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Short Communication

Differential regulation of *Dlx* gene expression by a BMP morphogenetic gradient

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ABSTRACT Three members of the vertebrate Distal-less gene family, Dlx3, 5 and 6, are transcribed in early gastrula embryos of *Xenopus laevis*. This expression is confined to ectoderm and is excluded from the presumptive neural plate region. Expression of all three genes is dependent upon BMP signaling, with significant differences in how the three genes respond to the BMP antagonist chordin. This correlates with the different expression domain boundaries *in vivo* for Dlx3 compared to Dlx5 and 6, suggesting that BMP signal attenuation could be the primary factor in determining these different patterns in the gastrula ectoderm.

KEY WORDS: *Xenopus, ectoderm, Dlx3, Dlx5, Dlx6.*

The *Distal-less* (Dlx) family of vertebrate homeodomain factors comprises six genes in mouse and human (Stock *et al.*,1996), and seven or more in Zebrafish (Ellies *et al.,* 1997). In *Xenopus*, there are probably six genes as well, although this has not been firmly established (Papalopulu and Kintner., 1993; Dirksen *et al.,* 1994; T. Luo, unpublished data). The mammalian Dlx genes are organized into three pairs, each linked to one of the four Hox clusters (Stock *et al.*,1996). The nomenclature of Dlx family members has been confusing, particularly in nonmammalian vertebrate species such as *Xenopus*. In this report we follow a nomenclature corresponding to that used in human and mouse for Dlx genes (Beanan and Sargent, 2000): "Dlx3" is used instead of "Xdll-2" (Papalopulu and Kintner, 1993) or "Xdll2" (Dirksen *et al.,* 1994); "Dlx5" instead of Xdll3 (Papalopulu and Kintner, 1993) and "Dlx6" instead of "Xdll" (Asano *et al.,* 1992).

Individual Dlx genes are expressed in partially overlapping regions, most notably in the forebrain, limbs, otic vesicles, and branchial arches. Gene targeting experiments have revealed important roles for Dlx1, Dlx2 and Dlx5 in mouse forebrain (Anderson *et al.,* 1997), dentition (Thomas *et al.,* 1997) and craniofacial development (Qiu *et al.,* 1997; Acampora *et al.,* 1999; Depew *et al.,* 1999), and for Dlx3 in placental differentiation (Morasso *et al.,* 1999). In addition, Dlx3 has been implicated in the early patterning of the anterior neural plate in *Xenopus* and in the control of epidermal cell differentiation in mouse (Feledy *et al.,* 1999; Morasso *et al.,* 1996). Dlx5 is initially expressed in the anterior neural fold in mouse, chick and *Xenopus*, defining the rostral limit of the neural plate in these species (Papalopulu and Kintner, 1993; Yang *et al.,* 1998; Pera *et al.,* 1999). In mouse embryos at the late streak stage, Dlx5 expression extends laterally towards the posterior, demarcating prospective neural crest (Yang *et al.,* 1998). Dlx3 has been shown to be under positive regulation by bone morphogenetic protein (BMP) signaling in *Xenopus* embryos (Feledy *et al.,* 1999), while induction of Dlx5 by BMPs has been demonstrated in murine osteoblastic cells by Miyama *et al.,* (1999).

There is evidence suggesting that in the early *Xenopus* embryo, the antagonism of BMP signaling results in a graded response as opposed to a simple binary mechanism (Wilson *et al.,* 1997), and morphogenetic gradients based on BMP signaling have been proposed as a mechanism for establishing spatial patterns in mesoderm of *Xenopus* embryos (Knecht and Harland, 1997; Wilson *et al.,* 1997; Dosch *et al.,* 1997), while similar models have been invoked to explain shifts in dorsoventral marker gene expression in zebrafish embryos with mutations in BMP signaling pathway components (Nguyen *et al.,* 1998). These findings suggest the possibility that regulatory factors might mediate developmental responses to morphogenetic signals and gradients based on BMPs. For example, ectodermal cells located adjacent to the Spemann organizer in Xenopus or the embryonic shield in zebrafish would be expected to experience a higher concentration of BMP antagonist (or alternatively a longer temporal duration of exposure to an invariant concentration) than ectodermal cells located somewhat further away. Thus graded exposure to partially antagonized BMP signaling might be expected to occur at the boundaries between neural plate and epidermis laterally and anterior neural fold and epidermis rostrally.

Abbreviations used in this paper: BMP, bone morphogenetic protein; Dlx, distal-less; SSPE, standard saline phosphate EDTA.

