Universität Ulm
Abt. Biochemie
Leiter Prof. Dr. Dr. W. Knöchel
AG Prof. Dr. D Wedlich
Albert-Einstein-Allee 11
89081 Ulm

Specification of the Orthogonal Axes in *Xenopus laevis*: Maternal and Zygotic Genes Involved in Patterning the Dorsal Axis

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Dean: Professor Dr. Peter Gierschik

Expert 1: Professor Dr. Doris Wedlich

Expert 2: Professor Sally A. Moody, PhD

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Abbreviations

aa	amino acid
amp	ampicillin
bHLH	basic helix-loop-helix (family of transcription factors)
BMP	bone morphogenetic protein (signaling peptide)
bp	base pair
CFU	colony forming unit
CPE	cytoplasmic polyadenylation element
D1.1	dorsal animal midline blastomere at the 16-cell stage
DEPC	Diethylpyrocarbonate
Dig	digoxigenin
dNTP	deoxy ATP/CTP/GTP/TTP
dsh	dishevelled
DV	dorsal-ventral
e.g.	for example
eya	eyes absent (presumptive transcriptional co-activator)
FGF	fibroblast growth factor
Fig	figure
frz	frizzled (Wnt-receptor)
GFP	green fluorescent protein
H4	histone 4
HMG	high mobility group
kb	kilobase
LEF	lymphoid enhancer-binding factor (HMG box transcription factor)
MBT	mid-blastula transition (onset of zygotic transcription)
NAM	Normal Amphibian Medium (salt solution)
OD	optical density
PBS	phosphate buffered saline
PIF	activin A homologue (induces mesoderm)
rpm	revolution per minute

RT-PCR	reverse transcription succeeded by PCR
so	*Drosophila* sine oculis
Six	vertebrate homologues of so
TGF-β	transforming growth factor-β (signaling peptide)
UTR	untranslated region
UV	ultraviolet light
V1.1	ventral animal midline blastomere at the 16-cell stage
Wnt	signaling peptide
XWnt	*Xenopus* Wnt
Xfrzb	secreted *Xenopus* Wnt antagonist
XTcf	*Xenopus* T-cell factor (HMG box transcription factor)

1 Introduction

The formation of the body axes is an important initial step in development. Amphibians, particularly the South African clawed frog *Xenopus laevis*, are useful model organisms in which to study the initiation and the specification of the three body axes of the vertebrate: dorsal-ventral, anterior-posterior, left-right. As in invertebrates, the body axes are established early in development, after which embryonic cells build upon their spatial patterning to become arranged in orderly arrays of differentiated tissues. Axis determination and tissue patterning are intertwined processes that are regulated by a plethora of spatially and temporally restricted transcription factors, secreted and structural proteins and by their highly complex interactions. In addition, with the establishment of the orthogonal axes the embryo acquires a fate map. Hence, it is possible to predict during early cleavage stages which blastomeres and their progeny give rise to distinct tissues and organs of the tadpole (Moody, 1987a, b; Dale and Slack, 1987).

During the course of this dissertation two independent aspects of body axis development were investigated. Therefore, this work was subdivided into two parts. **Part (I)** focuses on the role of maternal genes in dorsal-ventral axis formation. **Part (II)** focuses on the characterization of a novel homeobox transcription factor that is involved in the patterning of the anterior-posterior axis in *Xenopus.*

1.1 **Part I**: Formation of the dorsal-ventral axis

The landmark experiment of how the vertebrate embryonic axis is formed was performed in the newt by Spemann and Mangold (1924). By transplanting the dorsal blastopore lip (the so-called Spemann Organizer) to a region fated to form ventral mesoderm, they showed that the transplanted tissue induced a second body axis (Spemann and Mangold, 1924). Hence, the Organizer contains all major factors needed to initiate gastrulation movements, to induce

dorsal-type mesoderm (notochord and somites), and to convert ectoderm to neural tissue. This experiment established the concept of induction, which is the ability of one tissue to influence the developmental pathway, e.g. differentiation, of another responding tissue region. Intense efforts have been undertaken to elucidate the formation of the Organizer and its molecular properties. Although the Spemann Organizer is essential as a molecular source for patterning the dorsal-ventral (DV) axis (reviewed in Harland and Gerhart, 1997), it has been demonstrated that patterning along the DV axis already occurs prior to the onset of zygotic transcription, which does not start until 6-8 hours after fertilization, (Newport and Kirschner, 1982a,b) by maternally provided mRNAs and proteins (Heasman, 1997; reviewed in Sullivan et al., 1999).

Of the known maternal dorsalizing molecules, β-catenin has been shown to be essential for the formation of the DV axis. This multifunctional protein is required for anchoring cadherins (transmembrane cell adhesion molecules) to the cytoskeleton (Ozawa et al., 1989; Kemler, 1993), and in addition it functions as a signal transduction component in the Wnt-signaling pathway (Fig.1). Depletion of β-catenin transcripts using antisense oligodeoxynucleotides resulted in embryos lacking an endogenous axis (Heasman et al., 1994). Wnts are secreted signaling molecules, functioning in critical developmental processes such as embryonic induction, generation of cell polarity and specification of cell fate (reviewed in Cadigan and Nusse, 1997). The activation of the Wnt-signaling cascade leads to the stabilization and concomitantly to the accumulation of β-catenin in the cytoplasm (Larabell et al., 1997). This accumulation triggers the translocation of β-catenin, in association with an HMG-box transcription factor of the LEF/Tcf family, from the cytoplasm into the nuclei on the dorsal side of the embryo (Schneider et al., 1996; Molenaar et al., 1996; Larabell et al., 1997). As a result, this complex activates the transcription of dorsalizing target genes after mid-blastula transition (MBT), the onset of zygotic gene transcription. These include the homeobox transcription factors *siamois* (Brannon et al., 1997) and *Xtwn* (Laurent et al., 1997), and

nodal-related 3 (*Xnr-3*), which encodes a TGF-β-type secreted signaling peptide (McKendry et al., 1997).

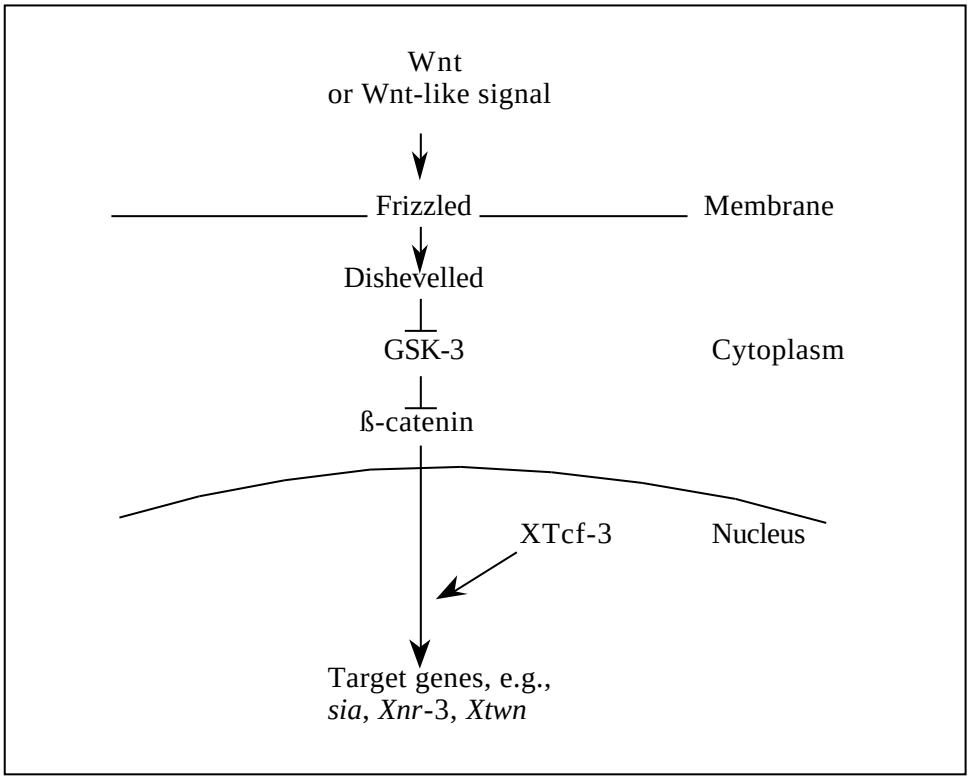


Fig.1: *Wnt* signaling pathway in dorsal axis formation in *Xenopus.* For simplification, additional factors involved are disregarded. The signaling cascade is activated by a Wnt/Wnt-like signal, which inhibits phosphorylation of β-catenin by glycogen-synthase-kinase-3 (GSK-3) resulting in the stabilization and accumulation of β-catenin in the cytoplasm. β-catenin then translocates into the nucleus and initiates in association with the HMG-box transcription factor XTcf-3 the transcriptional activation of dorsalizing target genes, such as *siamois* (*sia*), *Xenopus nodal-related-3* (*Xnr-3*) and *Xtwn*.

Except for *XWnt8b* none of the other known maternal *XWnts* that induces a complete secondary axis is expressed in a spatial pattern that could correlate with a possible function in activating the signaling cascade on the dorsal side of the embryo. However, although *XWnt8b* seems to be a likely candidate, *XWnts* per se might not be required for axis formation, even if they normally participate in this process, because overexpression of a dominant-negative *XWnt8* construct did not block endogenous axis formation (Hoppler et al., 1996). Furthermore, to date, a great number of factors that either activate or inhibit *XWnt* signaling are known and it is important to consider their temporal and spatial expression in regard to

the different roles of *XWnts* in distinct tissues (reviewed in Gradl et al., 1999). The complexity of Wnt signaling becomes even more evident with the most recent data demonstrating that Wnt and TGF-β signaling pathways interact during formation of the Spemann Organizer by sharing components of both pathways to activate transcription of *Xtwn* (Nishita et al., 2000).

1.1.1 Molecular asymmetry in the *Xenopus* embryo

In *Xenopus*, the initial egg's radial symmetry in the DV axis is abolished at fertilization. The DV axis is determined with sperm entry, which causes a rearrangement of the cortical cytoplasm (cortical rotation). The side toward which the vegetal pole cortex rotates thereby always becomes the dorsal side of the embryo (Gerhart et al., 1981). The subsequent specification of the DV axis requires the differential expression and activation of multiple genes that direct individual blastomeres and their lineages toward a dorsal or ventral fate. This fate decision is achieved by genes that either act as inducers or modify tissues to respond to inducing factors appropriately. The driving force of dorsal axis specification are molecular asymmetries that are maternal and zygotic in nature. These asymmetries can be established by either one or a combination of the following manners: by (1) mechanical translocation of maternal mRNAs and proteins (Miller et al., 1999; Rowning et al., 1997), (2) post-transcriptional (Schroeder et al., 1999), and (3) post-translational modifications (Thomsen and Melton, 1993).

Several molecules have been reported to be differentially expressed in *Xenopus* along the DV axis, as well as in the vegetal-animal axis (reviewed in Sullivan et al., 1999). For example, Epi1, a cell surface antigen present only on epidermal epithelium, was shown to be predominantly expressed in epidermal cells derived from ventral animal blastomeres (London et al., 1988). Furthermore, this DV polarized expression of Epi1 in animal blastomeres already exists at the 8-cell stage (London, et al., 1988). A pre-existent pattern along the DV axis in the animal hemisphere at the blastula stage (stage 8) also was revealed by Sokol and

Melton (1991). Prospective dorsal and ventral ectoderm regions isolated at stage 8 and cultured separately in a medium containing a mesoderm inducing activin A homologue (PIF) differentiated differently. The dorsal half developed eyes, neural tissue and notochord, whereas the ventral half developed ventrolateral mesodermal tissue such as epidermis, muscle and mesenchyme. Furthermore, the establishment of this differential competence to respond to a mesoderm-inducing factor depends on cortical rotation since prospective dorsal animal ectoderm isolated from embryos that were irradiated with UV light was no longer able to form dorsoanterior structures in the presence of PIF (Sokol and Melton, 1991). The pre-pattern in the animal hemisphere is established as early as the 8-cell stage. Ventral animal blastomeres dissected at the 8-cell stage formed atypical epidermis when cultured in activin A whereas dorsal animal blastomeres differentiated into dorsal mesodermal structures (Kinoshita et al., 1993). Also, only dorsal animal midline blastomeres (D1.1) (see Results Fig.3) but not ventral animal midline blastomeres (V1.1) of a 16-cell stage embryo elongated autonomously in a simple salt medium and differentiated notochord and cement gland (Gallagher et al., 1991). Interestingly, dorsal animal blastomeres of an 8-cell stage embryo do not yet exhibit this behaviour in the absence of a growth factor. However, they can be stimulated to elongate in a PIF containing medium (Hainski and Moody, 1996). Furthermore, the maternal RNA pool of D1.1 blastomeres is able to induce a secondary axis when ectopically expressed whereas overexpression of V1.1 RNA in ventral vegetal blastomeres does not elicit formation of secondary axial structures (Hainski and Moody, 1992).

The initial event that causes an asymmetric distribution of maternal dorsalizing factors is the rearrangement of the egg's cytoplasm due to cortical rotation during the first cell cycle of the fertilized egg (Gerhart et al., 1981; Kageura, 1997; Darras et al., 1997; Ding et al., 1998). The transport of two maternal members of the Wnt-signaling pathway, Dishevelled (Dsh) and β-catenin, to the future dorsal side was demonstrated to be mediated by a specific set of organelles that move along subcortical microtubule arrays (Miller et al., 1999; Rowning et al., 1997). Molecular asymmetries also can be established by differential activation or inhibition

of some mRNAs and proteins. For example, in the absence of a *Wnt*-like signal, maternal β-catenin protein becomes phosphorylated by glycogen synthase kinase-3 (GSK-3), which renders it unstable and targets it for degradation. Upon activation of the Wnt signaling cascade on the dorsal side of the embryo, GSK-3 function is inhibited leading to the stabilization of β-catenin in the cytoplasm, which in turn results in the activation of dorsalizing target genes. Although modifications of proteins can lead to molecular asymmetries, localization of mRNA or locally restricted (de-)activation of mRNAs is a more efficient way to localize protein products. Several activation mechanisms that render quiescent RNAs into the translatable pool are known (reviewed in Curtis et al., 1995). One of these regulative mechanisms is the elongation of the poly(A) tail of some mRNAs in the cytoplasm (reviewed by Richter, 1996). Uracil-rich sequence motifs located in the 3' untranslated region (3'UTR) of these mRNAs have been identified as cytoplasmic polyadenylation elements (CPE) that can direct the addition of adenyl-residues (Simon et al., 1992; Wickens et al., 1997). Additionally, the cytoplasmic poly(A) addition induces 5'cap ribose methylation, which was suggested to mediate the translational recruitment of maternal mRNA (Kuge and Richter, 1995). Simon et al. (1996) demonstrated that activin receptor (XAR7) mRNA, which contains two CPEs, undergoes cytoplasmic polyadenylation before MBT. Furthermore, deletion of the CPEs showed that these sequence elements are necessary for the induction of the phenotype resulting from overexpression of activin receptor mRNAs. The translational activation of maternal *XWnt11* mRNA also is regulated by a CPE (Schroeder et al., 1999). Additionally, its translation is spatially restricted and results in an asymmetric accumulation of XWnt11 protein along the DV axis with more protein being synthesized on the dorsal side of 32-64 cell stage embryos (Schroeder et al., 1999).

