Patterning of the embryo along the anterior-posterior axis: the role of the caudal genes

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SUMMARY

Patterning along the anterior-posterior axis takes place during gastrulation and early neurulation. Homeobox genes like Otx-2 and members of the Hox family have been implicated in this process. The caudal genes in Drosophila and C. elegans have been shown to determine posterior fates. In vertebrates, the caudal genes begin their expression during gastrulation and they take up a posterior position. By injecting sense and antisense RNA of the Xenopus caudal gene Xcad-2, we have studied a number of regulatory interactions among homeobox genes along the anterior-posterior axis. Initially, the Xcad-2 and Otx-2 genes are mutually repressed and, by late gastrulation, they mark the posterior- or anterior-most domains of the embryo, respectively. During late gastrulation and neurulation, Xcad-2 plays an additional regulatory function in relation to the Hox genes. Hox genes normally expressed anteriorly are repressed by Xcad-2 overexpression while those normally expressed posteriorly exhibit more anterior expression. The results show that the caudal genes are part of a posterior determining network which during early gastrulation functions in the subdivision of the embryo into anterior head and trunk domains. Later in gastrulation and neurulation these genes play a role in the patterning of the trunk region.

Key words: Xenopus, gastrulation, neurulation, Xcad gene, Otx-2, Hox, regulatory interaction

INTRODUCTION

Specification of axial positions along the embryonic axes involves the concerted action of multiple genes and it begins to be established during gastrulation. Embryonic manipulations and grafts performed in amphibia have shown that, the later the mesoderm invaginates during gastrulation, the more posterior character it exhibits (for review see Slack and Tannahill, 1992). Inhibition of gastrulation movements results in anterior-posterior axial truncations where the head region is most sensitive to the treatments (Gerhart et al., 1989). Explant experiments where anterior and posterior tissues were juxtaposed results in posterior development (Slack and Tannahill, 1992). These results and others have lead to the suggestion of ‘posterior dominance’ where the cells as they involute change from a posterior specification and acquire a more anterior specification (Slack and Tannahill, 1992). Molecular and mutational studies have identified prospective candidates for genes active in the specification of the anterior-posterior axis.

The vertebrate Hox genes, like their Drosophila cognates, the homeotic genes, have been implicated in the specification of axial positions along the anterior-posterior axis (McGinnis and Krumlauf, 1992; Krumlauf, 1994). Analysis of both loss-of-function and gain-of-function alleles of murine Hox genes support the proposed role of these genes in axial specification (Krumlauf, 1994; Macanochie et al., 1996; Kessel et al., 1990; Lufkin, 1992). One aspect of the regulation of Hox gene expression that has been studied in some detail is their responsiveness to retinoic acid (Macanochie et al., 1996; Conlon, 1995; Simeone et al., 1991, Papalopulu et al., 1991). It has been shown that anterior Hox boundaries of expression are sensitive to retinoic acid treatment (Kessel and Gruss, 1991; Kessel, 1992; Conlon and Rossant, 1992; Dekker et al., 1992). The correlation between the shift in the anterior boundaries of Hox expression and changes in identity of axial structures have led to the suggestion that the combination of Hox genes expressed in a specific region serves as an axial code, the Hox code (Kessel and Gruss, 1991; Kessel, 1992).

Along the anterior-posterior axis, the most rostral Hox expression is posterior to the midbrain-hindbrain boundary (Krumlauf, 1994), suggesting that the development of anterior head structures is under the regulation of other gene(s). Loss-of-function alleles of homeobox proteins outside the Hox clusters have resulted in the loss of anterior head regions. Among these genes is Otx-2, a homeobox gene expressed in the rostral brain regions and thought to define the anterior regions of the embryo (Bally-Cuif and Boncinelli, 1997).

Another family of homeobox genes outside the Hox clusters,
the caudal genes, has been suggested as part of the posterior determining region. In Drosophila and C. elegans, the caudal gene is localized to the posterior regions of the embryo (Mlodzik and Gehring, 1987; Hunter and Kenyon, 1996) and it regulates posterior gene expression (Rivera-Pomar et al., 1995; Schulz and Tautz, 1995; Waring and Kenyon, 1991). In vertebrates, a number of genes belonging to the caudal family have been isolated and characterized. All the genes studied exhibit a predominantly posterior localization during gastrulation and neurulation (Frumkin et al., 1993; Meyer and Gruss, 1993; Gamer and Wright, 1993; Northrop and Kimmelman, 1994; Marom et al., 1997). Recently, it has been suggested that the caudal genes play a role in the patterning of the anterior-posterior axis probably in part by regulating members of the Hox gene family (Subramanian et al., 1995; Pownall et al., 1996).

