Anterior-posterior axis formation in Xenopus laevis

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CHAPTER 5

Analysing the function of a hox gene: an evolutionary approach

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Introduction

General Background

Hox proteins are involved in the specification of positional identities along the anteroposterior (AP) axis in all bilaterian metazoans, including vertebrates and insects (Lawrence and Morata, 1994; Manak and Scott, 1994; McGinnis and Krumlauf, 1992)(. While insect *Hox* genes are contained in a single (sometimes broken) chromosomal cluster, *Hox* genes in tetrapod vertebrates are organised in four clusters located on different chromosomes. Hox clusters are thought to have arisen by tandem duplication of a single gene, followed, in vertebrates, by duplication of the cluster itself (Bailey et al., 1997; Greer et al., 2000; Ruddle et al., 1994; Schughart et al., 1989). As a consequence, *Hox* genes occupying the same relative position along the 5' to 3' chromosomal coordinate, named paralogous genes, share more similarity in sequence and expression pattern than do adjacent *Hox* genes on the same chromosome (Greer et al., 2000). This homology of sequence, position and function is also conserved in evolution. So much so, that a human Hox gene, (Hoxd4), can substitute for its orthologue, Deformed, in Drosophila development (McGinnis et al., 1990). A phenomenon of particular interest is that *Hox* genes located at the 3' end of a cluster are expressed and function earlier and more anteriorly than the subsequently more 5' located genes (Duboule and Morata, 1994; Gaunt, 1988; Gaunt and Strachan, 1996; Wacker et al., 2004). How this spatiotemporal colinearity in the expression of *Hox* genes is regulated is intriguing but, to date, not well understood.

The hox proteins are known to act as transcription factors, positively and negatively regulating transcription of targets via action of at least two conserved domains: the homeodomain, a DNA binding domain, and the hexapeptide, which interacts with extradenticle/pbx cofactors. Besides their being transcription factors, there is recent evidence that Hox proteins mediate protein-protein interactions and that the homeodomain is also a protein-protein interaction domain (Plaza et al., 2001).

Recent findings connect early hox expression in vertebrates to gastrulation. It was long known from classical and modern studies that the vertebrate A-P pattern arises during gastrulation (Mangold, 1933; Nieuwkoop, 1952; Spemann, 1931). Recently, the earliest *Hox* expression patterns were analysed in *Xenopus* gastrula embryos (Wacker et al., 2004). This revealed temporally colinear initiation of mesodermal expression of a sequence of *Hox* genes within a horseshoe-shaped domain in ventrolateral marginal zone mesoderm at different stages during gastrulation, followed by sequential dorsalisation of each *Hox* expression zone into a stable AP zone in axial mesoderm and the neural plate. Chick, mouse, and zebrafish embryos appear to show equivalent early hox expression patterns as far as data are available (Alexandre et al., 1996; Forlani et al., 2003; Gaunt and Strachan, 1996). Recent evidence in the chicken embryo show that internalisation of mesoderm cells into the gastrula is controlled by this collinear hox gene sequence (Iimura and Pourquie, 2006). In the fruitfly Drosophila, in contrast, the hox gene expression pattern is set up before gastrulation and is controlled by a specific hierarchy of transcription factors that is probably not conserved in the vertebrates.

Specific aims and strategy for this study

Little is known about the mechanisms whereby hox genes exert their functions. Specifically, not much is known about their transcriptional targets and how these act. Two things that are clear are that: hox genes mediate very different functions at different times during embryonic development and at different places in the embryo (Akam, 1998), and

that at least some hox functions are conserved across the animal kingdom (Manak and Scott, 1994). We decided, in this study, to focus on the very earliest functions of hox genes, that occur soon after their expression begins in the early embryo and that evidently involve specification of zones along the main body axis- a function that is conserved across the metazoans (Coletta et al., 1994). We also decided to study the same, functionally homologous hox gene in two very different animals; the fruitfly *Drosophila melanogaster* and the frog *Xenopus laevis*. This strategy allowed us to identify possible conserved early functions of this hox gene by detecting early targets that were conserved across these two species. In this study only overexpression was used to identify targets since a loss of function approach affects only a region in the embryo and the microarray technology might not be sensitive enough to identify hox targets.