A temporally regulated expression of genes also can be accomplished by either an inhibitory element in the 3'UTR of their mRNAs (Robbie et al., 1995) or a sequence motif that negatively controls the polyadenylation status of specific messages (Tian et al., 1999). A novel sequence element in the 3'UTR of *XWnt8* mRNA directs the deadenylation and thereby the subsequent degradation of this message (Tian et al., 1999).

Another regulatory mechanism for controlling the activation of quiescent RNAs is translational masking. Translation is repressed by sequestering mRNA into mRNP particles (Standart and Jackson, 1994). These mRNAs themselves are competent for translation, but protein components of the mRNPs prevent mobilization of the mRNAs to ribosomes. Sequences that direct masking in *Xenopus* oocytes have not been defined, but specific sequences in the 3'UTR are necessary for unmasking at the correct time in development (Bouvet and Wolffe, 1994).

1.2 **Part II:** *Six* homeobox transcription factors and their role in head patterning

After the establishment of the orthogonal axes, a number of signaling molecules, including members of the FGF, TGF-β, and *Wnt* gene families and a great number of transcription factors function in patterning the three primary germ layers, ectoderm, mesoderm, and endoderm. Many of these signaling peptides and transcription factors have dual functions and are involved in both patterning the ectoderm along the anterior-posterior axis and in signaling cascades determining the DV axis at earlier developmental stages.

The ectoderm gives rise to diverse tissues, for example, epidermis, the central nervous system (CNS), the peripheral nervous system (PNS) and the placodes (olfactory, lens, otic, and lateral line). Key players in the determination of neural and non-neural ectoderm are the secreted dorsalizing factors Noggin (Lamb et al., 1993), Chordin (Sasai et al., 1994; 1995), and Follistatin (Hemmati-Brivanlou et al., 1994), and their interaction with bone morphogenetic protein 4 (BMP4), which acts as an antineurogenic factor and directly induces epidermis (Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995; Zimmerman et al., 1996; Fainsod et al., 1997). Regions with high concentrations of BMP4 antagonizing molecules undergo neural differentiation whereas regions predominantly expressing BMP4 form epidermis. Cerberus is yet another secreted protein expressed in the anterior endoderm that neuralizes ectoderm by inhibiting BMP4 (Bouwmeester et al., 1996; Piccolo et al., 1999). In

addition, it induces most anterior head structures by antagonizing Nodal and Wnt signals, which participate in trunk formation (Bouwmeester et al., 1996; Piccolo et al., 1999).

A large number of homeobox containing transcription factors have been identified that play a role in the determination of body segment identity (reviewed in Bürglin, 1994; Scott et al., 1989), but not many homeobox genes have been characterized that are involved in the development and patterning of the most anterior head structures. *Six* homeobox genes are likely candidates to function, at least during early development, in head patterning since, e.g., overexpression of certain *Six* genes results in the formation of ectopic head structures (Oliver et al., 1996; Loosli et al., 1999). Additionally, they can modulate expression domains of anterior marker genes when misexpressed (Kobayashi et al., 1998).

The major sensory organs of the head develop from placodes which arise via inductive interactions between different regions of the neural tube and the adjacent ectoderm. These epidermal thickenings are first identified as a band, the 'primitive placodal thickening' that surrounds the neural plate and neural crest area (Fig.2) (Knouff, 1935). The primitive band later disappears locally in the regions between the definitive placodes, which are the lens placodes, the olfactory placodes, the otic placodes and four epibranchial placodes (contributing to the trigeminal, facial, glossopharyngeal and vagus ganglia) and a dorsolateral series of lateral line placodes. The lateral line system perceives mechanical and electrical stimuli and is only present in fish and amphibians. The ganglia associated with the trigeminal, facial, glossopharyngeal and vagus nerves are distinct from other sensory ganglia in that they originate from two different components, the ectodermal placodes and the cranial neural crest (Hamburger, 1961; D'Amico-Martel and Noden, 1983).

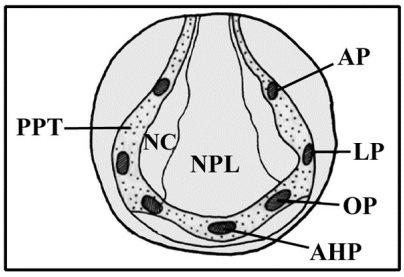


Fig.2: Head sensory organs develop from cranial placodes, which are epidermal thickenings located in the primitive placodal thickening (PPT) adjacent to the neural plate (NPL) and neural crest (NC). The placodal anlagen are: AP auditory placode; LP lens placode; OP olfactory placode; AHP adenohypophyseal placode (after Knouff, 1935).

Except for the lens placodes, which retain a strong epithelial character, all other placodes are neurogenic. The latter contribute to sensory receptors and their associated supporting cells, peripheral sensory neurons and some of them (olfactory and otic placodes) play a role in the morphogenesis of the vertebrate skull (Webb and Noden, 1993). The morphogenesis of placodes is characterized by two processes: invagination and delamination. Invagination of surface epithelium results in either a closed vesicle (e.g., lens and otic placodes) or in a larger cavity such as the olfactory placodes. Transformation of the epithelial mesenchyme into neuroblasts, or glia and non-sensory supporting cells takes place during the process of delamination. Placode-derived mesenchymal cells can either differentiate locally to form sensory neurons or sensory epithelia or they migrate to other parts of the embryo, as is the case for the lateral line placodes. The dorsolateral series of lateral line placodes consists of placodes giving rise to migratory receptor precursor cell populations (Stone, 1922; Landacre, 1927) that alternate with ganglion-forming placodes (reviewed in Winklbauer, 1989; Northcutt, 1992).

1.2.1 Molecular structure and functional domains of *Six* genes

The *Six* gene family constitutes a relatively new subclass of homeobox-containing transcription factors. A large number of vertebrate *Six* genes have been isolated from different organisms, many by their homology within the DNA binding domains to the *Drosophila sine*

oculis (*so*) gene, the first identified member of this family (Cheyette et al., 1994; Serikaku and O'Tousa, 1994). In addition to a diverged homeodomain, which is no more than 30% identical to previously described homeodomains (Oliver et al., 1995a), *Six* genes code for a highly conserved region of 115 aa, designated the Six domain (Cheyette et al., 1994). Among members of the *Six* subclass the homologies of the homeodomain and the Six domain are extremely high across species. The N- and C-terminal regions of Six proteins are more variable which might be, at least partly, due to alternative splicing. The DNA binding specificity of *Six* genes is not solely determined by the homeodomain but also by the C-terminal region of the Six domain, which was demonstrated for AREC3/Six4 in gel retardation assays (Kawakami et al., 1996a). This is similar to the POU homeobox transcription factors, in which both the POU domain and the homeodomain are necessary for the specific binding (Ingraham et al., 1990; Sturm and Herr, 1988).

The *so*/*Six* genes synergize with members of the *eyes absent* (*eya*) gene family, which are presumptive transcriptional co-activators, in activating target genes (Pignoni et al., 1997; Ohto et al., 1999). The interactions are specific and for an optimal transcriptional activation, the C-terminal region, which is the most heterologous among *Six* genes, is required (Ohto et al., 1999). Additionally, it was demonstrated that some Six proteins translocate certain Eya proteins into the nucleus (Ohto et al., 1999).

1.2.2 Expression and function of *Six* genes

Many homologues of *Drosophila so* have been isolated from different organisms within the past five years. Among those, the likely functional homologue of *so* is the vertebrate *Six3* (Oliver et al., 1995b; Seo et al., 1998a; Bovolenta et al., 1998; Loosli et al., 1998). Most of the *Six* genes were identified in mouse (*Six1-Six6*) (Oliver et al., 1995a,b; Kawakami et al., 1996a,b; Ohto et al., 1998; Jean et al., 1999), zebrafish (*Six3, Six6, Six7, Six8*) (Kobayashi et al., 1998; Seo et al., 1998a,b,c;), chicken (*cSix3, cSix4, Optx2*) (Bovolenta et al., 1998; Esteve and Bovolenta, 1999; Toy et al., 1998), *Drosophila* (*so*, *DSix3*, *DSix4*) (Cheyette et al., 1994; Serikaku and O'Tousa, 1994; Seo et al., 1999), and human (*Six1*, *Six3, Six5, Six6*) (Boucher et

al., 1995; 1996; Wallis et al., 1999; Gallardo et al., 1999). Only two members of the *Six* subclass have been described in *Xenopus* so far, *Six3* and the related *XOptx2* (Zuber et al., 1997; Zuber et al., 1999). A common feature of the heterologous *Six/so* genes is that they are expressed in restricted locations at specific developmental stages, suggesting a function in the differentiation of diverse tissues during early and late development. However, little is known about the molecular hierarchy of potential regulators and natural downstream target genes of this subclass of homeobox transcription factors in the various tissues in which these genes are expressed. A prominent function of known *Six* genes, deduced from their complex expression pattern, is their involvement in the development of head structures. Additionally, it has been demonstrated that some members also might function in myogenesis. *Drosophila so* and the mammalian *Six* genes have been studied in more detail. Since a thorough description of their dynamic expression patterns would extend beyond the topic of this work, their prominent features are summarized in Table 1.

Table 1: Prominent expression and function of *Drosophila so* and murine *Six* genes

	Organism	Expression	Function	References
sine oculis (so)	Drosophila	Eye disc Optic lobe	Development of the visual system	Cheyette et al., 1994
Six1	Mouse	Limb muscle, limb tendons	Patterning limb tendons	Oliver et al., 1995a
Six2	Mouse	Limb muscle, limb tendons	Patterning limb tendons	Oliver et al., 1995a
Six3	Mouse	Anterior neural plate, eye anlage	Eye and brain development	Oliver et al., 1995b
Six4 (AREC3)	Mouse	Neural tissue, particularly in the retina, skeletal muscle	Regulates the Na, K-ATPase α1 subunit gene, differentiation of neural retinal cells, possibly involved in myogenesis	Kawakami et al., 1996a; Ohto et al., 1998; Spitz et al., 1998
Six5 (DMAHP)	Mouse	Retina, skeletal muscle	Myogenesis	Kawakami et al., 1996b; Boucher et al., 1995; Murakami et al., 1998; Winchester et al., 1999
Six6/Optx2	Mouse	Embryonic eye field	Development of the visual system	Jean et al., 1999

The newly identified *Xenopus Six* gene presented in this work is likely the structural homologue of mammalian *Six1*. However, differences in the embryonic expression pattern discussed later implicate a possible shift of the predominant function for this gene determined by evolution.

Murine ***Six1*** is broadly expressed in head mesoderm and pharyngeal pouches during early stages, followed by expression in trunk axial and paraxial mesoderm (Oliver et al., 1995a). Later, the mesodermal expression persists and is joined by expression in the otic vesicle, Rathke's pouch and dorsal root ganglia. Transcripts of murine ***Six2*** also are detected early in head mesoderm, but unlike *Six1*, expression of *Six2* is not as widely distributed. Later in development both genes are expressed in a complementary fashion in the limb and limb tendons, with *Six1* largely restricted to the dorsal extensor tendons of the digits and *Six2* to the ventral flexor ones (Oliver et al., 1995a). At least one function of murine *Six1* is to activate myogenin through its MEF3 binding site and hence contribute to early steps of myogenesis (Spitz et al., 1998). Human *Six1* also is expressed in adult skeletal muscle (Boucher et al., 1996) but its role during the differentiation of this tissue has not yet been elucidated.

2 Rationale of experiments

Molecular asymmetries are the driving force in the determination and patterning of the three embryonic axes in *Xenopus laevis*. Many transplantation and explant studies demonstrated that maternal dorsalizing determinants are associated with the cortical cytoplasm in the vegetal pole of the fertilized egg and are translocated to the future dorsal side of the embryo before the first cleavage. In addition to mechanical translocation of maternal mRNAs and proteins, the spatially and temporally regulated expression of a number of genes that are either components of signaling pathways and/or modify tissues to respond to inducing factors appropriately is of critical importance.

For the elucidation of molecular asymmetries in the early *Xenopus* embryo, the aim of the first part (**Part I**) of the dissertation was to identify genes that are differentially expressed along the dorsal-ventral axis in animal blastomeres. Previous data suggest that there are maternal, dorsal axis inducing RNAs localized to dorsal animal midline blastomeres, which become translationally activated between the 8- and 16-cell stage (Hainski and Moody, 1996). It was of particular interest to: (1) investigate the translational activation mechanism of these dorsal-specific mRNAs and (2) identify these dorsalizing mRNAs in dorsal animal midline blastomeres of 16-cell stage embryos through sib selection techniques. If a novel gene is isolated, further characterization follows by determining its temporal and spatial expression pattern during development, and by investigating its biological role in misexpression studies.

The second part (**Part II**) of the dissertation focuses on the characterization of a novel homeobox-containing transcription factor, *XSix1,* which was isolated in a cDNA library screen independent from the one described in Part I. This screen was performed to specifically isolate and identify *Xenopus* homologues of the *Drosophila sine oculis* gene, which is the founding member of a relatively new subclass of homeobox genes (*Six* genes). To date, only two members of the *Six* gene family have been described in *Xenopus*, however, data

from other vertebrates strongly suggest that each organism expresses multiple *Six* genes. The elucidation of developmental functions of *Six* genes is still in the initial stages, but a general role in patterning head structures has been implicated. Thus, *Six* genes likely have important roles in patterning the anterior-posterior axis.

