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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). In flies, as in vertebrates, the order of the genes along the complex is the same as their order of expression along the anterior-posterior axis and their temporal pattern of expression (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 to 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*, pSP64TXcad-2ΔH3, was generated by digesting the pSP64Xcad-2 plasmid with *Hpa*I which cleaves within the homeodomain and *Acc*I 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 *Xba*I 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 *Xba*I, transcribed with T7 polymerase; *Otx-2* – plasmid pXOT30.1 (Lamb et al., 1993), cut with *Not*I, transcribed with T7 polymerase; *Hoxd-1 – labial* clone (Blumberg et al., 1991) cut with *Eco*RI, transcribed with T7 polymerase; *Hoxb-1* – clone CX19/21 (Godsave et al., 1994) cut with *Eco*RI, transcribed with SP6 polymerase; *Hoxb-3* – clone M71/12 (Godsave et al., 1994) cut with *Eco*RI, transcribed with SP6 polymerase; *Hoxb-4* – clone E16 (Gont and De Robertis, personal communication) cut with *Bam*HI, transcribed with T7 polymerase; *Hoxc-6* – the clone of XlHbox1 (Cho and De Robertis, 1990) cut with *Hind*III, transcribed with T7 polymerase; *Hoxb-9* – the clone of XlHbox6 (Cho et al., 1988) cut with *Eco*RI 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-

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

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

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 protuding forebrains 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 aproximately 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 *Xcad-2* gene. By removing an internal *Hpa*I-*Acc*I fragment from the *Xcad-2* cDNA, the open reading frame gets interrupted 2 amino acids downstream from helix-2 in the 'turn' region towards helix-3. The reading frame shifts and terminates after 9 amino acids. Capped RNA encoding the truncated *Xcad-2* protein, ΔH3, was also injected into *Xenopus* embryos (Table 1). Injection of low amounts of ΔH3 RNA resulted in microcephalic embryos with shortened axis (Fig. 1D). Injection of high amounts of ΔH3 mRNA (1.6 ng/embryo) showed the reverse phenotype of enlarged head structures and longer trunks. The phenotypes obtained with the ΔH3 RNA are very similar to those obtained with the antisense RNA suggesting that the ΔH3 RNA encodes for a dominant negative *Xcad-2* protein. In addition, these results further support the specificity of the phenotypes obtained.

An initial molecular analysis of the *Xcad-2* phenotypes observed was performed by studying the pattern of expression of the *Krox-20* gene (Bradley et al., 1992). The *Krox-20* gene is expressed in rhombomeres 3 and 5 during formation of the central nervous system. Injection of low amounts of *Xcad-2* sense RNA (0.2 ng/embryo) results in the decrease of *Krox-20* transcripts in rhombomere 3 preferentially (Fig. 2B). Further increase in the amount of mRNA injected resulted in the general down-regulation of *Krox-20* expression (Fig. 2C). Injection of *Xcad-2* antisense RNA initially resulted in the down-regulation of the rhombomere 5 expression (Fig. 2D). Further increase in the amount of antisense RNA injected results in the appearance of a second stripe of *Krox-20* expression (Fig. 2E). 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 enlargment 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

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

Pattern of expression	Per cent embryos exhibiting the phenotype *Prolactin* 1.6 ng/embryo	*Xcad-2* 0.2 ng/embryo	*Xcad-2* 0.4 ng/embryo	*Xcad-2* 0.8 ng/embryo	*Xcad-2* 1.6 ng/embryo
Otx-2					
normal	100	ND	59	33	38
reduced	0	ND	33	17	23
erased	0	ND	8	50	39
Hoxd-1					
normal	100	31	33	7	0
reduced	0	46	59	56	50
erased	0	23	9	37	50