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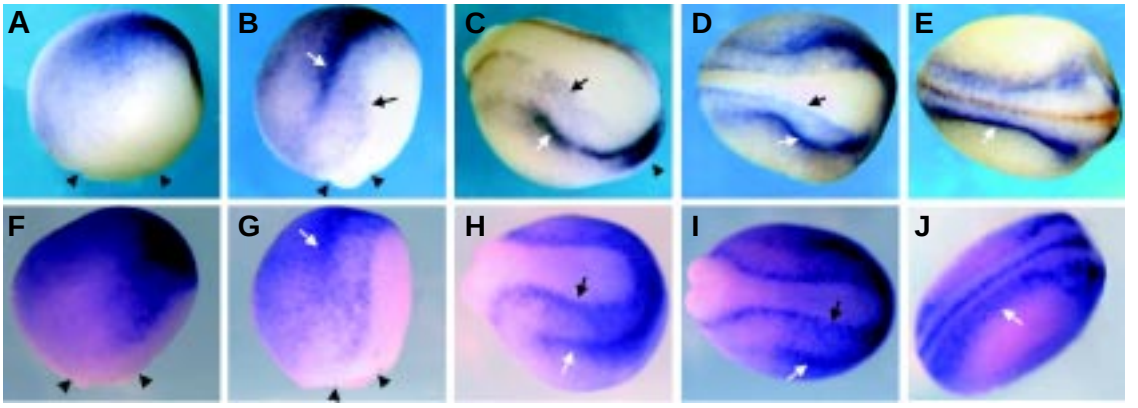

Fig. 1. Spatial expression patterns of Dlx5 and Dlx6. *Embryos were fixed and hybridized under conditions optimized for detection of epidermal transcripts. (A-E), Dlx5. (F-J), Dlx6.* **(A,F)** *Mid/late gastrula (stage 12) showing broad expression in ectoderm, except for the dorsal side (on the right) which is the posterior region of the prospective neural plate. The two black arrowheads indicate the edges of the yolk plugs.* **(B,G)** *Early neurula (stage 13). A stripe of relatively intense hybridization has appeared (white arrows in these and subsequent panels) lateral to the epidermal-neural plate boundary (black arrow). The yolk plugs are indicated by black arrowheads. Dorsal is to the right.* **(C,H)** *Mid neurula (stage 15). Anterior neural plate hybridization has resolved into anterior neural fold and cement gland (black arrowheads). Anterior is to the right.* **(D,I)** *Late neurula (stage 17). The intense hybridization stripes (white arrows) and epidermal-neural plate boundaries (black arrows) continue to be well-resolved. Anterior to right.* **(E,J)** *Early tailbud (stage 20). The neural tube has closed at this stage and the epidermal-neural plate boundaries have fused at the dorsal midline. The intense lateral stripe of expression clearly lies outside the neural tube for both Dlx5 and Dlx6 (white arrows). Anterior is to the right.*

We have analyzed the expression, regulation and function of Dlx genes in early *Xenopus* development, using whole mount *in situ* hybridization that is optimized for detection of transcripts in superficial ectoderm, and in animal cap overexpression experiments. This work has revealed novel features of the Dlx5 and Dlx6 expression pattern, and shows that, like Dlx3, these two genes are under the control of BMP signaling in the frog embryo. We also show that this control is quantitatively different from that of Dlx3, potentially accounting for the differences in expression and providing a mechanism by which the *Xenopus* genome might respond to a morphogenetic gradient during gastrulation.

Analysis of spatial expression for *Xenopus* Dlx6 has not been reported, and while whole mount *in situ* hybridizations for Dlx5 have been published (Papalopulu and Kintner, 1993), these did not detect transcripts prior to neurula stages, nor in the epidermis. We carried out optimized whole mount *in situ* hybridizations with probes for these genes, which revealed that both Dlx5 and Dlx6 have spatial expression patterns (Fig. 1), similar to but distinct from that which has been described for Dlx3 (Feledy *et al.,* 1999). At mid to late gastrula, Dlx5 and Dlx6 transcripts are detectable rather uniformly in the ectoderm, except in the most dorsal region where the RNAs are not detectable (Fig. 1 A,F). This negative region corresponds to the prospective neural plate, caudal to the anterior neural fold. By early neurula (stage 13) this pattern becomes more complex. A stripe of high-level expression forms in the anterior half of the embryonic epidermis, just outside the neural/epidermal boundary (Fig. 1B,G; white arrow). This stripe of high-level expression coincides with the anterior neural fold in the rostral part of the embryo, just posterior to a region of intense expression in the presumptive cement gland, first clearly visible at stage 15 (Fig. 1 C,H). By the end of neurulation, when the neural tube is completely closed (stage 20, Fig. 2 E,J), the stripe of relatively intense expression lies outside the neural tube.