3 Materials and Methods

3.1 **Reagents**

Acetic Acid (glacial)	Fisher Scientific, Fair Lawn, NJ
Acrylamide (40%)	Fisher Scientific, Fair Lawn, NJ
(Acrylamide: Bis-Acrylamide 19:1)	
AG 501-X8 Resin	Bio-Rad Laboratories, Hercules, CA
Agarose Low EEO	Fisher Scientific, Fair Lawn, NJ
Ammonium Persulfate	Fisher Scientific, Fair Lawn, NJ
Ampicillin	Fisher Scientific, Fair Lawn, NJ
Benzyl Alcohol	Fisher Scientific, Fair Lawn, NJ
Benzyl Benzoate	Acros Organics, NJ
Boric Acid	Fisher Scientific, Fair Lawn, NJ
BSA (Albumin, Bovine)	Sigma, St. Louis, MO
Chloroform	Fisher Scientific, Fair Lawn, NJ
Diethylpyrocarbonate (DEPC)	Sigma, St. Louis, MO
DTT (DL-Dithiothreitol)	Sigma, St. Louis, MO
DAB (3,3'-Diaminobenzidine)	Sigma, St. Louis, MO
EGTA	Sigma, St.Louis, MO
(Ethylene Glycolbis(2-AminoethylEther) N,N,N',N-Tetraacetic acid)	
EDTA (0.5M)	Gibco BRL, Grand Island, NY
(Ethylene Diamine Tetraacetic acid)	
Ethanol	AAPER, Shelbyville, KY
Ficoll	Sigma, St. Louis, MO
Formaldehyde Solution (37%, w/w)	Fisher Scientific, Fair Lawn, NJ
Guanidine Thiocyanate	Fisher Scientific, Fair Lawn, NJ

HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])	Sigma, St.Louis, MO
Hydrogen Peroxide (30%)	Fisher Scientific, Fair Lawn, NJ
Igepal CA-630	Sigma, St. Louis, MO
N-Lauroylsarcosine	Sigma, St. Louis, MO
Luria Broth Base	Gibco, Paisley, Scotland
2-Mercaptoethanol	Fisher Scientific, Fair Lawn, NJ
Methanol	Fisher Scientific, Fair Lawn, NJ
3-(N-Morpholino) Propane-Sulfonic Acid	Fisher Scientific, Fair Lawn, NJ
O.C.T. Compound	Sakura Finetek USA Inc., Torrance, CA
Paraformaldehyde Purified "Granular"	Electron Microscopy Sciences, Fort Washington, PA
Phenol:H_2O (3.75:1, v/v; pH 5.0)	Gibco BRL, Grand Island, NY
Phenol:$CHCl_3$:Isoamylalcohol (25:24:1; pH 7.0)	Fisher Scientific, Fair Lawn, NJ
RNase Inhibitor (from human placenta)	Boehringer Mannheim, Indianapolis, IN
Ribonucleoside Vanadyl Complexes	Sigma, St. Louis, MO
Select Agar	Gibco, Paisley, Scotland
Select Peptone 140	Gibco, Paisley, Scotland
Select Yeast Extract	Gibco, Paisley, Scotland
Sodium Citrate	Fisher Scientific, Fair Lawn, NJ
TEMED	Promega Corp., Madison, WI
Terrific Broth	Gibco, Paisley, Scotland
Triton	Fisher Scientific, Fair Lawn, NJ
Trizma Base	Sigma, St. Louis, MO

Other fine chemicals were purchased from Fisher and Sigma.

3.1.1 Kits

ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit	Perkin-Elmer Corp., Foster City, CA
DIG RNA Labeling Kit (SP6/T7)	Boehringer Mannheim, Indianapolis, IN
mMessage mMachine (Large Scale In Vitro Transcription Kits for Synthesis of Capped RNAs)	Ambion, Austin, TX
Page-Plus (concentrate 40%) Electrophoresis Matrix (contains urea)	Amresco, Solon, OH
Poly(A)Quik mRNA Isolation Kit	Stratagene, La Jolla, CA
PUREscript RNA Isolation Kit	Gentra Systems, Inc., Minneapolis, MN
QIAprep Spin Miniprep Kit	QIAGEN, Santa Clarita, CA
QIAquick Gel Extraction Kit	QIAGEN, Santa Clarita, CA
Random Prime Labeling Kit	Gibco BRL, Grand Island, NY
SMART PCR cDNA Library Construction Kit	Clontech, Palo Alto, CA

3.1.2 Molecular Weight Markers

Lambda DNA/HindIII Marker	Promega, Madison, WI
100 bp DNA Ladder	Gibco BRL, Grand Island, NY
Low DNA Mass Ladder	Gibco BRL, Grand Island, NY

3.1.3 Radioactive Material

^{32}P-α-dCTP (370 MBq/ml)	Amersham, Arlington Heights, IL
^{32}P-γ-ATP (370 MBq/ml)	Amersham, Arlington Heights, IL

3.1.4 Bacteria

ElectroMAX DH10B Cells	Gibco BRL, Grand Island, NY
MAX Efficiency DH5α Competent Cells	Gibco BRL, Grand Island, NY

3.1.5 Plasmids

pCS2+	Peter Hausen, MPI, Tuebingen, Germany
pDH105	Richard Harland, Berkeley, CA

3.1.6 Enzymes

Deoxyribonuclease I	Gibco BRL, Grand Island, NY
Superscript II RNase H^- Reverse Transcriptase	Gibco BRL, Grand Island, NY
T4 DNA Ligase	Gibco BRL, Grand Island, NY
T4 Polynucleotide Kinase	Gibco BRL, Grand Island, NY
E.coli Poly(A) Polymerase	Gibco BRL, Grand Island, NY
Phosphatase, alkaline, shrimp	Boehringer Mannheim, Indianapolis, IN

Restriction enzymes were purchased from Promega (Madison, WI) and New England Biolabs (Beverly, MA).

3.1.7 Equipment

Beckman Model J-21 Centrifuge	Beckman Instruments, Inc., Fullerton, CA
Beckman Model L3-50 Ultracentrifuge	Beckman Instruments, Inc., Fullerton, CA
Borosilicate Glass (O.D.: 1.0 mm, I.D.: 0.50 mm, 10 cm length)	Sutter Instrument Co., Novato, CA
Cell Porator *E.coli* Electroporation System	Gibco BRL, Grand Island, NY

Centri-Sep Spin Columns	Princeton Separations, Inc., Adelphia, NJ
Flaming/Brown Micropipette Puller	Sutter Instrument Co., Novato, CA
Foto UV System, Camera Systems and Filters	Fotodyne, Inc., Hartland, WI
Gel Dryer Model 583	Bio-Rad Laboratories, Hercules, CA
GeneAmp PCR System 9700	Perkin-Elmer Corp., Foster City, CA
GeneQuant II, RNA/DNA Calculator	Pharmacia Biotech, Cambridge, England
G24 Environmental Incubator Shaker	New Brunswick Scientific Co., Inc., Edison, NJ
Nitex #3-100-47 (Nylon Monofilament Screening Fabrics)	TETKO Inc., Briarcliff Manor, NY
Nylon Membrane (Hybond N+)	Amersham, Arlington Heights, IL
MTX-150 High Speed Micro Refrigerated Centrifuge	TOMY Tech. USA, Inc., Palo Alto, CA
PLI-100 (for RNA injections)	Medical Systems Corp., Greenvale, NY
Polaroid B/W Prints, Type 667, 3000 ISO	Polaroid Corp., Cambridge, MA
Storm 860 Phosphorimager	Molecular Dynamics, Sunnyvale, CA
Thermolyne Type 16500 Dri-Bath	Scientific Industries, Inc., Bohemia, NY

3.1.8 Antibodies and other Biologic Reagents

12/101	Developmental Studies Hybridoma Bank, University of Iowa
Sheep-anti-mouse Ig (peroxidase-linked)	Amersham, Arlington Heights, IL
Tor70	Richard Harland, Berkeley, CA
Goat Serum	Gibco BRL, Grand Island, NY

3.1.9 Sequencing

DNA sequencing was partly performed by Veritas, Inc., Rockville, MD.

3.2 Methods

3.2.1 Manipulation and Maintenance of Embryos and Explanted Tissues

3.2.1.1 Buffer and Solutions

100% Steinberg's solution, pH 7.4 (5 liter):

NaCl	17.00 g
KCl	0.25 g
$Ca(NO_3)_2 \times 4H_2O$	0.40 g
$MgSO_4 \times 7H_2O$	1.02 g
Trizma HCl	3.30 g
Trizma Base	0.48 g

Normal Amphibian Medium (NAM), pH 7.4:

10 ml NaCl	(10x)	(5.84 g NaCl / 100 ml)
1 ml KCl	(100x)	(190 mg KCl / 10 ml)
1 ml $CaCl_2 \times 2H_2O$	(100x)	(290 mg $CaCl_2 \times 2H_2O$ / 10 ml)
1 ml $MgCl_2 \times 6H_2O$	(100x)	(410 mg $MgCl_2 \times 6H_2O$ / 10 ml)
1 ml $NaHCO_3$	(100x)	(420 mg $NaHCO_3$ / 10 ml)

Add dH_2O to 100 ml final volume.

This solution is good to use for two days.

3.2.1.2 Obtaining Embryos by Natural Fertilization

Two adult females and two adult males are injected with 500-800 I.U. and 150 I.U. of human chorion gonadotropin (HCG), respectively, the previous evening. Eggs are collected the next day, dejellied in a 2% cysteine hydrochloride solution (pH 8.4) for 4 minutes and rinsed thoroughly with 10% Steinberg's solution.

3.2.1.3 Obtaining Embryos by Fertilization *in vitro*

Two adult females are injected with 500-800 I.U. of HCG the previous evening. One adult male is sacrificed the next day to prepare its testes. The testes are macerated in 100% Steinberg's solution and the suspension is distributed over the eggs which were massaged from the female into a petri dish. Fill the dish with 10% Steinberg's solution after 2-3 minutes. Leave the eggs undisturbed for approximately 90 minutes before they start cleaving and can be dejellied.

3.2.1.4 RNA Injections into Cleavage Stage Embryos

Prepare microinjection pipettes by pulling autoclaved borosilicate glass (O.D.: 1.0 mm, I.D.: 0.50 mm, 10 cm length) in a micropipette puller to obtain the appropriate needle tip size (approximately 10 µm). Transfer the embryos to a dish containing 3% Ficoll (made up with 100% Steinberg's solution) and clay which holds them in the right position. The injection volume is determined by measuring the diameter of the droplet expelled from the micropipette tip with a stage micrometer. The desired volume depends on the size of the blastomere that is targeted and can vary between 1-4 nl. The highest RNA concentration that embryos can tolerate is about 5-8 ng. After injection the embryos are cultured in 50% Steinberg's solution.

3.2.1.5 Isolation and Culturing of Embryonic Tissues

Blastomeres and animal caps are dissected from cleavage stage embryos in 100% Steinberg's solution using two fine forceps. Explants are transferred to agar-coated (2% agar) 24-well dishes filled with NAM (pH 7.4) and cultured until sibling embryos have reached the appropriate developmental stage.

When activin is added to NAM to a final concentration of 10 ng/ml, blastomeres are kept in this solution for 1.5 - 2 h before they are transferred to plain NAM.

3.2.2 Molecular Biology Techniques

3.2.2.1 Buffer, Solutions, and Media

Terrific Broth (TB) medium

12 g tryptone

24 g yeast extract

dH_2O to 1l; autoclave

Luria Broth (LB) medium

10 g tryptone

5 g yeast extract

5 g NaCl

dH_2O to 1l; autoclave

For LB agar plates add 15 g agar. Autoclave solutions and add appropriate antibiotic when solutions have cooled to approximately 50 °C.

Ampicillin

50 mg/ml; add to medium to a final concentration of 50 µg/ml.

TE buffer

10 mM Tris/HCl, pH 7.5; 1 mM EDTA

TAE electrophoresis buffer (50x)

242 g Tris base

57.1 ml glacial acetic acid

100 ml 0.5M EDTA (pH 8.0)

dH_2O to 1l

TBE electrophoresis buffer (5x)

54 g Tris base

27.5 g boric acid

20 ml 0.5M EDTA (pH 8.0)

dH_2O to 1l

Loading Buffer (10x)

30% (v/v) glycerol, 0.25% (w/v) bromphenol blue

Ethidium bromide

10mg/ml in TAE buffer

Phenol/Chloroform

Phenol TE-buffer saturated, pH 8.0; 0.1% 8-Hydroxychinolin

Phenol H_2O-saturated, pH 5.0; 0.1% 8-Hydroxychinolin

Chloroform with 1/25 volume Isoamyl alcohol

3.2.2.2 Separation of DNA Fragments by Agarose Gel Electrophoresis

Prepare the gel, using 1x TAE electrophoresis buffer and 1% (w/v) electrophoresis grade agarose by melting in a microwave oven. Let the gel cool to ~50°C, add ethidium bromide at 0.5 µg/ml and pour it into a sealed gel casting platform. Add 1/3 volume loading buffer to the DNA samples before loading the gel. Electrophorese at 60-100 V. The bromphenol blue dye from the loading buffer comigrates with ~0.5-kb fragments and serves to judge the migration distance of the DNA fragments. The gel can be photographed directly on a UV transilluminator.

Lambda DNA/HindIII marker and a 100 bp DNA ladder were used as DNA molecular weight markers.

Fragment sizes of the lambda DNA/HindIII marker in kb (the amount of each fragment when 1 µg marker is loaded is given in parentheses):

23.1 (0.47 µg); 9.4 (0.19 µg); 6.6 (0.136 µg); 4.6 (94.5 ng); 2.3 (47.2 ng); 2.0 (41.1 ng); 0.56 (11.5 ng), 0.12 (2.5 ng).

Fragment sizes of the 100 bp DNA ladder in bp: 100; 200; 300; 400; 500; 600; 700; 800; 900; 1000; 1500.

3.2.2.3 (Non-)Denaturing Polyacrylamide Gel Electrophoresis

DNA fragments (<1000 bp) also can be separated by (non-)denaturing polyacrylamide gel electrophoresis. Polyacrylamide gels containing high concentrations of urea as a denaturant are used for the resolution of short (<500-nucleotide) fragments of single-stranded DNA or RNA. Such gels can resolve fragments differing by only a single nucleotide in length. These gels are used for sequencing, analyzing mRNA structure by S1 analysis, ribonuclease protection assays, or primer extension. The amounts given in the table below are for a total volume of 50 ml:

Acrylamide Concentration	5%	8%	10%	20%
Urea	21 g	21 g	21 g	21 g
40% Acrylamide	6.25 ml	10 ml	12.5 ml	25 ml
H_2O	33.5 ml	29.6 ml	27.2 ml	14.7 ml
5X TBE	10 ml	10 ml	10 ml	10 ml
10% APS	350 µl	350 µl	250 µl	250 µl
TEMED	25 µl	25 µl	25 µl	25 µl

If preparing a denaturing polyacrylamide gel, dissolve urea completely and filter the gel mixture before use.