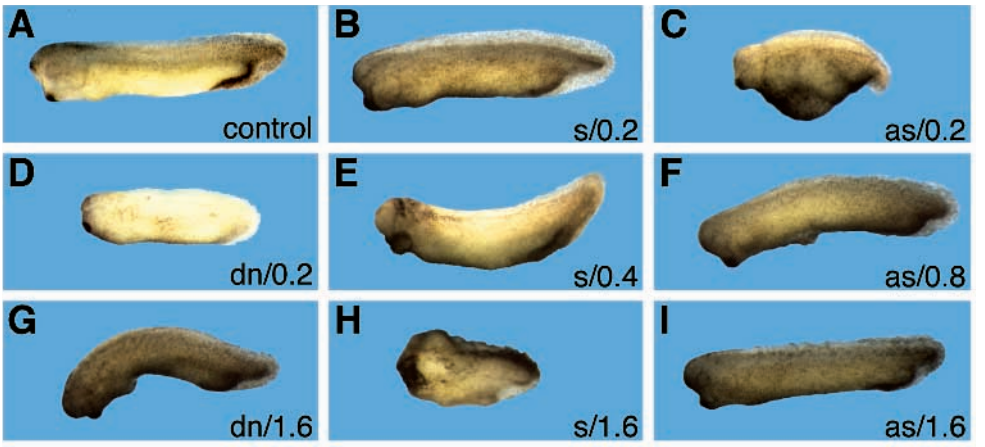


Fig. 1. Phenotypes induced by *Xcad-2* overexpression or partial loss of function. *Xenopus* embryos were injected at the 4-cell stage with *Xcad-2* sense (B,E,H), antisense (C,F,I) or the ΔH3 dominant negative (D,G) capped RNA at different concentrations. A sibling control uninjected embryo is also 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.

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 down-regulation 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

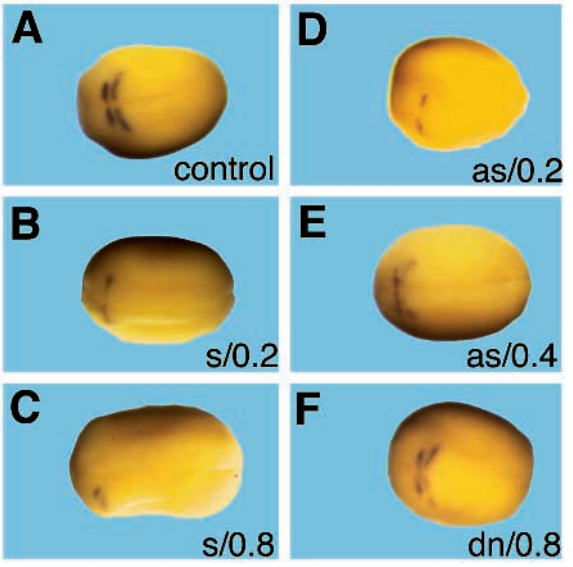


Fig. 2. Changes in the pattern of *Krox-20* expression as a result of *Xcad-2* overexpression and partial loss of function. Embryos injected with sense (s), antisense (as) or dominant negative (ΔH3; dn) *Xcad-2* were studied during neurulation to determine the effects on the *Krox-20* pattern of expression. (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.

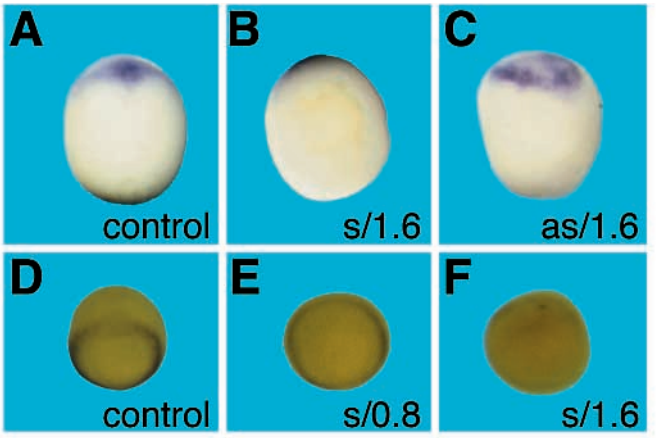


Fig. 3. The regulatory interactions between the *Xcad-2* and the *Otx-2* genes. Early neurula embryos injected with *Xcad-2* constructs were hybridized with an *Otx-2*-specific probe (A-C). *Xenopus* embryos were either uninjected control (A), injected with sense *Xcad-2* RNA at 1.6 ng (B) or antisense *Xcad-2* RNA also at 1.6 ng (C). Gastrula-stage embryos injected with *Otx-2* sense RNA were probed for *Xcad-2* expression (D-F). (D) Control uninjected embryo, (E) embryo injected with 0.8 ng *Otx-2* mRNA and (F) embryo injected with 1.6 ng *Otx-2* mRNA.

