996; Muhr et al., 1997). In doing this the \textit{Hox} gene expressing paraxial mesoderm is involved in the activation of the neural expression of an identical set of \textit{Hox} genes (Grapin-Botton et al., 1997). Our experiments indicate that the same is true for non-organizer mesodermal \textit{Hox} expression in Xenopus (shown for \textit{Hoxd-1} in figure 5).

Despite these similarities, there is variation in neural patterning among vertebrate species, particularly with regard to timing. The chick neural plate is already formed, when posteriorizing signals result in transformation (Muhr et al., 1999). In chick and mouse, paraxial mesoderm of a five to ten somite stage affects the positional value of neur ectoderm, when this is transplanted heterotopically (Grapin-Botton et al., 1997; Itasaki et al., 1996). In Xenopus, we already find a sensitive phase for transformation and associated ectodermal activation of at least some \textit{Hox} genes during gastrulation. The competence of amphibian ectodermal tissue to respond to transforming signals has been reported to end at midneurula stages (Nieuwkoop and Albers, 1990), long before the somites begin to form. In our experiments the ability of organizer/non-organizer mesoderm
interactions to establish axial patterning ends at the end of gastrulation. Therefore transformation appears to be completed earlier than in chick or mouse.

Gaunt has suggested models for AP patterning in chick and mouse (Gaunt et al., 1999; Gaunt, 2000). The foundation of his and our models is the formerly considered hypothesis that “the correct timing of activation of this gene family (i.e. Hox genes) is necessary in order to properly establish the various expression domains” (Duboule and Morata, 1994). Gaunt’s first model is based on a posteriorising morphogen gradient, which is not likely, because of the extensive movements of morphogen sources and responding tissues during gastrulation (compare Schohl and Fagotto, 2002). Gaunt’s second model (the timing model) is based on the “opening domain”, a restricted zone, where new Hox genes can be activated. A certain Hox pattern is defined as soon as cells leave this domain (Gaunt, 2000). In accordance with this second model we find such an opening domain, namely the non-organizer mesoderm. However, our experiments with UV ventralised embryos demonstrate that leaving the “opening domain” is not sufficient to make an AP pattern, since Hox gene expression under these conditions is only transient. The organizer transplantation experiments indicate that an additional signal for the stabilisation of an Hox expression domain is necessary. In Gaunt’s third model this stabilising signal is postulated to be an anterior to posterior spreading “wave of refractoriness” (Gaunt, 2000). In contrast we find that the stabilising signal is held steady, originating from the organizer during gastrulation (the organizer’s sphere of influence) and the responding cells continuously move into this stabilising zone.

Materials and methods

Handling and treating embryos

Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). In vitro fertilisation, embryo culture, operation techniques, protein and mRNA injection, and culture of recombined embryos and explants were carried out as previously described (Wacker et al., 2000; Winklbauer, 1990). Ventralisation with UV light (Scharf and Gerhart, 1983), dorsalisation with LiCl (Kao and Elinson, 1988), and the Wrap assay (Zoltewicz and Gerhart, 1997) were described previously.

Microsurgery was carried out using hair knives. Organiser tissue (or non-organizer mesoderm) was explanted and the epithelial layer removed. After keeping these explants for a few minutes in MBS, they were implanted into the marginal zone of host embryos. We used grafts and not “Einsteck” experiments, because we expected the position of the implant in the host to be important. For the Wrap assay the explants were placed between two animal caps, which had been cut immediately before to prevent curling. Wraps and transplanted embryos were cultivated in MBS for about 30 minutes and then transferred to 10% MBS. Embryos with grafts were fixed at stage 26 to 27. Wraps were fixed about 5 to 6 hours after preparing them, when control embryos reached stage 12.5 (i.e. late gastrulation). For the timed Noggin application: 200 nl recombinant mouse Noggin/Fc chimera protein (R&D systems) at 0.1 µg/µl was injected in the blastocoel of staged embryos. A similar approach has been successfully used for mesoderm induction (Cooke and Smith, 1989). Embryos were harvested at stage 26 to 27.