3.2.2.4 Isolation of DNA Fragments from Agarose Gels

Isolation of DNA Fragments from Low Gelling/Melting Agarose Gels (Wieslander, 1979)

Electrophorese the DNA fragments on a 1% low gelling/melting agarose gel. Cut out the target band with a scalpel on a UV transilluminator (310 nm) and place it in a sterile microfuge tube. Add 1/10 volume 5M NaCl and melt the gel slice at 65°C for 5 minutes. Extract the DNA with salt-saturated phenol and reextract once with chloroform. Recover the aqueous phase and precipitate the DNA with 2.5 volumes of EtOH.

Extraction of DNA Fragments using the QIAquick Gel Extraction Kit

Electrophorese the DNA fragments on a 1% low gelling/melting agarose gel or on a standard agarose gel. Cut out the target band with a scalpel and place it in a sterile microfuge tube. Add 3 volumes of buffer QG to 1 volume of gel (100 mg ~ 100 µl). Incubate at 50°C until the gel slice has completely dissolved. Add 1 gel volume of isopropanol to the sample and mix. Place a QIAquick spin column in a 2-ml collection tube. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 minute at 13,000 rpm. Discard flow-through, add 0.5 ml of buffer QG to QIAquick column to remove traces of agarose and centrifuge for 1 minute. To wash, add 0.75 ml of buffer PE to QIAquick column and centrifuge for 1 minute. Discard the flow-through and centrifuge for an additional 1 minute to completely remove ethanol from buffer PE. Place QIAquick column into a clean 1.5 ml microfuge tube. To elute DNA, add 30-50 µl buffer EB or dH_2O to the center of the column, let stand for 1 minute, then centrifuge for 1 minute.

3.2.2.5 Restriction Digest of DNA

A standard reaction mixture with a total volume of 10 µl is made by adding DNA that had been dissolved in dH_2O, 1 µl 10x buffer and 1 µl of the appropriate restriction enzyme. The reaction mixture is incubated at 37°C for a minimum of 2 hours or overnight. The digested DNA is analyzed on an agarose gel to confirm complete cutting.

3.2.2.6 Repairing 3’ or 5’ Overhanging Ends to Generate Blunt Ends

Producing Blunt Ends Using the Klenow Fragment

The Klenow fragment, which consists of the C-terminal portion of *E. coli* DNA polymerase I, is used to create blunt ends from 5’overhanging ends. A standard reaction mixture with a total volume of 10 µl consists of up to 2 µg DNA, 1 µl 10 mM dNTP, 1 µl 10x buffer (500 mM Tris-Cl, pH 7.6; 100 mM $MgCl_2$), and 1 µl Klenow fragment (3-5 U). Incubate at 37°C for 30 minutes. Stop reaction by either heating 10 minutes at 75°C or by adding 1 µl of 0.5M EDTA.

Producing Blunt Ends of 3’ Overhanging Ends Using T4 DNA Polymerase

To 2 µg DNA in TE add $MgCl_2$ to a final concentration of 5 mM, 1 µl 10 mM dNTP and 4 U T4 polymerase. Incubate 15 minutes at 12°C and stop the reaction by adding 2 µl of 0.5M EDTA.

3.2.2.7 Dephosphorylation of DNA Fragments Using Shrimp Alkaline Phosphatase (SAP)

Add 1/10 volume of 10x SAP buffer to DNA fragments. Use 1 U SAP for 50 ng DNA. Incubate for 1 hour at 37°C. Inactivate SAP by incubating for 15 minutes at 65°C.

3.2.2.8 Ligation

A standard ligation mixture is made by adding 4 µl of 5x ligase reaction buffer to 10-100 ng total DNA. The optimal insert:vector ratio (molar) is 3:1. Add 0.1 U T4 DNA ligase and bring the reaction volume to 20 µl. Incubate the reaction mixture at 23-26°C for 1 hour. Higher ligation efficiency is obtained when the reaction mixture is incubated at 14°C overnight.

3.2.2.9 Random Primed Labeling of DNA Fragments

25-50 ng DNA is denatured at 95°C for 5 minutes. Prepare reaction mixture with 15 µl buffer (contains hexamer primers), 2 µl of each dATP, dGTP, dTTP, 5 µl α-^{32}P-dCTP, and 1 µl Klenow fragment (DNA polymerase). Add the denatured DNA fragment and incubate the reaction at 37°C for 1 hour. The reaction is stopped by adding 5 µl stop-buffer. Denature the mixture again at 95°C for 5-10 minutes before adding it to the hybridization buffer. This method yields radioactively labeled DNA fragments of ~80-120 bp in length.

3.2.2.10 5'DNA Terminus Labeling (Phosphate Exchange Reaction)

Combine 5 µg DNA with 5 µl 5X exchange reaction buffer, 3 µl γ-^{32}P-ATP (10 µCi/µl), and 1-5 U T4 kinase. Add dH_2O to a final volume of 25 µl and incubate the reaction mixture at 37°C for 30 minutes. Stop the reaction by adding 1 µl of 0.5M EDTA and spin briefly.

3.2.2.11 First Strand cDNA Synthesis

A 20-µl reaction volume can be used for 1-5 µg of total RNA. Combine 1 µl oligo $(dT)_{12-18}$ (500 µg/ml) with 1-5 µg total RNA and add nuclease-free H_2O to 12 µl. Heat mixture to 70°C for 10 minutes and quick chill on ice. Collect the contents of the tube by brief centrifugation and add 4 µl 5x first strand buffer, 2 µl 0.1 M DTT, and 1 µl 10 mM dNTP mix. Mix gently and incubate at 42°C for 2 minutes. Add 1 µl (200 units) of reverse transcriptase (e.g. Superscript II from Gibco BRL) and incubate 50 minutes at 42°C. Inactivate the reaction by heating at 70°C for 15 minutes. The cDNA can now be used as a template for amplification in PCR.

3.2.2.12 Polymerase Chain Reaction (PCR)

The polymerase chain reaction is a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA. The following basic protocol is a general guideline. Optimal reaction conditions vary (cycle number, primer annealing temperature, concentration of *Taq* DNA polymerase, primers, $MgCl_2$, and template DNA) and need to be optimized for each template and primer set. A standard 50 µl reaction mixture using PCR Supermix (Gibco) contains 2-3 µl template DNA, 1 µl of each primer (10 µM), 1 µl ^{32}P-α-dCTP (optional), and 44-45 µl PCR Supermix (20 mM Tris-HCl, pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$, 200 µM dNTPs, 20 U recombinant Taq DNA polymerase/ml). Overlay the reaction mixture with a drop of mineral oil and place the tubes in a thermal cycler. Denature the DNA at 94°C for 4 minutes and perform 30 cycles of PCR amplification as follows: denature at 94°C for 30 seconds, anneal at 55°C for 1 minute, and extend at 72°C for 1 minute. Incubate for an additional 7 minutes at 72°C and maintain the reaction at 4°C.

3.2.2.13 Construction of a cDNA Library with the SMART PCR cDNA Library Construction Kit (Clontech)

Basic Principle

This PCR-based method for making a cDNA library is suitable when only small amounts of RNA are available. Total RNA (0.05-1.0 µg) or polyA^+ RNA (0.025-0.5 µg) is reverse transcribed using a modified oligo (dT) primer (oligo $(dT)_{30}N_{-1}N$; N = A, G, C, or T; N_{-1} = A, G, or C). The provided SMART oligonucleotide binds to the 7-methylguanosine cap structure present on the 5' end of all eukaryotic mRNAs and serves as an extended template at the 5' end for the reverse transcriptase. The resulting full-length single stranded cDNA contains in most cases the complete 5' end of the mRNA and the SMART oligonucleotide then serves as a PCR priming site in the subsequent amplification. The amplified cDNAs are blunt-ended with T4 DNA polymerase, followed by the ligation of the provided adaptors,

which have a pre-existing EcoRI “sticky end”, as well as NotI and SalI restriction sites. The pool of cDNAs is phosphorylated at the EcoRI sites using T4 polynucleotide kinase and size-fractionated to remove small (<0.5 kb) cDNA fragments, unincorporated primers and unligated adaptors. After the size-fractionation the cDNAs are ready to be ligated into a suitable EcoRI-digested, dephosphorylated vector.

For the construction of two plasmid cDNA mini-libraries the protocol above was slightly modified. In order to perform directional cloning of the cDNAs two oligo(dT) primers were synthesized for the construction of each library that contained an XbaI and a NotI restriction site respectively. Total RNA (0.5-1.0 μg) was isolated from hundreds of D1.1 blastomeres and reverse transcribed using either one of the synthesized oligo(dT) primer. After the adaptor ligation the cDNAs were cut with XbaI to create cDNAs with EcoRI and XbaI “sticky ends”. Following the size fractionation the cDNAs were ligated into the expression vector pCS2+. cDNAs for the second library were cut with SalI and NotI after the adaptor ligation to create the appropriate “sticky ends”. The cDNAs were size fractionated and ligated into the expression vector pDH105. Cells (ElectroMAX DH10B) were transformed by electroporation.

3.2.2.14 Transformation of Competent Cells

Electroporation

Solutions and Medium:

S.O.B. Media

20 g Tryptone

5 g Yeast Extract

0.584 g NaCl

0.186 g KCl

Add ddH_2O to 1l. Adjust pH to 7.0 with NaOH and autoclave.

2M Mg^{2+}-solution

20.33 g $MgCl_2x6H_2O$

24.65 g $MgSO_4x7H_2O$

Add ddH_2O to 100 ml. Autoclave or filter sterilize.

2M Glucose

36.4 g Glucose

Add dd H_2O to 100 ml. Filter sterilize.

S.O.C. Medium

Add 1 ml of 2M Mg^{2+}-solution and 1 ml of 2M glucose to 98 ml of S.O.B. medium.

50x TE buffer

12.11 g Tris-base

3.72 g Na_2EDTA

Dissolve Tris and EDTA in 100 ml dH_2O. Titrate with HCl to pH 7.4. Bring volume to 200 ml with dH_2O. Filter-sterilize or autoclave.

DNA used for electroporation into *E.coli* must be free of phenol, ethanol, and detergents. In addition, electroporation is more efficient if the DNA is in a buffer of very low conductivity, such as 0.5x TE buffer. Place the microelectroporation chambers on ice. Add 1 µl of DNA to 20 µl of ElectroMAX DH10B Cells and mix gently with the pipet tip. Pipet the DNA-cell mixture between the bosses of the microelectroporation chamber. Place the loaded chamber into the refrigerated single safe base. Apply ~2.5 kV to the chamber with the Cell-Porator *E.coli* Electroporation Pulser. Remove the sample from the electrode bosses, and place into a culture tube containing 1 ml of S.O.C. medium. Incubate samples for 1 hour in a shaker at 37°C and 225 rpm. Plate the cells on LB agar plates containing the appropriate antibiotic.

Heat Shock Transformation

Add 1 µl (1-10 ng) DNA to 100 µl competent cells (e.g., DH5α) and mix gently. Incubate DNA-cell mixture on ice for 30 minutes. Heat-shock cells 45 seconds in a 42°C water bath. Place on ice for 2 minutes. Add 900 µl of room temperature S.O.C. medium and shake at 37°C and 225 rpm for 1 hour. Dilute the reaction as necessary and spread 100-200 µl of this solution on LB agar plates containing the appropriate antibiotic.

3.2.2.15 Expansion of Plasmid cDNA Libraries

LB medium (2X)

20 g Tryptone

10 g Yeast Extract

10 g NaCl

dH_2O to 1l

2X LB medium with 12.5% glycerol (200ml); autoclave

This protocol for expansion of a plasmid cDNA library is adapted from the Superscript Plasmid System for cDNA Synthesis and Plasmid Cloning Kit from Gibco. Add 450 ml of 2X LB medium to 1.35 g SeaPrep agarose (FMC BioProducts) while stirring to avoid the formation of large clumps of agarose. Prepare 2-4 bottles. After autoclaving for 30 minutes the bottles are cooled in 37°C water bath for about 2 hours until the medium reaches 37°C. Add ampicillin to 200 µg/ml or carbenicillin to 50 µg/ml (preferred antibiotic since it reduces satellite formation). To each bottle add 4×10^5 to 6×10^5 CFU and mix thoroughly on a stir plate for 2 minutes. Place the bottles in an ice-water bath such that the water in the bath is at the same level as the medium in the bottle and incubate for 1 hour. Place the bottles in a gravity flow incubator set at 30°C for 40-45 hours without disturbance. Handle bottles of semi-solid agar containing suspended colonies gently. Pour contents of bottles into centrifuge

bottles and spin at 9500 rpm for 20 minutes at room temperature. Pour off supernatant and resuspend the cells in 20-25 ml 2X LB glycerol (12.5%). Prepare aliquots and store at -70°C.

3.2.2.16 Plasmid Preparation with QIAprep columns

The QIAprep plasmid preparation procedure uses the modified alkaline lysis method of Birnboim & Doly (1979). Bacteria are lysed under alkaline conditions, and the lysate is subsequently neutralized and adjusted to high-salt binding conditions in one step, ready for purification on the QIAprep silica-gel membrane. Centrifuge 1.5-3 ml of an overnight culture at 10,000 rpm at 4°C. Resuspend pelleted bacterial cells in 250 µl of Buffer P1 (contains RNase A). Add 250 µl of Buffer P2 and invert the tube gently to mix until the solution becomes viscous and slightly clear (do not let reaction exceed 5 minutes). Do not vortex, as this will result in the shearing of genomic DNA. Add 350 µl of Buffer N3 and invert the tube gently immediately, to avoid localized precipitation. Centrifuge at 14,000 rpm for 10 minutes. Place a QIAprep spin column in a 2 ml collection tube and apply the supernatants to the column. Centrifuge at 13,000 rpm 30-60 seconds. Discard the flow-through. To remove trace nuclease activity the column can be washed with 0.5 ml of Buffer PB followed by centrifuging for 30-60 seconds. Discard the flow-through. Wash QIAprep spin column by adding 0.75 ml of Buffer PE and centrifuging 30-60 seconds. Discard the flow-through, and centrifuge for an additional 1 minute to remove residual wash buffer. Place QIAprep column in a sterile 1.5 ml microfuge tube. To elute DNA, add 50 µl of Buffer EB (10 mM Tris-HCl, pH 8.5) or ddH_2O to the center of each column, let stand for 1 minute, and centrifuge for 1 minute.