Here we show that the Xcad-2 gene, a Xenopus caudal gene, plays a role in the specification of anterior-posterior axial positions during gastrulation and neurulation. The phenotype of Xcad-2 overexpression or partial loss of function was determined and the molecular basis for the phenotypes observed was studied. Overexpression of Xcad-2 results in embryos lacking anterior head structures and short axes while partial loss of function gives rise to embryos with enlarged heads and longer trunks. During early- to mid-gastrulation Xcad-2 appears to play a role in the specification of the trunk organizer by directly or indirectly interacting with anterior-head-specific genes such as Otx-2. Later during development, Xcad-2 plays a regulatory role on the expression of anterior and posterior Hox genes. The possible role of the caudal genes as members of the posterior signal in the embryo is discussed.

MATERIALS AND METHODS

Preparation of probes and capped RNA

The full-length Xcad-2 cDNA clone C11 (Blumberg et al., 1991) was subcloned into pSP64T vector (Krieg and Melton, 1984) in both orientations in order to prepare capped sense (pSP64Xcad-2) and antisense (pSP64Xcad-2as) RNA. The dominant negative construct of Xcad-2 plasmid with the clone of XlHbox1 (Cho and De Robertis, 1990) cut with XbaI which cleaves within the homeo-domain and Acc1 which cuts downstream of the homeobox. The ends of the DNA were polished and the plasmid was circularized. To prepare RNA from all three Xcad-2 plasmids they were digested with XbaI and SP6 RNA polymerase was used for transcription with the CAP-Scribe kit (Boehringer).

Probes were prepared with the Ribo-Max kit (Promega) using the digoxigenin or fluorescein RNA-labeling mixes (Boehringer) and subsequently cleaned using the RNA Easy kit (Qiagen). The probes used were: Xcad-2 – clone #73 (Blumberg et al., 1991), cut with XbaI, transcribed with T7 polymerase; Otx-2 – plasmid pXOT301 (Lamb et al., 1993), cut with NorI, transcribed with T7 polymerase; Hoxd-1 – labial clone (Blumberg et al., 1991) cut with EcoRI, transcribed with T7 polymerase; Hoxb-1 – clone CX19/21 (Godsave et al., 1994) cut with EcoRI, transcribed with SP6 polymerase; Hoxb-3 – clone M71/12 (Godsave et al., 1994) cut with EcoRI, transcribed with SP6 polymerase; Hoxb-4 – clone E16 (Gont and De Robertis, personal communication) cut with BamHI, transcribed with T7 polymerase; Hoxc-6 – the clone of XIHbox1 (Cho and De Robertis, 1990) cut with HindIII, transcribed with T7 polymerase; Hoxb-9 – the clone of XIHbox6 (Cho et al., 1988) cut with EcoRI transcribed with T7 polymerase.

Capped sense RNA for the Otx-2, Hoxd-1 and Hoxb-4 genes was prepared by subcloning the full-length cDNA clones into the pSP35T plasmid (Amaya et al., 1991).

Preparation of embryos

Xenopus laevis frogs were purchased from Xenopus I (USA). Fertilizations and injection of embryos were performed as previously described (Fainsod et al., 1994). Embryos were staged according to Nieuwkoop and Faber (1975).

Whole-mount in situ hybridization

Whole-mount in situ hybridizations using single probes were performed as previously described using digoxigenin labeled RNA probes (Fainsod et al., 1994). For double whole-mount in situ hybridization, the embryos were hybridized with both probes at the same time using the standard conditions (Fainsod et al., 1994). Immunodetection was performed in two stages, first the embryos were moved into MABT (100 mM Maleic acid, 150 mM NaCl, 0.1% Tween-20, pH 7.5) with the addition of 2% Boehringer blocking reagent (Boehringer) and 20% heat-inactivated goat serum. In this same solution, an alkaline-phosphatase-conjugated first antibody was added. After overnight incubation and subsequent washes, staining was performed with magenta-phos (5-bromo-6-chloro-3-indolyl phosphate p-toluidine salt; Fluka). The alkaline phosphatase enzyme was inactivated in 100 mM glycine pH 2.2, 0.1% Tween-20 followed by refixation in 4% paraformaldehyde. For the detection of the second probe, the embryos were blocked again as before and the second antibody, also alkaline phosphatase conjugated, was incubated overnight. The second alkaline phosphatase reaction was performed with BCIP alone to obtain a turquoise color. The embryos were refixed and photographed.

RESULTS

Phenotypes of Xcad-2 ectopic overexpression, partial loss of function and dominant negative effects

The caudal genes in vertebrate embryos remain localized to the posterior end throughout gastrulation and early neurulation. This expression pattern raises the possibility that the caudal genes play a role in the specification of the anterior-posterior axis. In order to study the role of the Xcad-2 during embryogenesis, ectopic overexpression of this gene was achieved by injection of capped RNA. Xenopus embryos at the 4-cell stage were injected in all four blastomeres in the equatorial region. The phenotypes were scored at stage 33.