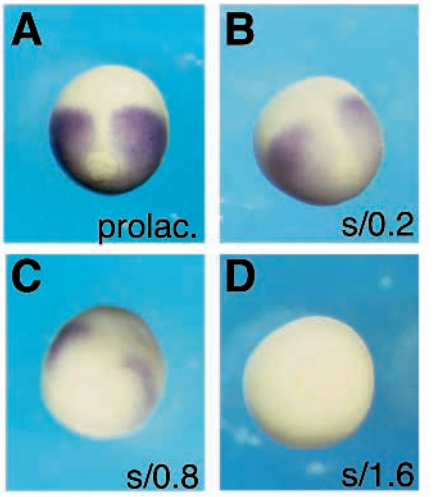


Fig. 4. Dose-response of *Hoxd-1* to the gradual increase in the levels of *Xcad-2*. *Xenopus* embryos were injected at the 4-cell stage and hybridized with an *Hoxd-1*-specific probe at stage 12. (A) Control embryo injected with *prolactin* mRNA. (B) Embryo injected with 0.2 ng of sense *Xcad-2* RNA. (C) Injection of 0.8 ng of *Xcad-2* results in the almost complete elimination of *Hoxd-1* expression. (D) Elimination of *Hoxd-1* expression was observed in embryos injected with 1.6 ng of *Xcad-2* sense RNA.

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 RNA/embryo (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 *Xcad-2* antisense RNA (0.4 ng) results in the widening of the *Hoxb-3* stripe of expression (Fig 5C) suggesting that transcripts of this gene are appearing in neighboring regions. Further increase in the amount of antisense RNA injected (1.6 ng) restores the *Hoxb-3* expression to almost normal expression in the rhombomere thus exhibiting a reversing phenotype. In these same embryos, *Hoxb-3* expression in the neural crest cells is undetectable (not shown). Thus with respect to *Hoxb-3*, injection of sense or antisense RNA gave opposite effects.

At about stage 25, *Hoxb-4* (*Xhox-1A*; Harvey et al., 1986) expression can be detected in four populations of neural crest cells (Fig. 5D). Injection of low amounts of capped *Xcad-2* sense RNA (0.2 ng) resulted in a strong down-regulation of *Hoxb-4* expression. Further increase in the amount of *Xcad-2* mRNA eliminated all *Hoxb-4* expression (Fig. 5E). 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

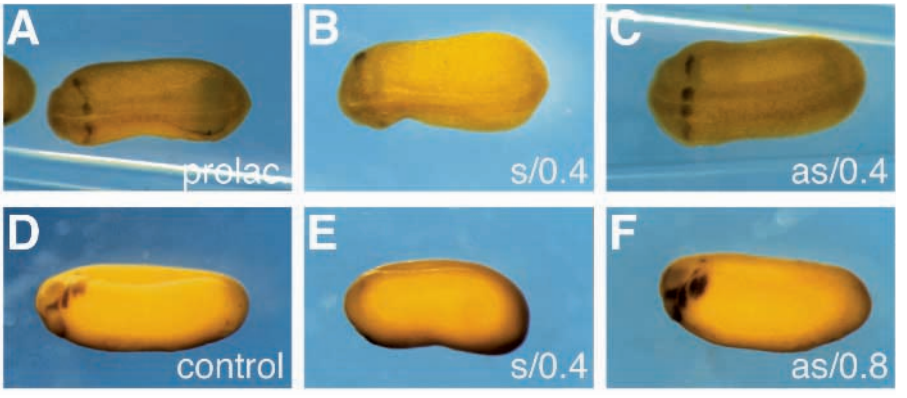


Fig. 5. The response of *Hoxb-3* and *Hoxb-4* to changes in the levels of *Xcad-2*. Embryos injected with *Xcad-2*-derived RNAs were probed either with the *Hoxb-3* (A-C) or *Hoxb-4* (D-F) probes. Control embryos were injected with *prolactin* RNA as a control (A) or uninjected (D). Embryos injected with 0.4 ng sense *Xcad-2* RNA are shown for both probes (B,E). Injections with 0.4 and 0.8 ng antisense *Xcad-2* RNA are shown for the *Hoxb-3* (C) and *Hoxb-4* (F) hybridizations, respectively.

studied: *Hoxc-6* (*XlHbox1*; Cho et al., 1988) and *Hoxb-9* (*XlHbox6*; 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 (Pownall 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 (Pownall et al., 1996; Thompson and Slack, 1992; Isaacs 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* (Pownall 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.