3.2.2.17 Sib Selection (after Lemaire et al., 1995)

Sib selection is a functional assay for isolating dorsal axis inducing genes by screening pools of cDNAs for their ability to induce secondary (par)axial structures. After the construction of a plasmid cDNA library, a specified number of colonies (10,000 - 100,000) is plated on

LB agar plates (containing the appropriate antibiotic). The colonies are scraped off the plates (5 ml TB medium for 100 mm petri dishes; 10 ml TB medium for 150 mm petri dishes) the next day. 800 µl of the bacterial suspension are added to 200 µl sterile glycerol, vortexed thoroughly and stored at -80°C. The remaining bacterial suspension is added to 20 ml TB (+ ampicillin) and grown for 6-8 hours at 37°C and 220 rpm. After a plasmid midi-preparation, the pooled cDNAs are linearized and used for in vitro synthesis of capped RNAs. When screening for dorsalizing messages, the RNA pools are injected into the ventral vegetal blastomeres of cleavage stage *Xenopus* embryos. The injected embryos are cultured and scored for induction of secondary (par)axial structures. The bacterial stock of the RNA pool with the highest axis inducing activity is replated in a higher dilution, and new pools of RNA are obtained as described above. This procedure is repeated until cDNA inserts of single colonies can be isolated and characterized.

3.2.2.18 Dideoxy (Sanger) DNA Sequencing

For the preparation of the template DNA, standard primers (SP6, T3, T7, M13) or gene specific primers are used in the dideoxycycle sequencing protocol recommended by Perkin Elmer and outlined in the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit. This method uses a thermostable Taq polymerase to incorporate dye-coupled dideoxynucleotides into extending strands of DNA. In preparation for sequencing, the DNA sample is purified using Centri-Sep spin columns. Hydrate the spin columns with distilled H_2O for 30 minutes at room temperature and spin the column at 3000 rpm for 2 minutes to remove interstitial fluids before applying the products of the sequencing reaction. Spin the column at 3000 rpm for 2 minutes and dry the sample in a vacuum centrifuge. Resuspend the DNA in loading buffer composed of 25 mM EDTA (pH 8.0) in deionized formamide with 50 mg/ml blue dextran. Denature the DNA at 90°C for 3 minutes before loading. Apply samples to a 6% denaturing polyacrylamide gel and electrophorese according to the ABI 377 manual for seven hours. Sequence data is automatically collected as the chemoluminescence passes

through a laser beam contained in the automated system and the detected dye-terminator is recorded and analyzed by the ABI software.

3.2.3 Good Laboratory Practices when Working with RNA

Gloves are to be worn throughout the procedures to protect the RNA from degradation by RNases. Prepare all buffer and solutions with DEPC-treated H_2O. To prepare DEPC-treated H_2O add 1 ml DEPC to 1l H_2O, let it stir overnight at room temperature and autoclave it the next day. Glassware and spatulas are baked at 180°C for at least 2 hours. The gel electrophoresis apparatus is treated with 50 mM NaOH or 10% SDS for at least 20 minutes and rinsed thoroughly with DEPC-treated H_2O before running a gel.

3.2.3.1 RNA Isolation from Embryos and Explanted Tissues

Denaturing Solution:
4M guanidinium thiocyanate
25 mM sodium citrate, pH 5.2
0.5% sodium N-laurylsarcosine
0.1M β-mercaptoethanol

Other Solutions:
3M sodium acetate, pH 5.0
Phenol H_2O-saturated (3.75:1, v/v; pH 5.0)
Chloroform

This protocol for isolation of total RNA is adapted from the Micro-Scale Total RNA Separator Kit from Clontech. Dissolve up to 25 mg of tissue in 500 µl denaturing solution by vortexing. Pass the lysed cell suspension through a 26-30 gauge syringe twice, to shear genomic DNA. Add 200 µl 95% ethanol and incubate the cell suspension on ice for 5

minutes. After spinning at 10,000 rpm at 4°C for 15 minutes redissolve the pellet in 150 µl of denaturing solution by vigorous vortexing. Extract the RNA by adding 1/10 volume of 3M sodium acetate and an equal volume of phenol:chloroform (10:4). Add 2.5 volumes of 95% ethanol and precipitate the RNA overnight at -20°C. Resuspend the dried pellet in an appropriate volume of DEPC-treated H_2O.

Isolation of total RNA with Purescript RNA Isolation Kit

Add tissue (5-10 mg) to 300 µl Cell Lysis Solution and homogenize quickly with a microfuge tube pestle. Add 100 µl Protein-DNA Precipitation Solution to the lysate, invert gently and place the tube into an ice bath for 5 minutes. Centrifuge at 15,000 rpm for 3 minutes. Transfer the supernatant to a new, sterile microfuge tube and centrifuge for 3 minutes. Pour the supernatant into a sterile microfuge tube containing 300 µl 100% isopropanol. Invert gently 50 times and centrifuge at 15,000 g for 3 minutes. Wash with 70% EtOH and resuspend the dried pellet in DEPC-H_2O.

If a low RNA yield is to be expected, add glycogen (1 µl 20 mg/ml glycogen per 600 µl isopropanol) as an RNA carrier to the isopropanol.

3.2.3.2 Purification of Poly(A+) RNA from Total RNA using Oligo(dT) Cellulose Push Columns

Heat total RNA at 65°C for 5 min and subsequently place it on ice. Add 10X sample buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 5M NaCl) to a final concentration of 1X. Prepare the oligo(dT) cellulose columns by repeatedly pushing 200 µl of high salt buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 0.5M NaCl) slowly through the column. Apply total RNA to the column and push through in the same manner. Collect the flow-through in a sterile Eppendorf tube and reapply it to the column. After two washes with 200 µl of high-salt buffer and three washes with 200 µl of low-salt buffer (10 mM Tris-HCl, pH 7.5; 1 mM

EDTA; 0.1M NaCl) the poly(A+) RNA is eluted with four 200-µl aliquots of elution buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) that has been preheated to 65°C. If the RNA concentration is too dilute for subsequent use, precipitation will be required. Add 10X sample buffer to a final concentration of 1X and mix well. Add 2.5 volumes of ice-cold 95% EtOH and store at -20°C overnight. Resuspend the precipitated RNA in an appropriate volume of DEPC-H_2O.

3.2.3.3 Post-transcriptional Polyadenylation of Poly(A-) RNA In Vitro (Wormington, 1991)

Solutions and Buffers (prepare RNAse-free):

TE Buffer:

10 mM Tris-HCl, pH 7.5

1 mM EDTA

RNA Extraction Buffer (TNES):

100 mM Tris-HCl, pH 7.5

300 mM NaCl

10 mM EDTA

2% (w/v) Sodium Dodecyl Sulfate (SDS)

2 X Poly(A) Polymerase Buffer:

100 mM Tris-HCl, pH 8.0

500 mM NaCl

20 mM $MgCl_2$

2 mM $MnCl_2$

All reagents should be added to a sterile 1.5 ml microcentrifuge tube at 4°C in the following order:

25 µl	2 X poly(A) polymerase buffer
1 µl	100 mM dithiothreitol (DDT)
50 units	placental ribonuclease inhibitor
1 µl	2.5 µg/µl BSA in H_2O
1 µl	2.5 mM ATP in TE
5 µl	RNA at 0.5-1.0 µg/µl in H_2O

Add H_2O to a final volume of 50 µl, then add 3 units of *E. coli* poly(A) polymerase. *E. coli* poly(A) polymerase adds 100-325 adenylate residues to poly(A-) RNA in vitro. Mix the components gently and incubate the reaction for 5 minutes at 37°C. Add 50 µl of TNES and extract the polyadenylated RNA with phenol-chloroform. Precipitate the RNA with 2.5 volumes of EtOH at –20°C and resuspend the pellet in DEPC-H_2O.

3.2.3.4 *In vitro* Transcription for Synthesis of Capped RNAs

All reagents should be added to a sterile 1.5 ml microcentrifuge tube at room temperature (Spermidine in the transcription buffer can lead to precipitation of the template DNA if the reaction is assembled on ice) in the following order:

x µl	nuclease-free dH_2O for a final reaction volume of 20 µl
2 µl	10X transcription buffer
10 µl	2X ribonucleotide mix
1 µl	(optional) [α-^{32}P]-labeled ribonucleotide
1 µg	linearized template DNA
2 µl	10X enzyme mix

Mix contents gently and incubate the reaction at 37°C for at least 1 hour. Add 1 µl of RNAse-free DNase 1 (2U/µl) and incubate at 37°C for 15 minutes. Stop the reaction by adding 115 µl of nuclease-free dH_2O and 15 µl of ammonium acetate stop solution. Extract the RNA with phenol/chloroform. Precipitate the RNA by adding 1 volume of isopropyl alcohol.

3.2.3.5 DNase-Treatment of RNAs

A standard reaction mixture with a total volume of 10 µl is made by adding 1 µl 10X DNase-buffer and 1 µl DNase to 1 µg RNA. The mixture is incubated at room temperature for 15 minutes and the reaction is stopped by adding 1 µl 25 mM EDTA (RNase-free) followed by a final incubation at 65°C for 15 minutes to heat inactivate the enzyme.

3.2.3.6 Northern Blot Hybridization of RNA Denatured by Glyoxal/DMSO Treatment

0.1M Na-phosphate-buffer, pH 7.0:

3.9 ml 1M NaH_2PO_4; 6.1 ml 1M Na_2HPO_4; add DEPC-treated H_2O to 100 ml.

Phosphate-buffer:

0.5M Na_2HPO_4 x $7H_2O$; adjust pH to 7.2 with ~ 2 ml 85% H_3PO_4

Transfer buffer:

75 mM NaOH

(Pre-)Hybridization buffer:

Dissolve 14 g SDS in 60 ml DEPC-treated H_2O. Add 100 ml 0.5M phosphate-buffer and 3 g BSA. Add DEPC-treated H_2O to 200 ml.

Wash buffer:

Dissolve 5 g SDS in DEPC-treated H_2O and 20 ml 0.5M phosphate-buffer. Add DEPC-treated H_2O to 500 ml.

Glyoxal:

Deionize with ion exchange resin AG 501-X8 (pH >5.0). Glyoxal denatures RNA and prevents the formation of secondary structures during electrophoresis. Furthermore it yields better results when the RNA is transferred to the nylon membrane.

Loading Dye:

50% glycerol; 10 mM Na-phosphate-buffer; 0.25% bromphenol blue

Agarose Gel Electrophoresis

Combine 5.4 µl glyoxal, 16 µl DMSO, 3 µl 0.1M Na-phosphate-buffer and 5.4 µl RNA (up to 10 µg) in a sterile microcentrifuge and incubate at 50°C for 1 hour. An RNA molecular weight standard needs to be treated alike. In the meantime prepare a 1-1.4% agarose gel with 10 mM Na-phosphate-buffer. Cool the RNA samples on ice before adding loading dye. Electrophorese at 60 V and change buffer (10 mM Na-phosphate-buffer) every 30 minutes to avoid a pH gradient. If the pH rises above 8, the glyoxal dissociates from the RNA. Following the electrophoresis, stain the gel (or the marker lane only) with ethidium bromide and photograph it with a ruler so that the band positions can later be identified on the membrane.

RNA Transfer onto a Nylon membrane

Place an object that can serve as a platform in a glass dish filled with 7.5 mM NaOH. Soak Whatman 3MM paper with 7.5 mM NaOH and cover the platform by leaving two sides of the paper submerged in the transfer buffer. Place the gel on the platform and cover everything

around the gel with plastic wrap. Cut the nylon membrane (Hybond N+) to the size of the gel, soak it in transfer buffer and position it on the gel. Soak three pieces of Whatman 3MM paper in transfer buffer and place them on the nylon membrane. Assemble a stack consisting of Whatman 3MM paper and paper towels. Place a glass plate on top of the stack and apply pressure overnight. Capillary action transfers the RNA onto the nylon membrane. UV-crosslink (254nm; 0.12 J/cm^2) the RNA to the nylon membrane.

Hybridization

Incubate the membrane in pre-hybridization buffer rotating at 65°C for 4 hours. Replace the solution with hybridization buffer and add the radioactive labeled probe which had been denatured previously at 95°C. The hybridization is done at 55-65°C overnight (16 hours). The membrane is washed 4 times at 65°C for 30 minutes each. Use hybridization buffer for the first wash and wash buffer for the following rounds. The signal on the membrane can be detected by phosphorimager analysis or autoradiography.

3.2.3.7 Isolation of Polysomes

Polysome Buffer:

300 mM NaCl; 20 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$; 0.5% Igepal CA-630; 10 mM Ribonucleoside Vanadyl Complexes

Extraction Buffer:

4M guanidinium thiocyanate; 50 mM Tris-HCl, pH 7.6; 5 mM EDTA; 0.5% sodium N-laurylsarcosine; 0.1M β-mercaptoethanol

STE-Buffer:

10 mM Tris-HCl, pH 7.6; 5 mM EDTA; 1% SDS

The following protocol for isolating polysomal RNA is adapted from McGrew et al. (1989) and Wormington (1991). RNA that is associated with polysomes and hence is being translated can be separated from non-translated RNA by centrifugation in a sucrose gradient. To avoid overloading the gradient, RNA from no more than 50 cleavage stage embryos (or oocytes) should be loaded on the sucrose solution.

Homogenize up to 50 cleavage stage embryos or oocytes in 750 µl polysome buffer and centrifuge at 12,000 rpm for 15 minutes at 4°C. The supernatant is loaded on a sucrose gradient, which is composed of 5 ml 35% and 4 ml 10% sucrose in polysome buffer (SW41 tubes). Centrifuge at 29,000 rpm for 2 hours at 4°C using a Beckman SW 41 swinging bucket rotor. Dissolve the pellet in 0.5 ml extraction buffer, extract with phenol/chloroform, precipitate and resuspend the precipitated RNA in STE buffer. Repeat phenol/chloroform extraction and precipitate again. The RNA in the supernatant/sucrose gradient is precipitated with 2 volumes ethanol for 30 minutes at 4°C and treated by analogy. The purified RNA is resuspended in nuclease-free H_2O and can be used for Northern analyses or RT-PCR. A control experiment must be performed using polysome buffer to which EDTA has been added to a final concentration of 20 mM.

3.2.3.8 RNA Labeling with Digoxigenin-UTP or Fluorescein-12-UTP by *in vitro* Transcription

10X NTP Digoxigenin-UTP labeling mixture
10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-UTP, pH 7.5

10X NTP Fluorescein-12-UTP labeling mixture
10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM fluorescein-12-UTP, pH 7.5

The reaction mixture with a total volume of 20 µl is made by adding 2 µl 10x NTP labeling mixture, 2 µl 10X transcription buffer, and 1 µl RNase inhibitor to 1µg linearized and purified template DNA. Bring volume to 18 µl with DEPC-H_2O and add 2 µl RNA polymerase (SP6 or T7). Incubate for 2 h at 37°C. To remove the DNA template add 2 µl DNaseI (RNase-free) and incubate for 15 min at 37°C. Add 2 µl 0.2 M EDTA solution to stop the reaction. The RNA is precipitated by adding 2.5 µl 4 M LiCl and 75 µl prechilled 95% EtOH.