The phenotypes observed from Xcad-2 injection were dose dependent (Table 1). Relatively low amounts of Xcad-2 RNA (0.2-0.4 ng/embryo) resulted in embryos with enlarged head structures including forebrain and cement gland and longer trunks (Fig. 1A, B, E). Increasing the amount of Xcad-2 mRNA (0.8 ng/embryo) results in the opposite effect as evidenced by microcephalic embryos and reduced trunk length (not shown). At the highest amounts of sense RNA injected (1.6 ng/embryo), the embryos exhibit a loss of anterior head structures and severe shortening of the anterior-posterior axis (Fig. 1H).

To further characterize the role of Xcad-2 during embryogenesis, we also performed injections of antisense Xcad-2 RNA (Table 1). Injection of antisense RNA has been recently shown to be capable of partially reducing the levels of gene activity during gastrulation (Steinbeisser et al., 1995). Injec-
tions of low amounts of antisense RNA (0.2-0.4 ng/embryo) resulted in microcephalic embryos with shortened trunks (Fig. 1C). Further increase in the amount of antisense RNA injected (0.8-1.6 ng/embryo) resulted in the reversal of the phenotype such that the embryos exhibit protruding fore-brains and elongated trunks (Fig. 1F.I). The extreme reversal in the phenotypes observed with the sense and antisense RNA from elongated to shortened but in the opposite direction, suggest that indeed the phenotypes observed are due to changes in the levels of Xcad-2 expression. Lineage-tracing experiments using β-galactosidase mRNA included with the experimental RNA indicate that approximately the same number and population of cells are labeled, regardless of the amount and type of RNA injected (data not shown). These observations further strengthen the suggestion that the opposite phenotypes are a response to the amount of Xcad-2 gene product.

The murine caudal homologue, Cdx-1, has been mutated by homologous recombination (Subramanian et al., 1995). This mutation expresses a protein truncated at the beginning of helix-3 of the homeodomain which is unstable, as it could not be detected by immunological analysis. In an attempt to study the effect of the truncated protein on embryonic development, a similar truncated protein was designed for the Xenopus Xcad-2 gene (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996) and overexpression in Xenopus embryos (Blitz and Cho, 1995; Pannese et al., 1995). To better understand the head phenotype observed in Xcad-2 injections, changes in the Otx-2 pattern of expression were studied. Injection of Xcad-2 mRNA resulted in down-regulation of Otx-2 expression (Fig. 3B). This second stripe of expression joins with the rhombomere 3 expression suggesting either some loss of rhombomere 4 or a change in identity. Injection of the Xcad-2 dominant negative ΔH3, gave similar results (Fig. 2F). Xcad-2 overexpression causes the loss of head structures and Krox-20 activity. Partial loss of function results in the enlargement of anterior brain structures and malformations in the hindbrain as evidenced by Krox-20 expression.

### Interactions between Xcad-2 and Otx-2

The Otx-2 gene is one of the genes known to be required for head formation as evidenced from mutations in mice (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996) and overexpression in Xenopus embryos (Blitz and Cho, 1995; Pannese et al., 1995). To better understand the head phenotype observed in Xcad-2 injections, changes in the Otx-2 pattern of expression were studied. Injection of Xcad-2 mRNA resulted in down-regulation of Otx-2 expression (Fig. 3B). Injection of

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### Table 1. Distribution of phenotypes in embryos injected with the different Xcad-2 derived RNAs

<table>
<thead>
<tr>
<th>Type of injection</th>
<th>Amount injected (ng/embryo)</th>
<th>Phenotype (number of embryos)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enlarged head and long trunk</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduced head and short trunk</td>
</tr>
<tr>
<td>uninjected</td>
<td>–</td>
<td>58               0             0</td>
</tr>
<tr>
<td>sense Xcad-2</td>
<td>0.2</td>
<td>20               26            0</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>23               12            21</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>3                6             40</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>0                0             46</td>
</tr>
<tr>
<td>antisense Xcad-2</td>
<td>0.2</td>
<td>18               3             33</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>27               16            8</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>32               19            0</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>11               41            0</td>
</tr>
<tr>
<td>ΔH3</td>
<td>0.2</td>
<td>28               2             7</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>22               14            2</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>33               6             0</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>32               23            0</td>
</tr>
</tbody>
</table>

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### Table 2. The effect of Xcad-2 injection of the patterns of expression of Otx-2 and Hoxd-1