We chose the Drosophila gene Antennapedia, which was cloned by the Gehring lab in the '80's (Garber et al., 1983), as one of the first hox genes ever cloned. As its functional homologue, we chose the Xenopus hox gene Hoxc6, which was the 3rd hox gene and 1st vertebrate hox gene ever cloned, by E. de Robertis in the Gehring lab (Carrasco et al., 1984), and was screened out as a homeodomain sequence orthologue of Antennapedia. It is the first gene of this paralogous group to be expressed in development, being expressed halfway through gastrulation: the other genes of this paralogue group being expressed at mid neurulation (Hoxb-6) or not during early embryonic development (Hoxa-6)(HJ personal communication).

Results

First, we tested for true functional homology between Drosophila Antennapedia and Xenopus hoxc6. Their homeobox domains of these two hox genes show high (91%) sequence homology and they are expressed in homologous axial positions. Their anterior expression boundary coincides with the anterior part of the future thorax (Burke et al., 1995). Their early ectopic expression in fly and frog gives comparable axial phenotypes. Antennapedia ectopic expression posteriorises the Drosophila embryo, converting the antennal disc to a first thoracic segment leg disc and posteriorising the larval morphology of the head segments to that of the T2 thoracic segment. Hoxc6 ectopic expression posteriorises the Xenopus embryo, suppressing development of the head, and enlarging the thoracic part of the body.

If Hoxc-6 is overexpressed at a higher level it also shows a block in gastrulation movements. This effect is also seen in Drosophila. The gastrulation defect in Drosophila was in germ band retraction. That in Xenopus starts as a failure in forming a blastopore and involves a complete block of involution movements. It is being further characterised.

We tested functional homology first by determining if ectopically expressing Hoxc6 in the early Drosophila embryo could give the same specific axial phenotype as ectopically expressing Antennapedia. This it did; both an antenna to leg transformation and transformation of head segments in the larva to T2 thoracic segments. Another Xenopus hox gene, Hoxa7, which has considerable homeobox sequence homology with Antennapedia, failed to do this. Second, we determined if the morpholino loss of function phenotype of Hoxc6, which causes anteriorisation and loss of segmentation in the Xenopus embryo, can be rescued by Antennapedia. It can, and it cannot be rescued by the similar but modified Drosophila hox gene, fushi tarazu.

Our strategy for comparing the early targets of these two hox genes was to express them ectopically in their respective early embryos and harvest and compare mRNA from the experimental and control embryos to detect their transcriptional targets. Antennapedia was expressed in Drosophila embryos under control of the Nullo promoter, which drives expression at preblastoderm and blastoderm stages and then turns off. Total mRNA was harvested from experimental and control embryos from Drosophila embryos at two stages. At 100-130 min (stage4, syncytial blastoderm), just before endogenous hox gene expression begins. At 150-180 min.(stage 5, cellular blastoderm), just after Antennapedia expression starts. In Xenopus, Hoxc6 mRNA was microinjected into the zygote and total mRNA was harvested from injected embryos and controls at three later stages. At the beginning of gastrulation, St. 10.25, just before the first Hox expression starts. At mid gastrulation, st. 11, after 3' anterior Hox genes have started expression but before expression of Hoxc6 starts. At the end of gastrulation, st. 12, just after Hoxc6 expression has started. The Drosophila and Xenopus stages chosen here were thus closely comparable, in terms of the stages of Hox expression and expression of Antp/Hoxc6 The situation is complicated by the fact that these embryos display heterochrony. In Drosophila, gastrulation starts after the beginning of hox expression, at 180 AED. In Xenopus, gastrulation starts before hox expression.
To select for up- or down-regulated genes, only those probesets that showed a foldchange higher than 1.5 and scored a p-value lower than 0.05 in an unpaired t-test were considered.
The targets obtained differed between Drosophila and Xenopus. There were many more in Drosophila (194 at 100-130 min and 1761 at 150-180 min) than in Xenopus (23 at early gast, 100 at mid gast and 198 at late gast). Of all of these putative targets, 1519 in Drosophila were positive and 630 were negative and 83 of the Xenopus targets were positive and 195 were negative. 23 of the positive targets (28% in Xenopus and 1.5% in