3.2.3.9 Whole Mount in situ Hybridization

Hybridization buffer:

50% deionized formamide; 5X SSC; 100 µg/ml heparin; 1X Denhardt solution; 0.1% Tween 20; 0.1% CHAPS; 5 mM EDTA

tRNA:

50 mg/ml; store at -20°C.

RNase A:

10 mg/ml in TE; heat at 95°C for 10 minutes and store at -20°C.

RNase T:

10 U/µl in 0.1 M Na-acetate, pH 5.2; heat at 95°C for 2-3 minutes and store at -20°C.

20X SSC:

3M NaCl; 0.3M Na-citrate, pH 7.0; add DEPC-treated H_2O to 1l.

100X Denhardt solution:

1 g BSA/ 50ml; 1 g polyvinylpyrollidone; 1 g Ficoll 400.

MEMPFA:

0.1M MOPS, pH 7.4; 2 mM EGTA; 1 mM $MgSO_4$; add Paraformaldehyde freshly to a final concentration of 4%.

Lamb serum:

heat inactivate at 55°C for 30 minutes and store at -20°C.

20X PBS:

2.74M NaCl; 74 mM KCl; 170 mM Na_2HPO_4; 30 mM KH_2PO_4; pH 7.3.

PTW:

1X PBS; 0.1% Tween 20

Proteinase K:

20 mg/ml; store in aliquots at -20°C.

Triethanolamine:

9.285 g/500 ml sterile H_2O; sterile filter before use.

Paraformaldehyde:

Dissolve 32 g paraformaldehyde in 370 ml dH_2O at 60-65°C, adjust to pH 7.3 and add dH_2O to 400 ml. Store in aliquots at -20°C.

MAB-buffer:

100 mM maleic acid; 150 mM NaCl; pH 7.5

Alkaline Phosphatase Buffer:

10 ml 1M Tris-Cl (pH 9.5); 2.5 ml 2M $MgCl_2$; 2 ml 5M NaCl; 100 µl Tween 20; 2 mM Levamisole; add H_2O to 100 ml.

Fixing of embryos:

Embryos (preferably albino) are fixed at different developmental stages in 1X MEMPFA for a minimum of 2 hours on a nutator. The vitelline membrane can be removed manually from gastrula and neurula stage embryos to allow better staining. After fixing, the embryos are dehydrated through an ethanol series (25% EtOH, 50% EtOH, 75% EtOH) and stored in 100% EtOH at -20°C until use.

Hybridization:

Rehydrate embryos to PTW (75% EtOH, 50% EtOH, 25% EtOH/75% PTW; each step for 5 minutes). Wash embryos 3 x 5 minutes in PTW followed by an incubation in 2 ml PTW containing 10 µg/ml proteinase K for 15 minutes. Rinse 2 x 5 minutes in 0.1M triethanolamine (pH 7-8). Add 12.5 µl acetic anhydride/50 ml triethanolamine and after 5 minutes repeat acetic anhydride addition. Wash 2 x 5 minutes in PTW. Refix for 20 minutes with 4% paraformaldehyde in PTW. Rinse in PTW and follow with 4 x 5 minutes in PTW. Remove all but 1 ml PTW and add 250 µl hybridization buffer. Once embryos have settled, replace with 0.5 ml hybridization buffer. Place embryos at 60°C in a shaking incubator for 10 minutes. Replace hybridization buffer and prehybridize 4-6 hours at 60°C. Replace the buffer with hybridization buffer containing the digoxigenin- or fluorescein-labelled RNA (1µg/ml) and incubate at 60°C overnight. Remove the hybridization buffer containing the RNA probe the next day and store the probe at -20°C for subsequent use. Add 0.5 ml hybridization buffer and incubate at 60°C for 10 minutes. Wash 3 x 20 minutes with 2X SSC at 60°C. Wash in 2X SSC with RNase A (20 µg/ml) and RNase T (10 U/ml) at 37°C for 30

minutes (optional). Wash once in 2X SSC for 20 minutes at room temperature and wash twice in 0.2X SSC at 60°C for 30 minutes.

Antibody Incubation and Chromogenic Reaction:

Wash twice in 1X MAB for 15 minutes at room temperature followed by an incubation in 1X MAB + 2% BMB blocking reagent for 1 hour. Replace the MAB-BMB solution with 1X MAB + 2% BMB + 20% lamb serum and incubate at room temperature for 1 hour. Replace the blocking solution with 1X MAB + 2% BMB + 20% lamb serum containing a 1:2000 dilution of the anti-digoxigenin alkaline phosphatase antibody and incubate at room temperature for 4 hours. To remove excess antibody, wash embryos 5 x 30 minutes in 1x MAB. Wash twice, 5 minutes each at room temperature with alkaline phosphatase buffer. Replace last wash with BM purple and let the reaction continue until satisfied with staining intensity. Stop chromogenic reaction by replacing BM purple with PTW. Fix embryos with MEMPFA at room temperature for 2 hours (or overnight at 4°C) and dehydrate gradually through an ethanol series. Embryos can be stored in 100% ethanol at -20°C.

Bleaching of Embryos:

Bleaching Solution:

1% hydrogen peroxide

5% formamide

0.5X SSC

When pigmented wild type embryos are used for whole mount in situ hybridization, the embryos can be bleached after the chromogenic reaction to distinguish the purple staining from the black pigmentation of the embryos caused by intracellular melanin. Wash embryos that have been fixed in MEMPFA in 0.5X SSC for 5 minutes at room temperature. Fill vial with bleaching solution and place it on aluminum foil under a fluorescent light for ~1 hour or

until the pigment is gone. Dehydrate embryos gradually through an ethanol series and store at -20°C.

3.2.3.10 Whole Mount Immunostaining

Solutions and Buffers:

10X MEM-solution:

1M	MOPS, pH 7.4
20 mM	EGTA
10 mM	$MgSO_4$ x $7H_2O$

Add paraformaldehyde to a final concentration of 4% to 1X MEM-solution freshly before use.

Phosphate Buffer:

Solution A: 0.2 M Na_2HPO_4 x $7H_2O$

Solution B: 0.2 M NaH_2PO_4 x H_2O

0.1M Phosphate Buffer pH 7.4:

405 ml Solution A

95 ml Solution B

add dH_2O to 1l

PBS (Phosphate Buffered Saline):

Add 9 g NaCl to 0.1 M Phosphate buffer, pH 7.4 and add dH_2O to a final volume of 1l.

PBT:

0.1M PBS

2 mg/ml BSA

0.1% Triton

Goat Serum

1% DAB

30% H_2O_2

Embryos are fixed in 1X MEMPFA for a minimum of 2 hours on a nutator. Wash fixed embryos in 1X MEM solution and transfer embryos to PBT for 15 minutes. Replace PBT with 10% goat serum in PBT for 1 hour at room temperature, followed by an overnight incubation with a muscle-specific monoclonal antibody (12/101, diluted 1:10) or a notochord specific monoclonal antibody (Tor 70, diluted 1:5) at 4°C. Embryos are washed 4 x 30 minutes with PBT and incubated in a 1:100 dilution of peroxidase-linked, sheep-anti-mouse Ig overnight at 4°C. Embryos are washed 4 x 30 minutes with PBT followed by an incubation in 0.5% diaminobenzidine (DAB) in PBT for 30 - 45 minutes at 4°C. The color reaction is initiated with the addition of 1 µl 15% H_2O_2 in PBT. Embryos are dehydrated in 100% methanol and cleared in benzyl benzoate:benzyl alcohol (2:1).

3.2.4 Techniques to Study Histology

3.2.4.1 Sectioning

Embryos are fixed in 0.1M PBS; 3% sucrose; 4% paraformaldehyde for a minimum of two hours on a nutator. After two washes, 5 minutes each, in 0.1M PBS they are transferred to 0.1M PBS; 5% sucrose and kept in this solution at 4°C at least overnight. One hour before sectioning replace the solution with 0.1M PBS; 15% sucrose. Embryos are embedded in

O.C.T. compound and placed in the cryostat. Once the O.C.T. is frozen the tissue can be cut. The temperature should be adjusted according to the desired thickness of the sections. For this work sections of 20 µm were cut at -11°C, sections of 14 µm were cut at -14°C. Sections are mounted on slides, which had been subbed in 2% gelatin and 0.08% chromium potassium sulfate, and can be used for immunostaining or in situ hybridization. The sections are washed in 0.1M PBS and then coverglass mounted with 90% glycerol; 10% 0.1M Tris (pH 7.4), or dehydrated gradually in an EtOH series, incubated in toluene for 5 minutes, and coverglass mounted with Permount (Fisher).

3.2.5 Primer Sequences

The following primer sequences were used for PCR analyses performed for this work.

In chapters 4.1.1 and 4.1.2:

XWnt-8b, 227bp

F: GAA GTG ACT TGA ACA TCC ATT CT

R: TTG CTG GAG AAA GGA ATC CTG TA

D7.1 (Schroeder and Yost, 1996)

F: GAC GTT TGG GCT GCA ATA TGG

R: CTT CTT CAC AAG GAG GGC TTT G

In chapters 4.4.2 and 4.4.4:

XSix1, 271bp

F: GGG CCC CTC ACC TCC AGC

R: ATC CTT GTG GTA TCT CC

myogenin, 147bp (Nicolas et al., 1998)

F: CCA GCC CTT ATT TCT TTT CAG ACC A

R: AAT CCC TGA GCC CTG TAA TAA AAC C

H4

F: AGG GAC AAC ATC CAG GGC ATC ACC

R: ATC CAT GGC GGT AAC GGT CTT CCT

4 Results

Part I

4.1 Differential gene expression and identification of dorsal-specific RNAs

4.1.1 Localized expression of *XWnt-8b* and its possible function in dorsal-ventral axis specification

As described previously, explant studies demonstrated that animal blastomeres are already biased towards a more dorsal or ventral fate at early cleavage stages. For example, dorsal animal midline blastomeres (D1.1) of the 16-cell stage embryo elongate in culture autonomously whereas ventral animal midline blastomeres (V1.1) do not (Gallagher et al., 1991; Hainski and Moody, 1996). This effect suggests that maternal mRNAs and proteins with either ventralizing or dorsalizing activities are asymmetrically distributed in blastomeres of the animal hemisphere. An RT-PCR based differential screen was performed to identify candidate molecules localized to dorsal animal midline blastomeres (D1.1) at the 16-cell stage that could account for the elongation of D1.1 blastomeres. Therefore, V1.1 and D1.1 blastomeres were dissected from embryos displaying stereotypic radial cleavage patterns in which the third cleavage furrow does not extend into the vegetal half of the embryo (Fig.3) to avoid contamination with localized maternal mRNAs that induce mesoderm (Weeks and Melton, 1987; Ku and Melton, 1993; Gimlich and Gerhart, 1984). Embryos were selected at the 2-cell stage if the first cleavage furrow bisected the pale equatorial region in the animal hemisphere (Fig.4) to allow reliable identification of dorsal and ventral animal blastomeres at the 8-cell stage (Klein, 1987; Masho, 1990). RNA was extracted from the dissected blastomeres and subjected to RT-PCR. Of the tested maternal molecules known to have dorsal axis inducing activity, only transcripts of *XWnt-8b* were found to be localized. Interestingly, *XWnt8b* whose maternal component was shown to induce a secondary dorsal axis was found to be enriched in ventral animal (V1.1) blastomeres (Fig.5). The predominant presence of *Xwnt-8b* transcripts in the ventral half of the animal hemisphere suggests that this

molecule might function in specifying the dorsal-ventral axis by modifying tissue to promote a ventral bias already at early cleavage stages.

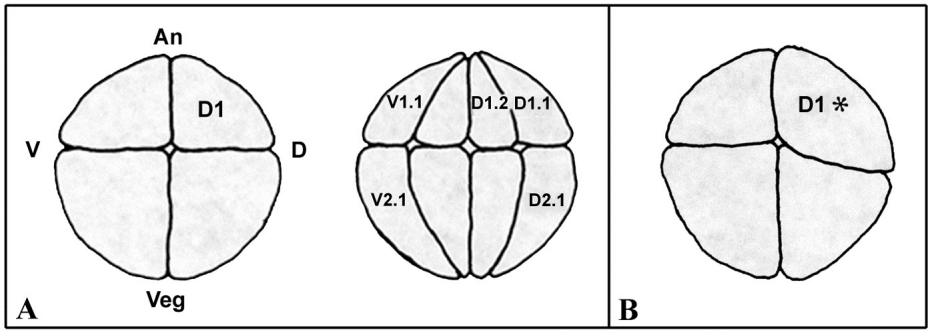


Fig.3: Standard cleavage patterns were selected to maintain consistency in blastomere identification across all experiments. (A) An 8-cell stage and a 16-cell stage embryo with standard cleavage pattern are shown. Blastomere nomenclature is that of Hirose and Jacobson (1979). (B) An 8-cell stage embryo with irregular cleavage pattern in which the third cleavage furrow dips into the vegetal hemisphere. The dorsal animal blastomere (D1*), therefore, contains vegetal-equatorial cytoplasm. An: animal; D: dorsal; V: ventral; Veg: vegetal.



Fig.4: *Xenopus laevis* embryos at early cleavage stages. Clockwise from top left: 2-cell, 4-cell, 8-cell, and 16-cell stage embryos. View is onto the animal pole. The lighter pigmented, future dorsal side is bisected by the first cleavage furrow at the 2-cell stage. The dorsal side can be distinguished well from the darker pigmented future ventral side.

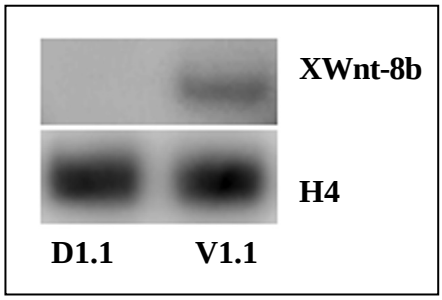


Fig.5: *XWnt-8b* transcripts are enriched in ventral animal midline blastomeres (V1.1). Total RNA was isolated from V1.1 and D1.1 blastomeres at the 16-cell stage and used for RT-PCR analyses to determine the localization of *XWnt-8b* transcripts. *H4* was used as a loading control.