<table>
<thead>
<tr>
<th>Pattern of expression</th>
<th>Per cent embryos exhibiting the phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prolactin 1.6 ng/embryo</td>
</tr>
<tr>
<td>Otx-2</td>
<td></td>
</tr>
<tr>
<td>normal</td>
<td>100</td>
</tr>
<tr>
<td>reduced</td>
<td>0</td>
</tr>
<tr>
<td>erased</td>
<td>0</td>
</tr>
<tr>
<td>Hoxd-1</td>
<td></td>
</tr>
<tr>
<td>normal</td>
<td>100</td>
</tr>
<tr>
<td>reduced</td>
<td>0</td>
</tr>
<tr>
<td>erased</td>
<td>0</td>
</tr>
</tbody>
</table>
the amount injected in ng/embryo are antisense; dn, dominant negative) and the length of the trunk are shown. The type of RNA (s, sense; as, antisense; dn, dominant negative) and the amount injected in ng/embryo are marked.

up to 0.4 ng/embryo of Xcad-2 capped RNA resulted in 58% of the embryos with the normal Otx-2 pattern of expression, 33% with reduced expression and 8% where expression of this gene was absent (Table 2). Injection of Xcad-2 mRNA in amounts ≥0.8 ng/embryo resulted in a reduction in the number of embryos with normal Otx-2 patterns of expression to about one third. This dose-response and the observation that, even at the highest amounts of RNA injected, more than 30% of the embryos retain the normal pattern of expression raised the possibility that Otx-2 exhibits a threshold in its response to Xcad-2 levels (Table 2). To corroborate that the effects observed are indeed due to changes in the levels of Xcad-2, injections of Xcad-2 antisense RNA were performed. Partial loss of function of Xcad-2 results in the expansion of the Otx-2 pattern of expression (Fig. 3C). These results support the observation that Otx-2 is under the regulation of the caudal genes but as opposed to the phenotypes, the response of Otx-2 is monotonic.

In order to test whether the initial division of the embryo into anterior and posterior regions becomes translated at the molecular level into regulatory interactions between anterior- and posterior-specific genes, we tested whether Otx-2 also regulates the expression of Xcad-2. Injection of 0.8 ng/embryo of Otx-2 sense RNA in the marginal zone resulted in the downregulation of Xcad-2 expression (Fig. 3E). Further increase in Otx-2 RNA injected to 1.6 ng/embryo resulted in the complete elimination of the Xcad-2 signal (Fig. 3F). Together these results provide evidence that the anterior- or head-specific genes like Otx-2 and the posterior genes represented by Xcad-2 establish mutually exclusive regulatory interactions.

The role of Xcad-2 in the hindbrain and trunk regions

As the anterior-posterior axis is established and the embryo elongates, the regions of Xcad-2 and Otx-2 expression are separated to the ends of the embryo. To test whether the phenotypes observed with differing levels of Xcad-2 expression can be explained in part by interactions with the Hox genes, their patterns of expression were studied in injected embryos.

The Hoxd-1 gene is expressed in Xenopus embryos from mid-gastrulation as a ring around the blastopore whose dorsal aspect is stronger and wider than the ventral expression (Xhox.lab1; Blumberg et al., 1991; Sive and Cheng, 1991). Hoxd-1 expression exhibits an anterior boundary at about the middle of the early anterior-posterior axis and expression decreases towards the blastopore. Injection of Xcad-2 capped RNA resulted in the suppression of Hoxd-1 expression (Fig. 4A-D). This down-regulation was dose-dependent (Table 2) and spreads from posterior to anterior. Injection of 0.2 ng of Xcad-2 mRNA resulted in the suppression of expression in the region close to the blastopore (Fig. 4B). Higher amounts of injected RNA (0.4-0.8 ng/embryo) further restricted the Hoxd-1 expression to a narrow stripe almost dividing the embryo in half along the anterior-posterior axis (Fig. 4C). High amounts of Xcad-2 sense RNA (1.6 ng) eliminated the Hoxd-1 expression (Fig. 4D). These results suggest that the response to Xcad-2 is gradual and monotonic over the whole range of concentrations. A sibling control uninjected embryo is also shown (A). The effects on the formation of the head and the length of the trunk are shown. The type of RNA (s, sense; as, antisense; dn, dominant negative) and the amount injected in ng/embryo are marked.

Fig. 1. Phenotypes induced by Xcad-2 overexpression or partial loss of function. Xenopus embryos were injected at the 4-cell stage with Xcad-2 sense (B,E,H), antisense (C,F,I) or the ΔH3 dominant negative (D,G) capped RNA at different concentrations. A sibling control uninjected embryo is also shown (A). The effects on the formation of the head and the length of the trunk are shown. The type of RNA (s, sense; as, antisense; dn, dominant negative) and the amount injected in ng/embryo are marked.