A upregulated targets

Xenopus Probe Set ID	Xenopus gene symbol	Closest Drosophila protein
Xl.23887.1.A1_at Xl.23887.2.S1_at	MGC85390	Rab40 CG1900-PA
Xl.4337.1.A1_at	MGC114680	
Xl.3464.1.A1_at	MGC81121	AnnIX
Xl.5440.1.S1_at	MGC53060	Cyclin G CG11525
Xl.16876.1.S1_at Xl.11586.1.S1_at Xl.8458.1.S1_at	LOC495072	CG4080
Xl.15204.1.A1_x_at	MGC64386	CG14536
Xl.5778.1.S1_at	herpud1-a	
Xl.7556.1.S1_at	PRICKLE	prickle CG12833
Xl.8630.1.S1_s_at Xl.8630.1.S1_at	MGC53542	polo CG12306-PB
Xl.13019.1.S1_at	MGC53580	CG8519
Xl.6421.1.A1_at	MGC68457	CG9214 Tob
Xl.12348.1.A1_at	MGC154560	CG3198
Xl.24275.1.S1_at	MGC82057	CG4210
Xl.8057.1.A1_at	MGC80135	CG5850
Xl.23579.1.S1_at	LOC495834	cnc
Xl.11215.1.A1_at	LOC495047	CTP synthetase CG6854
Xl.16040.1.S1_at	MGC52759	CG5991
Xl.15887.1.S1_x_at	MGC53394	CG9399

B downregulated targets

Xenopus Probe Set ID	Xenopus gene symbol	Closest Drosophila protein
Xl.3370.1.S1_at	HoxD1	labial
Xl.21639.1.S1_at	hoxa9-A	Abdb
Xl.14513.1.A1_at	dlx3	dll
Xl.838.1.S1_at	dlx3-a	
Xl.18401.1.S1_at	Dlx5	
Xl.262.1.S1_at	MGC130625	
Xl.16449.1.S1_at	MSX1	Dr (Drop) / msh (Muscle segmentation homeobox)
Xl.1268.1.S1_at	Otx2	oc (orthodenticle)
Xl.1047.1.S1_at Xl.21652.1.S1_s_at	lim5/Lhx5	Lim1
Xl.647.7.S1_at	PAX6 MGC5253	ey, toy
Xl.1041.1.S1_at	Xgli3	Ci
Xl.23634.1.S1_at	znf703-A (FLJ14299)	Zinc finger protein Noc
Xl.8440.1.S1_at	MGC53782+ ESR	deadpan [dpn]
Xl.15572.1.A1_at	XHR1-B	Enhancer of split mgamma (E(spl)mgamma
Xl.54.1.S1_at	hen2-a	CG33323 [Fer1] (CG10066 on chip)
Xl.492.1.S1_at	id2	emc:
Xl.5479.1.A1_at	MGC81442	Ssb-c31a: Single stranded-binding protein c31A / RNA
Xl.3789.1.S1_at	cebpa	slbo (Slow border cell, CG4354, CCAAT/enhancer-binding
Xl.12378.1.S1_at	dsipi	bun (bunched / shortsighted)
Xl.16557.1.S1_at	rpe65	ninaB: CG9347
Xl.6879.1.S1_at	SAS6 MGC83825	DSas-6 (CG15524):
Xl.1156.1.S1_at	xNLR-1	TARTAN (CG11280)
Xl.4294.1.A1_at	LOC398291:	lea / roundabout2 (CG5481)
Xl.866.1.S2_at	Sonic hedgehog	hh
Xl.30.1.S1_at	kit-A *xKrk1*	-VEGFR [Pvr = CG 8222]
Xl.24090.1.A1_at	kl1-A	
Xl.115.1.S1_at	LOC397974, Xron	Receptor protein tyrosine kinase ALK (CG8250)
Xl.24403.1.S1_at	MGC68696	Cyp18a1
Xl.13377.1.A1_at	kctd15	CG10440
Xl.7103.1.S1_at	LOC431791	CG4074
Xl.16320.1.S1_at	anxa9	AnnX
Xl.11134.1.S1_at	hatching gland-like Xhel	tld