To test whether *XWnt-8b* might counteract dorsal development and inhibit the autonomous elongation of D1.1 blastomeres, 50 pg of *XWnt-8b* RNA were injected into each dorsal animal quadrant at the 2-cell stage. At the 16-cell stage D1.1 blastomeres were dissected and placed in explant culture until siblings reached stages 23/24. Control, uninjected explants elongated modestly in the absence of growth factors (Fig.6A). Explants from V1.1 blastomeres did not elongate but instead formed crenulated balls (Fig.6B). The overexpression of *XWnt-8b* in dorsal animal midline blastomeres significantly inhibits the frequency of elongation by D1.1 explants (Fig.6C; Fig.7). If endogenous *XWnt-8b* functions as an inhibitory molecule, then co-culture of V1.1 blastomeres with D1.1 blastomeres should inhibit explant elongation of animal blastomeres. Indeed the frequency of elongation was significantly reduced (Fig.6D). To demonstrate whether this inhibition was caused specifically by *XWnt-8b*, the D1.1 blastomere's ability to respond to Wnt signaling was impeded by overexpression of *Xfrzb*. *Xfrzb* has been shown to block *XWnt-8* function (Leyns et al., 1997). Explant co-cultures of D1.1 (overexpressing *Xfrzb*) and V1.1 blastomeres elongated significantly compared to uninjected D1.1 and V1.1 blastomere co-cultures (Fig.7). There is no significant difference between the frequencies of elongation when explant co-cultures of D1.1 (overexpressing *Xfrzb*) and V1.1 blastomeres are compared to D1.1 control explants, indicating that blocking the effect of *XWnt-8b* restores the ability of D1.1 blastomeres to elongate.

In a separate assay based on the early (pre-MBT) dorsal axis inducing effects of *Wnts* it was tested if *Xfrzb* indeed blocks *XWnt-8b* function. Ectopic expression of *Wnts* before MBT elicits the formation of a secondary axis, which can be suppressed by co-injection of a *Wnt* antagonist. Embryos injected unilaterally with 30 pg *XWnt-8b* RNA into a ventral vegetal blastomere at the 8-cell stage developed a complete secondary axis, whereas unilateral coinjections of *XWnt-8b* (30 pg) and *Xfrzb* (430 pg) resulted in a normally developed embryo (Fig.8).

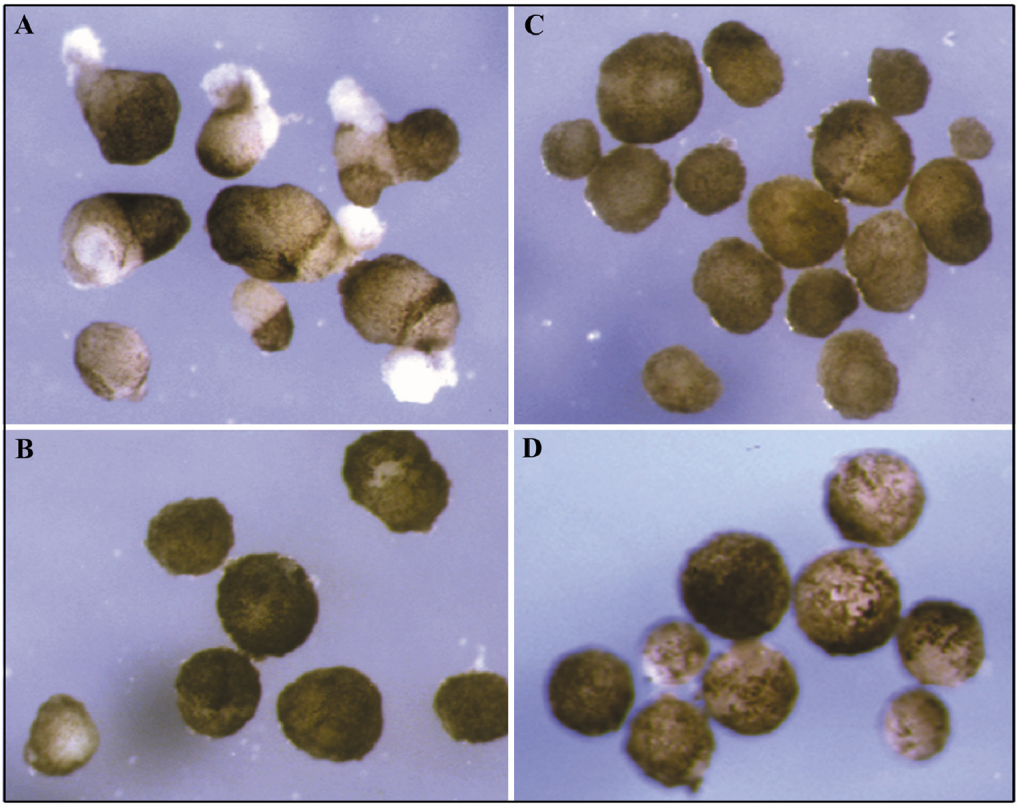


Fig.6: Blastomere explant cultures demonstrate a possible function for *XWnt-8b* in inhibiting the induction of a dorsal program on the ventral side of *Xenopus* embryos. (A) Dorsal animal midline blastomeres (D1.1) elongate autonomously, whereas ventral animal midline blastomeres (V1.1) do not (B). (C) Overexpression of *XWnt-8b* in D1.1 blastomeres inhibits elongation. (D) Elongation also is inhibited in co-cultures of D1.1 with V1.1 blastomeres, possibly by endogenous *XWnt-8b* signaling from the ventral side.

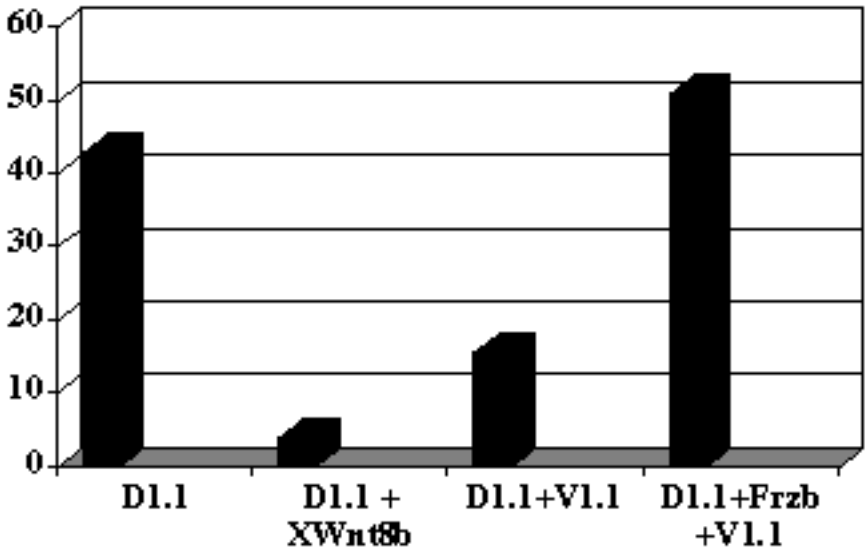


Fig.7: The ability of blastomere explants to elongate was tested and the frequency of explant elongation is given in percent. The frequency of dorsal animal midline blastomeres (D1.1) to elongate is inhibited significantly by overexpression of *XWnt-8b* in these blastomeres ($p<0.001$). Native *XWnt-8b* may inhibit elongation of D1.1 blastomeres in co-cultures of D1.1 with V1.1 blastomeres ($p<0.001$). The effect of *XWnt-8b* can be blocked by overexpression of *Xfrzb* in D1.1 blastomeres. In co-cultures of D1.1 (+ *Xfrzb*) with V1.1, elongation is restored ($p<0.001$). There is no significant difference between the frequencies of elongation when comparing co-cultures of D1.1 (+*Xfrzb*) and V1.1 with D1.1 control explants ($p>0.25$).

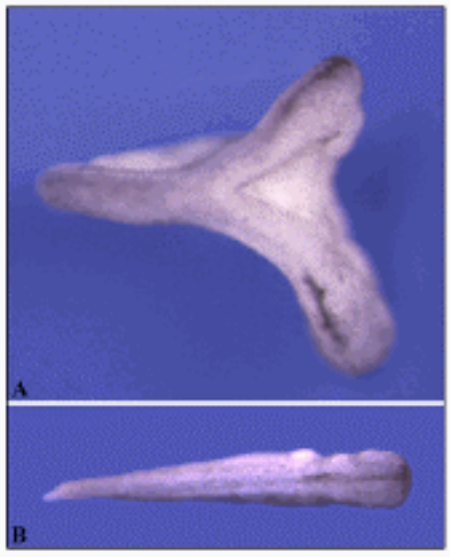


Fig.8: The ability of *Xfrzb* to block dorsal axis induction by *XWnt-8b* is demonstrated. Embryos were injected with *XWnt-8b* into a ventral vegetal blastomere and the induction of a complete secondary axis is shown in (A). Co-injection of *Xfrzb* with *XWnt-8b* results in the development of a normal embryo (B).

4.1.2 Is *XWnt-8b* being translated during early cleavage stages?

In order to support the hypothesis that maternal *XWnt-8b* elicits the above described effects in explant cultures (see Fig.6), it was investigated whether *XWnt-8b* transcripts are in the translatable RNA pool at cleavage stages. Polysomal and non-polysomal (supernatant) RNA pools were extracted from whole embryos that had been collected at the 16- to 32-cell stages. RNA that is associated with polysomes was separated from non-translated RNA by centrifugation in a sucrose gradient. Fig.9 shows total RNA of the different fractions that were isolated. RT-PCR was performed to investigate the presence of *XWnt-8b* in the polysomal or non-polysomal RNA pool (Fig.10). D7.1 RNA is known to be translated maternally (Smith et al., 1988) and was used as a positive control. The results showed that endogenous *XWnt-8b* can not be detected in association with polysomes at the 16- to 32-cell stages.

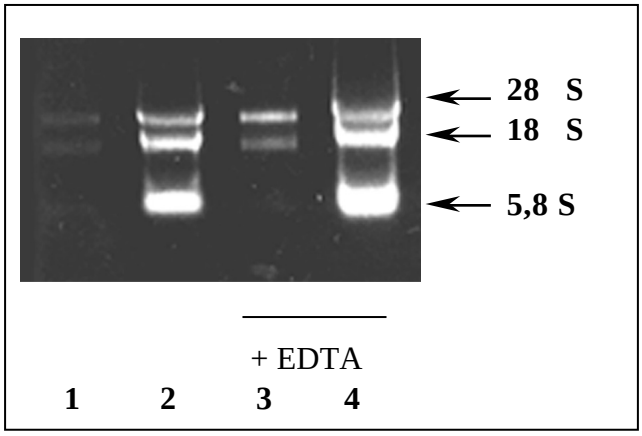


Fig.9: Total RNA was isolated from 16- to 32-cell stage embryos and RNA associated with polysomes was separated from non-polysomal RNA in a sucrose gradient. 4 µl of each RNA fraction were loaded onto the gel. Lanes 1 and 3: RNA from the polysomal fraction; lanes 2 and 4: RNA from the non-polysomal fraction. RNAs in lanes 3 and 4 were isolated in the presence of EDTA to dissociate translated RNAs from polysomes. Arrows point to the separated ribosomal RNAs (28S, 18S, and 5,8S rRNA).

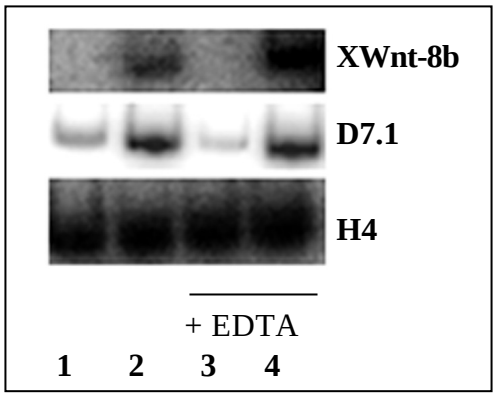


Fig.10: Polysomal RNA (Lanes 1 and 3) and non-polysomal RNA (Lanes 2 and 4) from whole embryos was subjected to RT-PCR to determine if *XWnt-8b* transcripts are associated with polysomes between the 16- and 32-cell stages. *XWnt-8b* transcripts only were detected in the non-polysomal RNA fraction. D7.1 RNA is known to be translated during early cleavage stages and was used as a positive control. The weak signal for D7.1 in Lane 1 can be explained by the initial lower concentration of RNA used for RT-PCR. H4 was used as a loading control.

These results can not confirm that maternally expressed, endogenous *XWnt-8b* is translated at early cleavage stages to inhibit a dorsal program on the ventral side. However, these data also do not completely exclude a function for maternal *XWnt-8b* in establishing a ventral bias in animal blastomeres during early cleavage stages. The polyadenylation status of *XWnt-8b* transcripts and hence their association with polysomes needs to be determined at different time points, from the unfertilized egg until MBT, before zygotic transcription begins. Furthermore, sufficient XWnt8b protein may have already been synthesized by the time that the polysomal preparations were made.

4.2 Post-transcriptional activation of dorsal-specific mRNAs

4.2.1 Isolation and manipulation of D1 RNA

Dorsal animal blastomeres dissected at the 16-cell stage (D1.1) elongate autonomously when cultured in a simple salt medium. Additionally, total RNA isolated from D1.1 blastomeres induces secondary axial structures whereas RNA isolated from their 8-cell mother (D1) does not (Hainski and Moody, 1992; 1996). Since the RNAs that affect this change in fate are

maternal and not newly transcribed (Newport and Kirschner, 1982a,b), they must be present in this lineage at both stages, but not translatable at the 8-cell stage. If translational inactivation of these dorsalizing RNAs at the 8-cell stage was due to a masking protein, then RNA extracted from D1 blastomeres should also possess axis inducing activity, since the RNA solution is free of protein contamination after a phenol/chloroform extraction. Another possibility for translational inactivity could be the presence of these dorsalizing RNAs in D1 blastomeres in a de-adenylated form.

Because maternal messages can be activated by cytoplasmic polyadenylation (Simon et al., 1992) it was tested whether the dorsalizing messages localized to dorsal animal midline blastomeres can be rendered active by elongation of their poly(A) tail *in vitro*. For each experiment 600-700 D1 blastomeres were dissected. It is important that blastomeres are only dissected from embryos displaying stereotypic radial cleavage patterns as described above (Fig.3). The dissected blastomeres were collected in a guanidine thiocyanate denaturing solution. Total RNA was extracted and separated into poly(A+) and poly(A-) RNA fraction using oligo(dT) cellulose push columns. An aliquot of the poly(A-) RNA fraction was used to elongate the poly(A) tail *in vitro* in order to activate possible dorsalizing messages.