Fig. 2. Changes in the pattern of Krox-20 expression as a result of Xcad-2 overexpression and partial loss of function. Embryos injected with sense (s), antisense (as) or dominant negative (ΔH3; dn) Xcad-2 were studied during neurulation to determine the effects on the Krox-20 pattern of expression. (A) Control uninjected embryo. Embryos injected with 0.2 ng (B) or 0.8 ng (C) of Xcad-2 mRNA exhibit elimination of Krox-20 expression. Injection of 0.2 ng (D) or 0.4 ng (E) of antisense Xcad-2 RNA shows the reorganization of the head region. (F) Embryo injected with 0.8 ng of dominant negative Xcad-2 RNA, which results in a similar phenotype as antisense RNA injections.
RNAs injected. *Hoxb-1* (Dekker et al., 1992) was tested in a similar manner and overexpression of *Xcad-2* resulted in the suppression of *Hoxb-1* expression in rhombomere 4 (not shown).

*Hoxb-3* (Dekker et al., 1992) at the stages studied is expressed as a narrow stripe in the hindbrain probably corresponding to rhombomere 5 and in the neural crest emanating from it (Fig. 5A). Injection of *Xcad-2* sense RNA elicited a dose-dependent suppression of *Hoxb-3* expression from the lowest amounts injected. Expression of *Hoxb-3* in the hindbrain was completely absent after injections of 0.4 ng *Xcad-2* mRNA (Fig. 5B); interestingly, expression of *Hoxb-3* in neural crest cells continued. Further increase to 0.8 ng of *Xcad-2* mRNA resulted in the elimination of *Hoxb-3* expression (not shown). Injection of low amounts of *Xcad-2* antisense RNA (0.2 ng) resulted in an increase in the levels of *Hoxb-4* transcripts and the four neural crest populations became restricted to a narrower region along the anterior-posterior axis. Injection of 0.8 ng of antisense RNA resulted in further increase in the level of *Hoxb-4* expression (Fig. 5F). At the highest amounts of *Xcad-2* antisense RNA injected (1.6 ng), expression of *Hoxb-4* became almost extinguished. In the case of *Hoxb-4*, the response to *Xcad-2* overexpression is dose-dependent and monotonic while partial loss of function of *Xcad-2* induces a reversing effect as a function of the amount of RNA injected.

Two *Hox* genes from regions more 5' in the clusters were...
studied: Hoxc-6 (XHbox1; Cho et al., 1988) and Hoxb-9 (XHbox6; Sharpe et al., 1987). In both cases, overexpression of Xcad-2 by mRNA injection resulted in the anterior shift of their rostral boundary of expression (Fig. 6). The position of the anterior boundary of expression was determined relative to the total length of the embryo (the head end was determined as 1). In both instances, shifts in the boundaries of expression could be observed from the lowest amounts of Xcad-2 sense RNA injected (0.2 ng/embryo; Fig. 6A,B). At high amounts of RNA (1.6 ng/embryo), the expression of the Hoxc-6 and Hoxb-9 can reach the anterior end of the embryo but these embryos exhibit a severely malformed anterior-posterior axis (Figs 1H, 6A,B). Hoxb-9 also exhibited an up-regulation in its level of expression (Fig. 6D). The effect of partial loss of function of Xcad-2 on the pattern of Hoxc-6 and Hoxb-9 expression was also determined by measuring the position of the anterior boundary. In the case of Hoxb-9, injection of antisense RNA resulted in the posterior shift of the anterior boundary of expression (Fig. 6B). This result suggests that the position of the Hoxb-9 anterior boundary of expression is dependent on Xcad-2. Injection of antisense Xcad-2 RNA had no effect on the position of the expression of Hoxc-6 (Fig. 6A), suggesting that the regulatory effect of Xcad-2 on Hoxc-6 might be indirect.

As both the caudal and the Hox genes are homeobox genes, it is possible that the latter also regulate the former. To test the possibility that the Hox genes regulate in part the expression of Xcad-2, capped sense RNA was prepared from the Hoxd-1 and the Hoxb-4 genes. This RNA (up to 1.6 ng/embryo) was injected into Xenopus embryos and the pattern of Xcad-2 expression was studied by in situ hybridization. In all injection experiments performed, no change in the Xcad-2 pattern of expression could be observed (not shown).

Regulatory interactions between Otx-2 and the Hox genes

As the anterior and posterior domains separate, the Hox genes are up-regulated in the gap between them. This observation suggests that the Hox genes may fulfill some of the regulatory functions previously performed by Xcad-2. To test this hypothesis, we examined the interaction among some of the Hox genes themselves and with Otx-2. The pattern of expression of Otx-2 was studied in embryos injected with either Hoxd-1 or Hoxb-4 mRNA. Overexpression of both genes resulted in dose-dependent effects on the expression of Otx-2 (Fig. 7A-C). At low amounts of injected sense RNA (0.2 ng/embryo), a slight down-regulation of Otx-2 expression can be observed (not shown). Higher amounts of injected RNA resulted in the reduction of the Otx-2-expressing region (Fig. 7B,C).