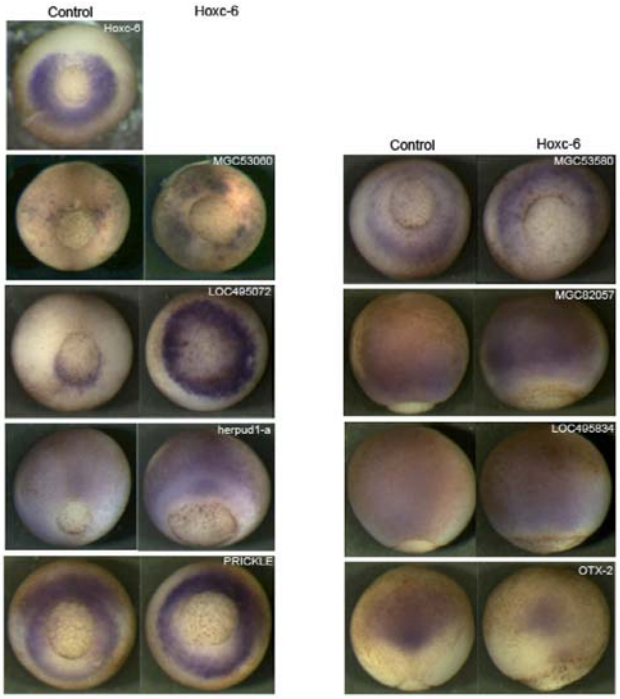
Table 1 Conservedly regulated probesets in Xenopus and Drosophila
A shows the conservedly upregulated Xenopus probesets in the first column. Probesets that detect the same gene are placed in one cell. The second column shows the Xenopus gene symbols. MGC85390 and MGC114680 are placed in one cell because they represent two alleles of the same gene of the pseudotetraploid *Xenopus laevis*. The third column shows the closest orthologues of *Drosophila melanogaster*.
B shows the conservedly downregulated Xenopus probesets in the first column. Probesets that detect the same gene are placed in one cell. The second column shows the Xenopus gene symbols. The third column shows the closest orthologues of *Drosophila melanogaster*.

Dorosophila) and 29 of the negative targets (15% in Xenopus and 4.6% in Drosophila) were conserved (table 1).
The conserved targets are interesting because they have a strong chance of mediating the known conserved functions of hox genes. Some of these functions are known to be mediated at least partly by protein-protein interactions between hox proteins and other proteins. But if transcriptional regulation is involved, we should have identified relevant

genes in this screen. The earliest known conserved function, which is underway during the stages during which we harvested our target mRNA's, is specification of regions and structures along the body axis. We classified our conserved targets into functional groups to try to identify target groups that might contribute to this function.

The first group we detected (11 probesets (ps)) was a group of transcription factors which were negative targets and most of which are known to be expressed anteriorly in the early embryo. These included the anterior neural plate genes Otx2, XHR-1B, Gbx-2, Pax6, several members of the dlx family of transcription factors, and the hindbrain gene Hoxd1.

The second conserved group (7 ps) was a group of genes that were concerned either with regulating or with mediating cell movement or cell polarity or cytoskeleton/cell adhesion. These were both positively and negatively regulated targets. The evident cell movement event during the developmental stages coinciding with or close to when our targets were harvested is gastrulation. These movement control genes ranged from Prickle, a regulating transcription factor, to effector genes including E-Cadherin.



The third conserved group (2 ps) were concerned with cell cycle regulation, perhaps reflecting a growth regulatory role for group 6 hox genes. Of these two genes, Polo kinase is concerned with mitosis and DNA damage checkpoints in G2/M. Cyclin G1 inhibits the G2/M transition.

Besides these groups, there were a couple of other groups. Ubiquitinylation genes (3 ps), perhaps reflecting a need to modify proteins for protein-protein interactions. Small GTPase signalling molecules (4 ps) contained both up and downregulated targets.

Besides the conserved targets described above, there were other targets belonging to each of the three major target gene

Fig. 1 In situ hybridisation with probes detecting Xenopus probesets that are conservedly regulated in Drosophila. Only probes that detected a up or downregulation are shown. In each panel the left embryo is showing the wildtype expression, the right embryo was injected with Hoxc6 mRNA. Hoxc6 wildtype expression is shown to indicate its normal expression domain. Shown are stage 12 embryos in vegetal view or dorsal view (probes MGC82057, LOC495834, and Otx2)