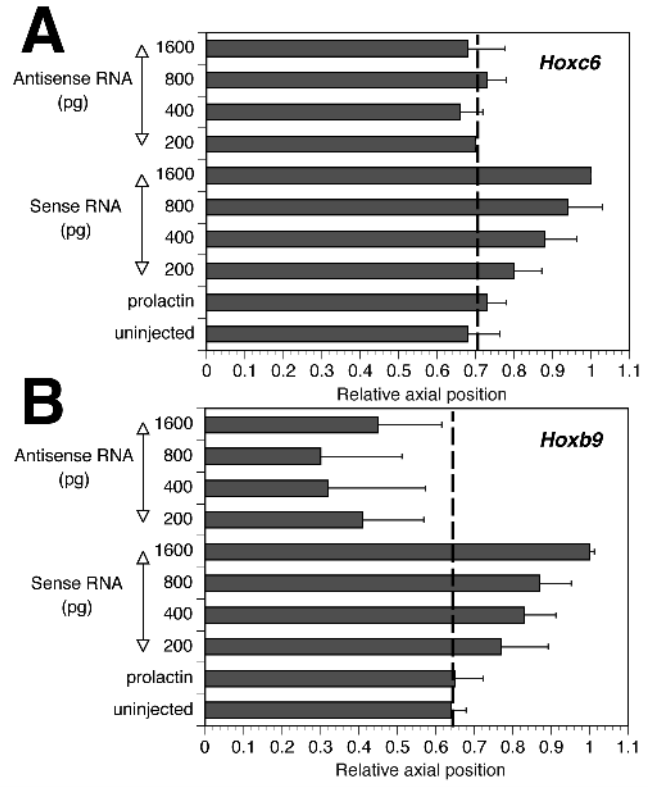
The head and trunk organizers

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; Matsuo 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 3′ *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



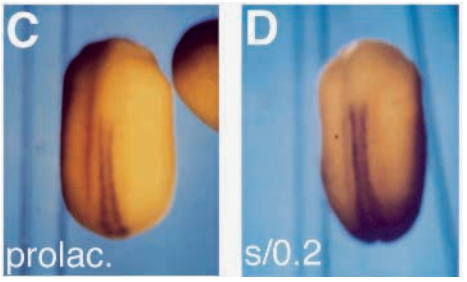


Fig. 6. The effect of *Xcad-2* on more posterior (5′) *Hox* genes. The effect of the injection of *Xcad-2* sense and antisense RNA on the *Hoxc-6* (A) and *Hoxb-9* (B) genes was studied by determining the position of their anterior boundaries of expression. The position of the anterior boundary of expression 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.

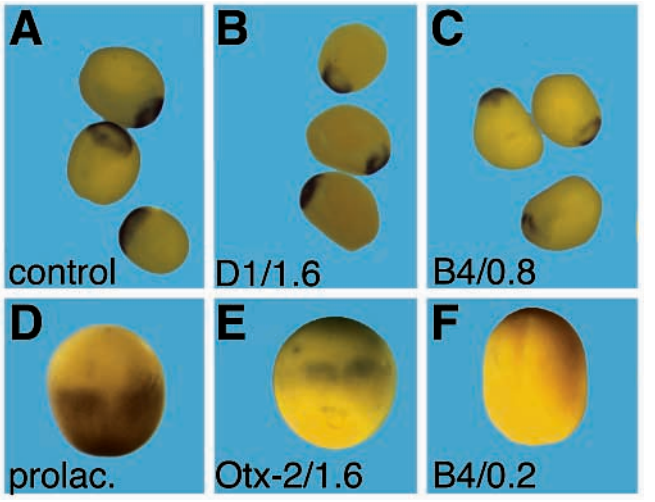


Fig. 7. The interaction between the *Otx-2* and the *Hox* genes. Injected embryos were hybridized with the *Otx-2* (A-C) or *Hoxd-1* (D-F) probes. To study the response of the *Otx-2* gene embryos were injected with 1.6 ng of sense *Hoxd-1* RNA (B) and 0.8 ng of *Hoxb-4* mRNA (C). The response of *Hoxd-1* to injection of 1.6 ng of *Otx-2* mRNA (E) and 0.2 ng of *Hoxb-4* sense RNA (F) is shown.