The effects of Otx-2 on Hox genes were tested using the Hoxd-1 probe. Injection of Otx-2 sense RNA resulted in the down-regulation of Hoxd-1 over the whole range of amounts of mRNA injected. At high amounts of RNA (1.6 ng/embryo), low expression of Hoxd-1 can be observed restricted to the region where the anterior boundary of expression should be localized (Fig. 7E).

The effect of Hoxb-4 overexpression on the Hoxd-1 pattern of expression was also studied. In embryos injected with low amounts of Hoxb-4 mRNA, expression of Hoxd-1 is almost absent (Fig. 7F). Increasing the amount of Hoxb-4 RNA injected results in a dose-dependent restoration of the Hoxd-1 expression (not shown). In embryos injected with high amounts of Hoxb-4 RNA (1.6 ng/embryo), the pattern of Hoxd-1 expression appears normal (not shown).

**The spatial relationship between the Otx-2, Hoxd-1 and Xcad-2 genes**

The regulatory interactions elucidated for Xcad-2 and its interactions with other anterior-posterior patterning genes were based mostly on overexpression induced by mRNA injection. To provide support for whether the suggested interactions occur in the developing Xenopus embryo, a comparative analysis of the spatial patterns of expression was performed by double whole-mount in situ hybridization. Transcripts of both genes are detectable by early gastrula stages, with Otx-2 having already migrated to the anterior regions while maintaining some expression on the dorsal lip of the blastopore (Fig. 8A). In the same embryos, Xcad-2 is lateral and ventral along the marginal zone but exhibits a dorsal gap of expression. By about stage 11-11.5, Otx-2 is no longer present in the dorsal lip region and the gap of Xcad-2 begins to close (Fig. 8B). At stage 12, Otx-2 marks the anterior (head) region while Xcad-2 covers the posterior region of the embryo (Fig. 8C). A gap between the regions of Xcad-2 and Otx-2 expression appears. In late gastrulation, the pattern remains but the gap between the ends widens (Fig. 8D).

The gap formed along the anterior-posterior axis between the Otx-2 and Xcad-2 regions of expression is reminiscent of the strong region of expression of Hoxd-1 (Fig. 4A). The weak Hoxd-1 expression appeared to map to the overlap with Xcad-2 while strong expression appeared to localize to the gap between Xcad-2 and Otx-2. To verify these impressions, we performed a double in situ hybridization. During mid-gastrulation, the anterior expression of Hoxd-1 clearly localized to the Xcad-2-free region (Fig. 8E). Whole-mount in situ hybridization with the Otx-2 and Hoxd-1 probes in parallel showed that, indeed, the latter almost completely fills the region between Otx-2 and Xcad-2. (Fig. 8F). A small gap remains between the Otx-2 and the Hoxd-1 regions of expression, which probably maps to the junction between the mid- and hindbrain.

**DISCUSSION**

**The vertebrate caudal genes as posterior determinants**

The vertebrate caudal genes from their onset of expression are restricted to the posterior region of the embryo. In Xenopus, as in other vertebrates, caudal expression initiates in the region where gastrulation is taking place. With the onset of neurulation, expression of these genes takes up a posterior position where the caudal transcripts will continue until the genes are down-regulated along the midline (Marom et al., 1997; Fainsod, Epstein, Pillemer, Gont, Blumberg, De Robertis, Yisraeli and Steinbeisser, unpublished data). As in Drosophila and C. elegans, the vertebrate caudal genes also appear to be part of the posterior determinants in the embryo. To help elucidate the role of these genes, we ectopically overexpressed Xcad-2 in frog embryos; embryos with anterior head trunca-
tions and reduced trunk length were obtained. A similar phenotype has been described for dorsal overexpression of the Xcad-3 gene (Ponnall et al., 1996). This phenotype is in agreement with the suggestion that the vertebrate caudal genes are part of the posterior determining region in the embryo. Overexpression of several FGF genes in the frog embryo also results in a similar phenotype where head structures are diminished and the body axis does not elongate as much (Ponnall et al., 1996; Thompson and Slack, 1992; Isaac et al., 1994). The similarity in the phenotypes obtained by both the FGF and the caudal genes suggests a relationship between these two gene families. It has been shown that disruption of the FGF signaling pathway preferentially eliminates Xcad-3 expression from the dorsal regions of the embryo (Northrop and Kimmelman, 1994; Northrop et al., 1995). In addition, overexpression of eFGF causes the up-regulation of Xcad-3 (Ponnall et al., 1996). These observations suggest that some of the phenotypes observed by FGF overexpression may be the result of changes in the pattern of expression of the caudal genes.