classes above that were non conserved targets either in Xenopus or Drosophila Eg: Antp/hoxc6 conservedly repress the expression of the brain genes Hoxd1, Otx2, Lim5, and, PAX6. Hoxc6 but not Antennapedia represses the brain genes XANF1, XANF2, Xgbx2. In Drosophila several cylins are upregulated wheras in Xenopus only cyclin G1 is upregulated. Besides the conserved functions above, there were interesting non conserved classes of targets typical either for Drosophila or Xenopus. These include; RNA binding proteins (Xenopus) and notch pathway genes (Xenopus).
To confirm the microarray results, and obtain spatial information about the expression of the putative target genes, we did in situ hybridisations with probes that detect the 23 probesets that are upregulated in both Drosophila and Xenopus. In Drosophila In Xenopus 17 probes were generated which represent the 23 conserved probesets upregulated upon Hoxc-6 overexpression. In situ hybridisation was done on stage 12 and tailbud stage embryos. Of the 17 probes 7 were clearly upregulated and the other probes were either to weak to detect or gave a strong general staining that made a conlusion about upregulation impossible (Fig 1). For the targets where we can clearly see an upregulation, we see this in the domain were hoxc-6 is normally expressed, suggesting a need for cofactors that are expressed in a hoxlike manner. Next to the upregulated genes we also examined the expression of Otx-2 in Hoxc-6 overexpressed embryos. This master gene for head development shows a clear downregulation.

Conclusions

We introduce a novel strategy for using functional genomics to investigate a developmental mechanism. This is to identify targets of the same developmental control gene in two widely different metazoan animals and to focus on the target genes and processes that are conserved between them as a route to understanding conserved developmental functions. The gene and animals chosen were: Antennapedia (Drosophila) and its orthologue hoxc6 (Xenopus). We first established that these two genes are functionally homologous and then examined the classes of common targets.
In Drosophila more targets were found than in Xenopus. This was true for all stages examined.
We don't know the reason why there were more targets in Drosophila than Xenopus. A technical difference is hard to rule out, but a possibility is that Drosophila has many specific hox functions that are not conserved in vertebrates. The greater conservation of negative than of positive targets indicates that the conserved functions are mainly to do with suppression rather than activation of gene activity.
Repression of mediators of head development was expected because this is a known function of hox genes including Antennapedia ref. This function is known from many other studies and it is interesting that some aspects of it work at another level: protein-protein interactions, e.g. between Antennapedia and Eyeless/Pax6 (Plaza et al., 2001; Plaza et al., 2008). We show here that this function can also act at the level of regulation of transcript abundance. Interestingly, this suppressive action is not restricted to more anterior genes. The very posterior hox gene Hoxb9/abdB is also a conserved negative target, perhaps supporting Ed Lewis and Walter Gehring's hypothesis that phenotypic suppression by Antennapedia reflects maintenance of a thoracic ground state. The anterior transcription facors repressed by Hoxc6 in Xenopus include several early brain factors, raising the possibility that this repressive function occurs exclusively in the developing nervous

system, in vertebrates. This suggests that one early function of Antennapedia/Hox6 is suppression of the development of the anterior part of the body and that this function is partly executed at the transcriptional level.

There is clear evidence that vertebrate gastrulation plays a role in setting up the A-P axis. See above. On the one hand, dorsal movement of progressively older gastrula mesoderm, expressing progressively more 5' hox genes, allows organiser signals to progressively mediate fixation of and transmission to neurectoderm of a 3' to 5' sequence of mesodermal hox codes in the Xenopus embryo (Wacker et al., 2004). On the other hand, the 3' to 5' identity of the hox codes expressed by a mesodermal cell determines the time during gastrulation at which it undergoes an EMT and ingresses into the embryo (in the chicken) (Iimura and Pourquie, 2006). The importance of gastrulation for axial patterning is less clear in Drosophila. Regulation of mediators of cell movement control and specifically of cell movements during gastrulation is not surprising because recent evidence shows that gastrulation and A-P patterning are intertwined in vertebrates. Notably that gastrulation movement, specifically the timing of the EMT needed for mesodermal ingression, is controlled by Hox genes. We have shown that both Antennapedia and hoxc6 regulate gastrulation and that their targets include a key gastrulation regulator: prickle. Prickle is known to be important for gastrulation in vertebrates (Takeuchi et al., 2003), and is a target of another hox gene; zebrafish hoxa1a (Rohrschneider et al., 2007). A cell polarity regulator, cap'n collar, was not previously studied in vertebrates, but is also a conserved target. Cap n' collar regulates cell polarity in Drosophila. It is known to be a target of another Drosophila hox gene; Deformed (Harding et al., 1995). These genes are thus an expected class of targets, giving confidence in the method. Perhaps the most surprising conclusion here is that, besides being found in vertebrates, where they are expected, hox gastrulation targets are found in Drosophila, where they were not expected. This suggests that at least elements of the vertebrate A-P patterning mechanism that acts during gastrulation, are conserved in Drosophila. This opens the possibility that, in addition to the known non conserved maternal effect-gap-segmentation gene hierarchy that sets up a hox pattern prior to gastrulation, Drosophila has a conserved patterning mechanism that either resets or reaffirms the pattern during gastrulation or is now non functional (atavistic).