Dlx5 and Dlx6 appear to be excluded to similar extents from the neural plate, but both seemed to intrude somewhat closer to the midline than what had been observed for Dlx3. To investigate this further, double *in situ* hybridizations with Dlx3/5/6 and chordin probes were carried out with carefully matched stage 14 embryos. As shown in Fig. 2, it is evident from these data that Dlx3 expression (Fig. 2 A,D) is significantly more restricted to ventral ectoderm, compared to Dlx5 or Dlx6 (Fig. 2 B,E; C,F). This boundary difference, and the presence of the lateral stripes for Dlx 5 and Dlx6, are the primary distinctions among these genes in the gastrula/early neurula embryo.

Could the different boundaries of Dlx3/5/6 expression be attributable to differential responsiveness of these genes to BMP signaling? To address this question, animal cap experiments were carried out in which increasing amounts of RNA encoding the BMP antagonist

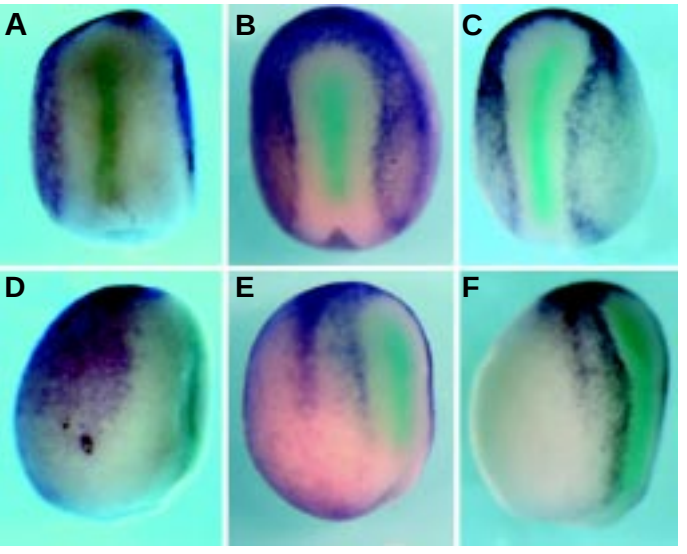

Fig. 2. Width of dorsal excluded regions for Dlx genes. *Stage-matched (st 14) embryos hybridized in situ with probes for chordin (blue/green) and Dlx3, 5 or 6 (purple).* **(A,D)** *Dlx3;* **(B,E)** *Dlx5;* **(C,F)** *Dlx6. The patterns are similar, but the distance between the midline (chordin signal) and Dlx3 is clearly greater than for either Dlx5 or Dlx6. A-C, dorsal view. D-F, lateral view.*

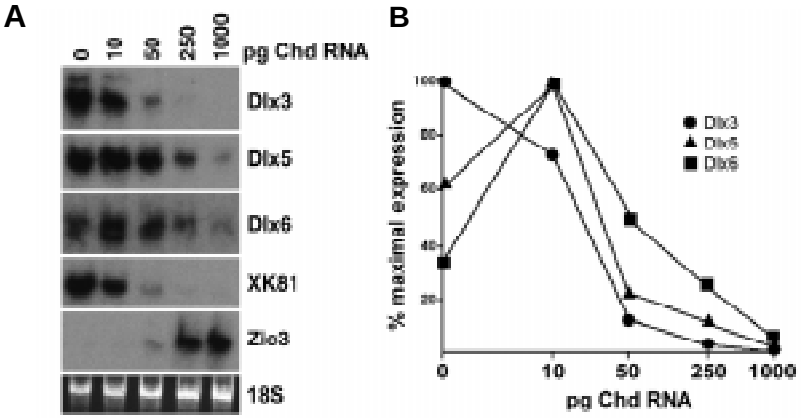

Fig. 3. Differential regulation of Dlx gene expression by BMP signaling. *Fertilized eggs were injected with 10-1000 pg of RNA encoding chordin, animal caps removed at stage 7-8, cultured until sibling embryos reached stage 12 and processed for RNA and Northern analysis.* **(A)** *Northern blots using probes for Dlx3, Dlx5, Dlx6, XK81 (epidermal keratin) and Zic3 (neural pre-pattern gene). Ethidium bromide staining of the 18S ribosomal RNA band is shown as a control for equal loading of lanes. Dlx3 and XK81 are almost completely repressed by 50 pg chordin RNA while Dlx5 and Dlx6 are much more refractory (see below).* **(B)** *Summary of densitometry analysis of multiple exposures of Northern blots from three separate experiments. Dlx5 and 6 require several-fold higher RNA levels, respectively, to achieve the same degree of inhibition as Dlx3 (note the log scale for the x-axis). There is also a significant increase in expression of Dlx5 and Dlx6 in embryos injected with the lowest dose of chordin, relative to uninjected controls.*