As part of the analysis of the Xcad-2 gene, we also injected antisense RNA. These embryos were macrocephalic, with elongated trunks. These embryonic malformations appear to be opposite to those observed after overexpression of Xcad-2, suggesting that the effects observed are specific for the caudal genes. Injection of sense or antisense RNA both led to dose-dependent phenotypes. Overexpression of Xcad-2 exhibited a non-monotonic response depending on the amount of RNA injected: at low levels, macrocephaly and longer trunks, while at high levels the embryos exhibited anterior head truncations and had shortened trunks. Antisense Xcad-2 also behaved in a non-monotonic fashion and exhibited similar phenotypes but in the reverse order. These observations raised the possibility that the phenotypes obtained are at least in part the result of compound interactions with other regulatory genes whose expression responds to the levels of caudal expression.

Treatment of Xenopus embryos with retinoic acid also produces phenotypes in which anterior head structures are missing and the trunk is also affected (Durston et al., 1989). Experimental manipulation of the retinoid signaling pathway by injecting modified retinoic acid receptors or binding proteins (Godsave et al., 1994; Blumberg et al., 1997), the COUP-TF1 orphan receptor (Schuh and Kimelman, 1995) and either the modified thyroid hormone receptor v-erbA or treatment with citral (Schuh et al., 1993) result also in similar phenotypes. These observations raise the possibility of a regulatory interaction between the caudal genes and the retinoid signaling pathway.

The head and trunk organizers

Patterning along the anterior-posterior axis becomes evident during gastrulation when a distinction between the anterior head and the posterior head/trunk regions takes place. Transplantation of early or late gastrula dorsal lip regions gives rise to secondary axes with head and brain structures or trunk and tail structures, respectively. These results led to the proposal of the existence of a head organizer responsible for head formation and a separate trunk organizer responsible for body axis formation (Spemann, 1938). One of the genes probably active in the head organizer is the Otx-2 gene (Bally-Cuif and Boncinelli, 1997). Otx-2 is normally expressed initially in the organizer region and subsequently becomes localized to the anterior head region (Blitz and Cho, 1995; Pannese et al., 1995). In mice, loss of Otx-2 activity results in the loss of anterior head structures (Acampora et al., 1995; Matsuoi et al., 1995; Ang et al., 1996). In our injection experiments, we could show that, during gastrulation, Otx-2, the anterior gene, and Xcad-2, the posterior gene, repress each other, when overexpressed. Moreover, in the case of Otx-2, partial loss of Xcad-2 function results in the expansion of its expression domain. To better define the extent of overlap between Xcad-2 and Otx-2 expression during gastrulation, we performed a double in situ hybridization study of their expression patterns. In only one place in the Xenopus embryo do the two expression domains abut each other; along the blastopore, in the dorsal region where Xcad-2 encroaches on the organizer, Otx-2 and Xcad-2-expression regions come in close proximity. As gastrulation proceeds both expression regions separate, while Otx-2 becomes restricted to anterior-most regions and Xcad-2 continues to be expressed around the blastopore. Study of these two genes therefore has allowed us to see the separation of the anterior and posterior regions and the gradual formation of the trunk region. The gap between the expression areas of Otx-2 and Xcad-2 suggests the existence of yet another signaling pathway that separates what appears to be representatives of the head and trunk organizer.

The interactions between the Xcad-2, Otx-2 and Hox can explain in part the dimorphism of the phenotypes obtained at different levels of Xcad-2 activity. Low levels of Xcad-2 overexpression would result in the down-regulation of anterior Hox genes. Under these conditions, however, due to the apparent threshold in the effect of Xcad-2 on Otx-2, the latter is not down-regulated; the reduction of Y′ Hox genes allows it to expand posteriorly thus enlarging the head domain. High levels of Xcad-2 overexpression already repress both the Hox and Otx-2 genes, thus resulting in the loss of anterior structures. Small reductions in the level of Xcad-2 by antisense injections apparently affect mainly the anterior Hox genes which are up-regulated. This up-regulation allows them to encroach on the head region, repress Otx-2 and reduce its domain of expression. High levels of antisense Xcad-2 release the early repression on Otx-2, which apparently can overcome the up-regulated Hox genes and lead to an enlarged head region.