For fluorescence labeling stage 2 embryos were injected with 2 times 8 nl of lysine fixable tetramethylrhodamine dextran (10,000 MW; 5 ng/µl; Molecular probes) into the animal region.
**Templates for RNA synthesis**

We used the available Xenopus Hox probes, including orthologues of genes from all four mammalian Hox clusters. Time and place of their early expression depends principally on paralogue group number, rather than on differences among paralogues.

Antisense, DIG-labelled transcripts were prepared from the following plasmids: a 1312 bp *Hoxa-1* fragment (*Hoxa-1*); a 666 bp *Hoxb-1* fragment (*Hoxb-1*); *xHoxlab1* (*Hoxd-1*) (Sive and Cheng, 1991); a 708 bp fragment containing the complete *Hoxb-4* ORF (*Hoxb-4*); a 998 bp *Hoxc-6* fragment in *pGEMI* containing a part of the homeodomain and extending into the 3' UTR (*Hoxc-6*); *Xhox36.1* (*Hoxa-7*) (Condie and Harland, 1987); a 470 bp *Hoxb-9* fragment in *pGEM3* (*Hoxb-9*); EST: *dc40d10* (*Hoxd-13*); a 1400 bp *Krox-20* fragment (*Krox-20*) (Bradley et al., 1993); a 1500bp *Engrailed-2* cDNA (*En-2*) (Hemmati-Brivanlou et al., 1991); *pSP73Xbra* (*Xbra*) (Smith et al., 1991); *pNPG152* (*nrp-1*) (Richter et al., 1990).

**Detection of gene expression**

Whole mount in situ hybridisation (WISH) was performed as previously described (Harland, 1991), except that probe concentration was reduced to 40 ng/ml, hybridisation temperature raised to 65°C and antibody incubations done in 0.1 M Maleic acid, 0.15 M NaCl, 0.1% Tween-20, 1% blocking reagent (Roche), pH 7.5 with anti-Digoxigenin-AP, Fab fragments (Roche). Some embryos were cut with a razor blade and halves used for WISH with different probes. In other experiments embryos were cut after WISH.

RT-PCR was carried out in the exponential phase of amplification as previously described (Busse and Seguin, 1993). The following primers were used: *Hoxa-1*-up: tgcctggcaatcaatagtac; *Hoxa-1*-down: atgtggacctgtccctagcagc; *Hoxd-1*-up: agggaaacttggcacaactctcc; *Hoxd-1*-down: gtgcagtacatgggtgtcgc; *Hoxb-4*-up: cgctgctgaatcaaccgct; *Hoxb-4*-down: caggececcaaactgtgtgatc; *Hoxc-6*-up: cagagccagacgtggactatcatccagg; *Hoxc-6*-down: caaggtaactgtcacagtatggagatgatggc; *Hoxa-7*-up: cacggcgggggcttctct; *Hoxa-7*-down: gcgtcgggggtctggtcact; *Hoxb-7*-up: gctcgacgctgcctccttatcaat; *Hoxb-7*-down: tctcctgcttttcctggctgttag; *Hoxb-9*-up: tacactggctgttgccag; *Hoxb-9*-down: agegtgtaaccagttgctg

**Acknowledgments**

We thank H. Sive for *xHoxlab1*, R. Harland for *Xhox36.1*, L. Bradley for *Krox-20*, A. Hemmati-Brivanlou for *En-2*, J. Smith for *pSP73Xbra*, J. Graff for *tBR-64T*, and K. Richter for *pNPG152*. We thank Olivier Destrée and Rudi Winklbauer for critically reading the manuscript. S.A.W. was supported by Marie Curie individual fellowship #MCFI-2000-01750. A.J.D. acknowledges support from EU grants HPRN-CT-2000-00097 and QLG3-CT-2000-01625.

**References**