chordin were injected at stage 1, followed by northern blot assays for Dlx gene expression in isolated ectoderm. As shown in Fig. 3A, this treatment inhibits non-neural genes, such as the epidermal keratin XK81 and Dlx3, 5 and 6, while inducing the transcription of neural genes such as Zic3 (Nakata *et al.,* 1997). The negative effects on the three Dlx genes is not uniform, however, as summarized in the graphical representation shown in Fig. 3B. There are two key differences. First, compared to Dlx5 or Dlx6, Dlx3 is inhibited by several fold lower doses of chordin RNA; in other words Dlx3 expression is more sensitive to BMP antagonism than that of Dlx5 or Dlx6. Second, Dlx5 and Dlx6 are both stimulated by a low chordin dose, then inhibited by higher levels, whereas the inhibition of Dlx3 is monotonic. In this context it is interesting to note that Dlx5 and Dlx6 are linked, suggesting that the similar BMP responsiveness of the two genes could be due to common regulatory elements residing in the intergenic region that have been implicated in regulating expression of these genes in the forebrain (Zerucha *et al.,* 2000). The induction of Zic3 expression takes place primarily at and above chordin levels (i.e. 250 pg in this series of experiments) that significantly inhibit expression of all three Dlx genes, which is consistent with the hypothesis that Dlx gene expression may function in the early embryo to inhibit neurogenesis (Feledy *et al.,* 1999). The evidence presented in this paper supports the conclusion that BMP-based morphogenetic gradients can control the differential expression of Dlx homeodomain genes, suggesting a possible mechanism for linking the gradient to regionalized tissue specification.

Experimental Procedures

Isolation of Dlx cDNA clones

A stage 24 *Xenopus laevis* cDNA library was screened at low stringency (final wash: 2X SSPE at 55˚C) using as probe a radiolabeled mixture of inserts from cDNA clones for zebrafish dlx1, dlx4, dlx6 and dlx7 (Ellies *et al.,* 1997; a gift from M. Ekker). This screen resulted in the isolation of several dozen positive clones, which included Dlx3, Dlx5, and Dlx6, but no other members of the Dlx family. The Dlx5 cDNA clone differed in DNA sequence from the Xdll-3 cDNA clone isolated by Papalopulu and Kintner (1993) at four positions, none of which altered the predicted amino acid sequence, and the Dlx6 cDNA clone was identical to the published Xdll sequence (Asano *et al.,* 1992).

Embryo manipulation

Embryos were obtained from adult *Xenopus laevis* by hormone-induced egg laying and artificial fertilization using standard methods, and staged according to Nieuwkoop and Faber (1967). Following microinjection, embryos were cultured in 3% Ficoll-400/1X MMR until sibling embryos reached stage 7. For animal explants, approximately 2/3 of the pigmented ectoderm was dissected from stage 7-8 embryos and cultured in 0.3X MMR containing 50 μg/ml gentamicin until sibling embryos reached the desired stage.

In situ hybridization

Antisense probes labeled with digoxigenin or fluorescein were synthesized using an *in vitro* transcription kit (Boehringer-Mannheim, Indianapolis) according to the manufacturer's instructions. The Dlx3, Dlx5 and Dlx6 probes were made by subcloning open reading frames for these cDNAs in pBluescript 2KS+ (Stratagene), linearizing with EcoR1 (Dlx3) or XbaI (Dlx5, 6) and transcribing with T7 (Dlx3) or T3 (Dlx5, 6) polymerase. The Chordin probe was made by linearizing pBSSK- chdF2 (a gift from Dr. I. Dawid) with EcoR1 and transcribing with T7 polymerase. Whole mount *in situ* hybridizations were carried out according to Harland (1991), except that fixed embryos were incubated in 10 μg/ml proteinase K for 5 minutes, rinsed twice for 5 minutes in 0.1 M triethanolamine, pH7.5, then treated for 10 minutes in 0.1 M triethanolamine pH 7.5, 0.25% acetic anhydride (Sigma).

Microinjection and RNA analysis

Full-length capped transcripts encoding chordin were generated using pCS2+Chd for chordin (a gift from E. DeRobertis) linearized with Not1 and an SP6 mMessage Machine kits (Ambion, Inc., Austin TX) according to the manufacturer's instructions. The RNA was checked for concentration, integrity and size by methylmercury gel electrophoresis, and quantified by optical density at 260 nm. Synthetic RNA was injected into two sites in the animal hemisphere at the one-cell stage in a total of 10 nl. RNA extraction, methylmercury hydroxide gel electrophoresis and northern blot analysis for expression of Dlx3/5/6 (Feledy *et al.,* 1999, and this paper), XK81(Jonas *et al.,* 1985), Zic3 (Nakata *et al.,* 1997), were carried out as previously described (Sargent *et al.,* 1986). All preparations were tested and found to be free of contaminating mesoderm by hybridization with appropriate cDNA probes (data not shown).

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