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 down regulation 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 *Antennapedia*-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*

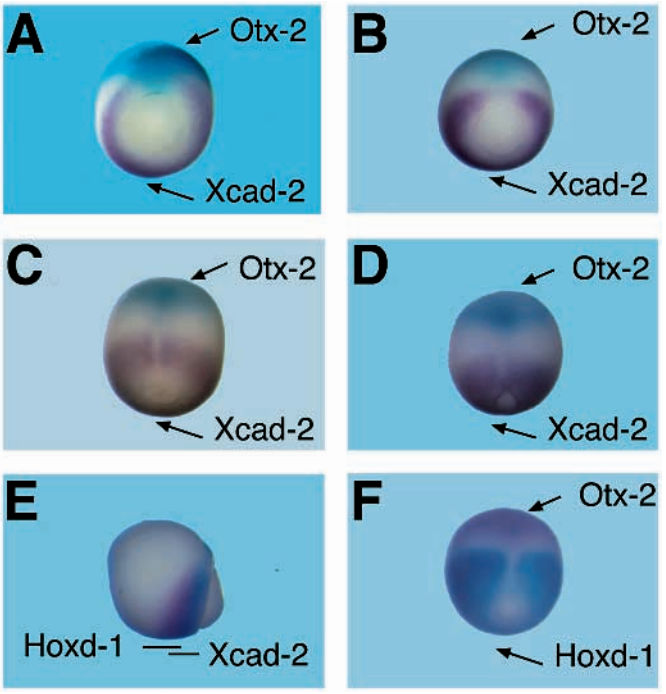


Fig. 8. Patterning of the anterior-posterior axis as evidenced from the patterns of expression if *Xcad-2*, *Otx-2* and *Hoxd-1*. Double in situ hybridizations were performed at different developmental stages to study the dynamics of the establishment of the anterior-posterior axis. The initial subdivision of the embryo into anterior and posterior domains and their distancing was evidenced from the analysis of the *Otx-2* (turquoise) and *Xcad-2* (magenta) of expression during stages 11 (A), 11.5 (B), 12 (C) and 12.5 (D). The partial overlap between the *Hoxd-1* (magenta) and *Xcad-2* (turquoise) was determine at stage 11.5 (E). The identification of *Hoxd-1* as one of the genes expressed in the gap between the anterior and posterior was determined by studying the patterns of expression of *Otx-2* (magenta) and *Hoxd-1* (turquoise) at stage 12.5 (F).

genes exhibit stronger activation in response to exogenous retinoic acid (Dekker et al., 1992). These responses to retinoic acid are opposite to those observed by overexpression of *Xcad-2*. During gastrulation in *Xenopus*, retinoic acid can be detected in the organizer region and subsequently during neurulation retinoic acid localizes to the posterior region of the embryo (Chen et al., 1994). While the early phase of the localization of retinoic acid is complementary to the localization of the *Xcad-2* transcripts, by late gastrulation and neurulation they overlap. The overlap between *Xcad-2* and the retinoic acid takes place at the stages during which the anterior-posterior axis becomes established and patterned. In contrast, both retinoic acid and *Xcad-2* appear to have opposite effects on the pattern of expression of the *Hox* genes. These observations taken together suggest that retinoic acid and the *caudal* genes establish a regulatory network whose function is to fine tune the expression of *Hox* genes along the anterior-posterior axis.

The model for induction and patterning of neural tissue has been has been termed the 'activation-transformation' model (Nieuwkoop, 1952). In this model, as neural induction takes place the induced tissue develops with anterior characteristics. 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 (Doniach, 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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