The effect of Antp/Hoxc-6 on cell cycle regulation was also not surprising, considering the accumulating evidence that hox genes are growth regulators and that many of them have important roles in particular cancers (Cillo et al., 2001). In the context of axial patterning, there are reasons to think that Hox genes may be involved in local growth control in the axis.

The conserved targets between Drosophila and Xenopus shed some light on the functions of hox genes that are conserved between these animals, but the groups of non-conserved targets also reveal some species specific hox functions. The effect of Hoxc-6 overexpression on delta-notch pathway genes is a confirmation of recent findings. It has been shown that Hox labial group knockdown disturbs segmentation in Xenopus (Peres et al., 2006). Knockdown of Hoxc-6 also leads to disturbed segmentation (N. Bardine in press.)

We suggest that the RNA binding proteins are important in vertebrates but not Drosophila. because vertebrate hox genes, in contrast to the widely spaced Drosophila hox genes, are tightly clustered in a way that puts limits on possibilities for unique transcriptional control,

and leads to such phenomena as polycistronic transcripts (Mainguy et al., 2007). Postranscriptional control is therefore neccessary and these targets may be involved in this.
In conclusion, we have identified a novel conservation method for the genomic analysis of hox gene function (and the functions of other regulatory genes). Our findings indicate that identifying and characterising functions that are conserved in evolution can deliver a rich harvest of new insights.

Material and methods

Embryo preparation, culture and treatment

Xenopus embryos were obtained by in vitro fertilisation using standard procedures, cultured in 1% MMR containing gentamycin (0.5 g/l) or 0.1% MBS (Sive et al., 1998) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). *Xenopus* embryos used in whole mount in situ hybridisation analysis were fixed in MEMPFA (Harland, 1991) for 4 h at room temperature or overnight at 4^0C, washed once in methanol and stored at -20^0C in fresh methanol.

Microinjection

For microinjection of Hoxc-6 and GFP mRNA, one-cell-stage embryos were transferred to 4% ficoll in 1% MMR or 2% ficoll in 0.1% MBS. After injection the embryos were cultured in 4% ficoll, 1% MMR for 1 hour and subsequently transferred to 1% MMR to culture the embryos until the appropriate stage. For microarray analysis GFP injected embryos (control) were compared to Hoxc-6 injected embryos.

Antp overexpression

To obtain overexpression of Antp in Drosophila, nullo-GAL4 was crossed to UASAntp. For microarray analysis nullo-Gal4/+ (control) embryos were compared to nullo-Gal4/UASAntp embryos.

Micoarrays

At the appropriate stage embryos (both Xenopus and Drosophila) were homogenised in Trizol (Invitrogen) and RNA was isolated according to the manufacturer. A subsequent purification was done using a RNeasy kit from Qiagen. The integrety of the extracted RNA was determined by capillary electrophoresis on a RNA6000 Bio analyzer (Agilent Technologies). RNA was labeled, hybridised to microarrays, and data was extracted according to the manufacturer (Affymetrix, Santa Clara, USA). For Dosophila DrosGenome 1 chips were used, for Xenopus Xenopus laevis Genome Array chips were used.
For the analysis of the data, the Genespring GX v7.2 software package (Agilent Technologies) was used .
The whole experiment was repeated three times to obtain three independent experiments.

In situ hybridisation

Whole-mount in situ hybridisation on *Xenopus* embryos was performed as previously described (Harland, 1991), except that probe concentration was reduced to 40 ng/ml, hybridisation temperature raised to 65°C and antibody incubations done in 0.1 M Maleic acid, 0.15 M NaCl, 0.1% Tween-20, 1% blocking reagent (Roche), pH 7.5 with anti-Digoxigenin-AP, Fab fragments (Roche). Analysis of the staining pattern was performed in PBS.
Probes were made by RNA polymerase transcription of plasmids containing an EST of the appropriate gene. Plasmids were obtained from Imagenes GmbH, Berlin.

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