Patterning of the trunk

Numerous results have suggested that the Hox genes play an important role in the patterning of the hindbrain and trunk regions (McGinnis and Krumlauf, 1992; Krumlauf, 1994; Kessel and Gruss, 1991; Kessel, 1992). The appearance of the gap between the Otx-2 and Xcad-2 regions of expression correlates in time with the earliest Hox expression as evidenced for Hoxd-1 (Fig. 4; Sive and Cheng, 1991). Interestingly, Hoxd-1 in Xenopus exhibits a bimodal pattern where the posterior expression, which overlaps with the caudal-positive regions, is weaker than the anterior-most expression, which would localize to the gap between Xcad-2 and Otx-2 at the same stage. These observations raise the possibility that, as the anterior and posterior embryonic domains separate, the Hox genes begin taking up their
positions along the hindbrain and trunk. Double in situ hybridization experiments using the Hoxd-1 and either the Otx-2 or Xcad-2 probes revealed that indeed the stronger expression of Hoxd-1 localizes to the region between the head and the posterior domains. Interestingly, a small gap remains between the Hoxd-1 and the Otx-2 expression suggesting the existence of yet another gene expressed in a ring-like fashion.

Overexpression of the Xcad-2 gene resulted in the downregulation of 3′ Hox genes, while injection of antisense RNA resulted in the increased expression of the same genes. High amounts of antisense RNA resulted in the repression of anterior Hox genes probably due to secondary interactions. This repression of 3′ Hox genes by Xcad-2, together with the normal pattern of Hoxd-1 expression (Sive and Cheng, 1991), raises the possibility that, although several of these genes have been shown to begin their transcription from the caudal end (Deschamps and Wijgerde, 1993), their up-regulation is achieved once caudal expression has retracted. These observations appear not to be true for all Hox genes; more posterior members of the family, Hoxc-6 and Hoxb-9, exhibit the opposite response and shift anteriorly with increased levels of Xcad-2. Further support for a role of the caudal genes in the regulation of Hox genes comes from experiments in mice, Xenopus and C. elegans. In mice mutant in the Cdx-1 gene, defects were described mainly in the cervical and thoracic vertebrae, correlating with changes in Hox patterns of expression (Subramanian et al., 1995). In Xenopus, injection of Xcad-3 mRNA can activate Hoxa-7 expression precociously or rescue its expression when FGF signaling has been inhibited (Pownall et al., 1996). In C. elegans, the caudal gene pal-1 has been suggested to be an activator of the Antenapedia-like homeotic selector gene mab-5 (Waring and Kenyon, 1991).

Expression of the Hox genes has also been shown to be under the regulation of retinoic acid (McGinnis and Krumlauf, 1992; Krumlauf, 1994). In a number of instances, it has been shown that retinoic acid treatment of mouse embryos results in changes in the Hox patterns of expression where the position along the complex determines the specific effect (Kessel, 1992; Conlon and Rossant, 1992). Depending on the time the treatment was administered, anterior (3′) Hox genes were rostrally induced while posterior (5′) genes were repressed. In Xenopus embryos, it was shown that 3′ Hox

![Fig. 6. The effect of Xcad-2 on more posterior (5′) Hox genes. The effect of the injection of Xcad-2 sense and antisense RNA on the Hoxc-6 (A) and Hoxb-9 (B) genes was studied by determining the position of their anterior boundaries of expression. The position of the anterior boundary of expression was determined relative to the total length of the embryo; the posterior end was determined as 0 length while the head end was determined as 1. Both RNAs were injected at four different concentrations, 200, 400, 800 and 1600 pg. Control uninjected and prolactin-injected embryos were used to determine the normal position of the anterior boundary of expression of both genes. Embryos injected with prolactin mRNA (C) or 0.2 ng of sense Xcad-2 RNA (D) are shown to illustrate the anteriorization of the anterior expression and the up-regulation of the Hoxb-9 gene.](image)

![Fig. 7. The interaction between the Otx-2 and the Hox genes. Injected embryos were hybridized with the Otx-2 (A-C) or Hoxd-1 (D-F) probes. To study the response of the Otx-2 gene embryos were injected with 1.6 ng of sense Hoxd-1 RNA (B) and 0.8 ng of Hoxb-4 mRNA (C). The response of Hoxd-1 to injection of 1.6 ng of Otx-2 mRNA (E) and 0.2 ng of Hoxb-4 sense RNA (F) is shown.](image)
This anterior neural tissue has to be ‘transformed’ to more posterior types. Retinoic acid has been shown to be able to promote this posterior transformation of the neural tissue (Blumberg et al., 1997; Papalopulu and Kintner, 1996; Yamada, 1994). Also FGF has been suggested as a posteriorizing signal (Doniaich, 1995) although recent results with a dominant negative FGF receptor have questioned this role (Kroll and Amaya, 1996). Interestingly, the caudal genes interact with both types of signaling molecules. These observations further support the role of the caudal genes as part of the ‘posterior signaling network’ and as part of the ‘transforming’ signal in the anterior-posterior patterning of the nervous system.

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