4.2.2 Injection of different D1 RNA fractions

About 4 ng of each D1 RNA fraction (either poly(A+) RNA, poly(A-) RNA or in vitro polyadenylated poly(A-) RNA) were injected bilaterally in a 4 nl volume into the ventral vegetal blastomeres of the 16-cell embryo (V2.1, see Fig.3) to assay for their ability to induce secondary axes. Injected embryos were raised until stages 30-36 (Nieuwkoop and Faber, 1967). The induction of ectopic paraxial structures was elucidated by whole mount immunostaining using the muscle-specific monoclonal antibody 12/101. The results depicted in Fig.12 show that in vitro polyadenylated D1 RNA had the ability to induce partial secondary axes whereas the poly(A-) RNA fraction had no effect. These data demonstrate that there are RNAs with dorsalizing activity located in 8-cell dorsal animal blastomeres that

can be activated via the elongation of the poly(A) tail. Some adenylation has already occured *in vivo* in the D1 RNA pool, because D1 poly(A+) RNA induced less developed secondary axial structures at a low frequency (Figs.11,12). These data demonstrate that a likely mechanism for the change in dorsal-inducing activity in the D1 lineage between the 8- and 16-cell stage is cytoplasmic polyadenylation. Furthermore, the activation of the dorsal-inducing RNA is not likely to occur all at once between the 8- and 16-cell stage, but is a gradual process that begins at least by the 8-cell stage (as evidenced by the effectiveness of the poly(A+) RNA fraction) and produces an effective concentration at the 16-cell stage (as evidenced by the effectiveness of the total RNA fraction).

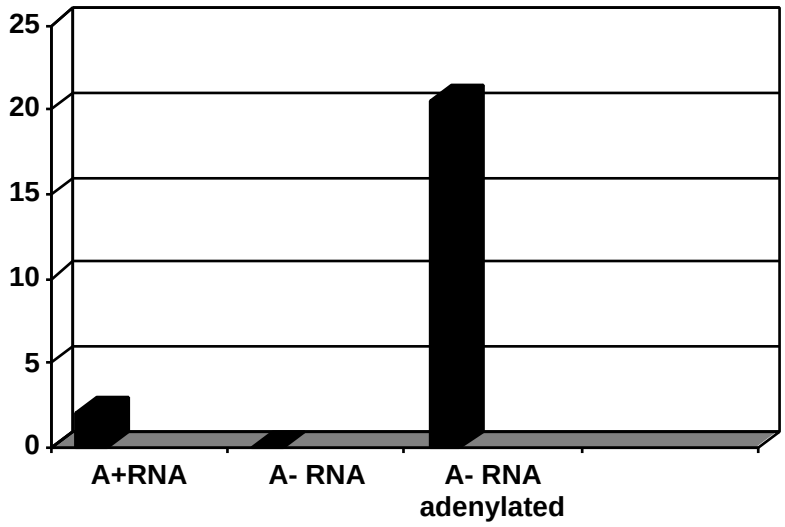


Fig.11: Percentage of embryos with secondary axes resulting from injection with different fractions of D1 RNA. D1 total RNA was separated into poly(A+) (A+ RNA) and poly(A-) (A- RNA) fractions and an aliquot of poly(A-) RNA was used for elongation of the poly(A) tail *in vitro* (A- RNA adenylated). About 8 ng of each RNA fraction was injected into ventral vegetal blastomeres and embryos with ectopic paraxial structures were scored.

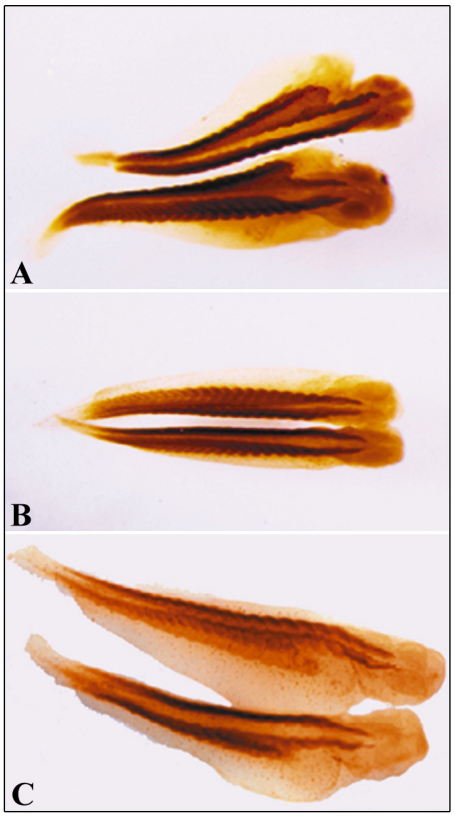


Fig.12: About 8 ng of different D1 RNA fractions (either poly(A+), poly(A-) or in vitro polyadenylated poly(A-) RNA) were injected into ventral vegetal blastomeres to assay their ability to induce secondary axes. Embryos were stained with the muscle-specific antibody 12/101. In vitro polyadenylated D1 RNA induced partial secondary axes (A), whereas D1 poly(A-) RNA had no effect (B). D1 poly(A+) RNA also had the ability to induce secondary paraxial structures which were less well developed (C).

4.3 Identification of dorsalizing mRNAs through sib-selection techniques

4.3.1 Construction of a D1.1 cDNA library and sib selection

To isolate and identify dorsalizing mRNAs that are active at the 16-cell stage and localized in the dorsal animal midline blastomeres (D1.1), a plasmid cDNA library was constructed using the SMART PCR cDNA Library Construction Kit from Clontech. Total RNA (0.5-1.0 µg) from 600-700 dissected D1.1 blastomeres was extracted and used for first strand cDNA synthesis. Blastomeres only were dissected from embryos whose third cleavage furrow did not extend into the vegetal hemisphere. A modified oligo(dT) primer containing an XbaI restriction site was used for first strand cDNA synthesis and subsequent PCR to allow directional cloning. The provided adaptors have a pre-existing EcoRI 'sticky end' which allowed subcloning the cDNAs into the expression vector pCS2+ via EcoRI and XbaI sites. Transformation was performed by electroporation followed by determining the titer of the library (3.1×10^4 CFU/ml). A total amount of about 24,000 CFU were plated on twelve LB (+ ampicillin) agar plates with each plate representing a pool of about 2000 CFU. Colonies were scraped off the plates with LB medium and used to grow cultures for the isolation of plasmid pools. An aliquot was used to prepare glycerol stocks of each pool. Each of the obtained 12 plasmid pools was linearized with SnaBI and used for synthesizing capped RNAs *in vitro* using SP6 polymerase. About 4-8 ng mRNA were injected bilaterally into ventral vegetal blastomeres (V2.1) at the 16-cell stage to assay for axis inducing activity of the different RNA pools. In addition, the RNA pools were mixed and co-injected with GFP RNA (green fluorescent protein) which serves as a lineage tracer. Injected embryos were raised at least until tail bud stages (stage 30-36) before they were fixed and analyzed. Of the 12 RNA pools tested, 5 had the ability to induce ectopic axial structures (pools 3, 8, 9, 10, and 11). The results of the first round of sib selection are summarized in Table 2.

Table 2: About 4-8 ng of the different RNA pools were injected into ventral vegetal blastomeres to assay for their axis inducing activity. The number of injected embryos and the percentages of embryos with partial secondary axes are presented.

RNA pool	Number of injected embryos	Induction of secondary axes (%)
1	47	0
2	29	0
3	37	22
4	48	0
5	31	0
6	43	0
7	29	0
8	51	47
9	55	45
10	51	19
11	77	26
12	17	0

Two of the 12 tested RNA pools, pools 8 and 9, each induced partial secondary axes in about half of the embryos. The induction of secondary anterior structures is clearly delineated in these embryos (Fig.13). In Fig.13B the green fluorescent protein is expressed in the ectopic axial structures indicating that the secondary structures are directly induced by the injected RNAs. Because of its high axis inducing activity, pool 8 was chosen for the second round of sib selection.

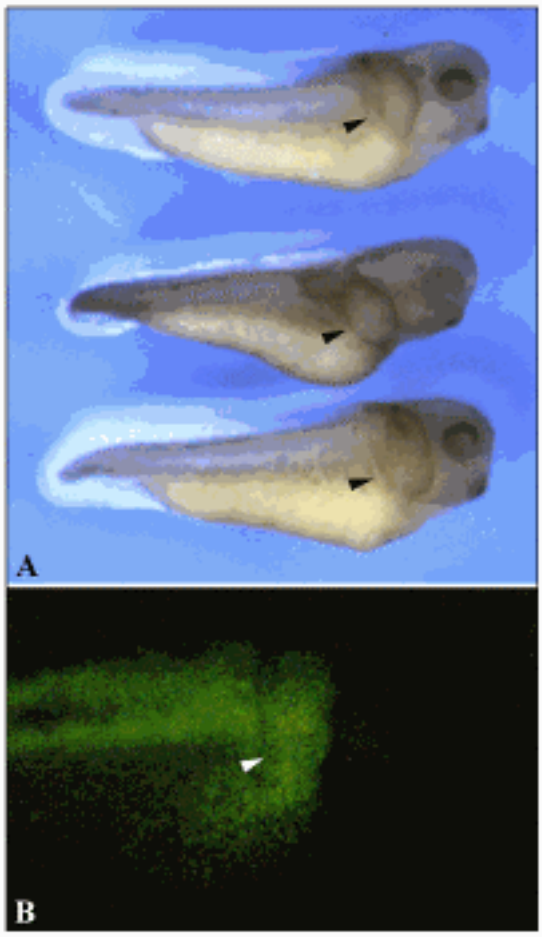


Fig.13: Sib selection to identify dorsalizing RNAs localized in dorsal animal midline blastomeres (D1.1). (A) Embryos were injected with about 4-8 ng RNA from pool 8 into ventral vegetal blastomeres and induction of secondary anterior structures (arrowheads) is seen. (B) Embryos were coinjected with a lineage tracer, green fluorescent protein (GFP) mRNA, which is expressed in the partial secondary axis (arrowhead) of an embryo from (A).

About 330 CFU of pool 8 were plated on each of six LB (+ ampicillin) agar plates (pool 8.1-8.6). Performing the above described procedure, 6 plasmid pools should have been transcribed and RNAs injected in order to determine the next pool with the highest axis inducing activity. However, several trials to transcribe RNA off the linearized plasmids *in vitro* were unsuccessful. Various methods were employed to determine the cause for this problem. The plasmid pools were subjected to PCR using primers for the SP6 and T7 promoter region flanking the subcloned cDNA inserts. However, no cDNA fragments were amplified raising the question of whether the SP6 promoter was defective since RNAs, too, could not be transcribed using the SP6 polymerase. In addition, sequencing could only be

performed using T7 polymerase and not SP6 polymerase, supporting the assumption that the SP6 promoter site was defective. Furthermore, sequence analyses revealed that the plasmid stock was contaminated with another plasmid. Restriction digests of plasmids obtained from single colonies also indicated that there had been a contamination since not all digests resulted in the same length for the vector.

To isolate potential axis inducing RNAs from pool 8, the cDNA inserts were excised with NotI. The cDNA inserts were then polished using the Klenow fragment, followed by ligating EcoRI adaptors. After a restriction digest with XbaI, the cDNAs were ligated into pCS2+, and transformed. Because of a low ligation efficiency (10%) single colonies were screened by PCR using primers for the SP6 and T7 promoter sites. This method allows one to screen a large number of colonies and to determine cDNA containing (positive) clones quickly. Positive clones were sequenced and further analyzed. Three cDNAs had homologies to *Xenopus laevis* enolase, mitotic phosphoprotein, and α-tubulin respectively. One cDNA was homologous to rat somatic cytochrome C. Another cDNA shared homologies with zebrafish tyrosine kinase ligand AL1. Many clones only contained vector sequences and some were not homologous to any sequences in the database. These unidentified clones were linearized and used to transcribe capped RNAs which were injected to assay for possible axis inducing activity. Since none of the tested RNAs induced secondary axes a new plasmid D1.1 cDNA library was constructed.

4.3.2 Construction of a new D1.1 cDNA library and sib selection

First, 600-700 D1.1 blastomeres were dissected anew and total RNA extracted as described above. First strand cDNA synthesis was performed using an oligo(dT) primer that contained a NotI restriction site. Amplified D1.1 cDNAs were subcloned into the expression vector pDH105 via SalI and NotI restriction sites (Fig.14).

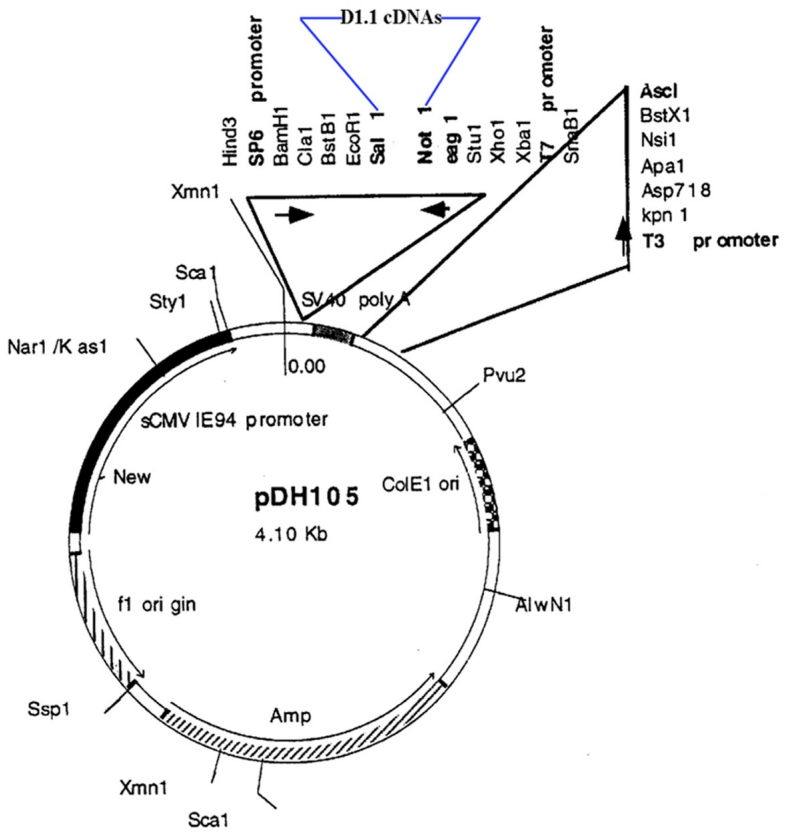


Fig.14: For the construction of a plasmid cDNA library, D1.1 cDNAs were directionally cloned into the expression vector pDH105 using SalI and NotI restriction sites.

After ligation the plasmids were transformed into DH10B cells by electroporation. The titer of the new D1.1 library was determined (1.9 x 10^5 CFU/ml) and 20 single colonies were picked to assay for ligation efficiency and insert length. Plasmid preparations of 20 colonies were digested with SalI and NotI, which demonstrated that 18 out of 20 colonies had a cDNA insert (90% ligation efficiency) (Fig.15). The average insert length is approximately 850 bp. This indicates that the library may not contain many full length cDNAs.

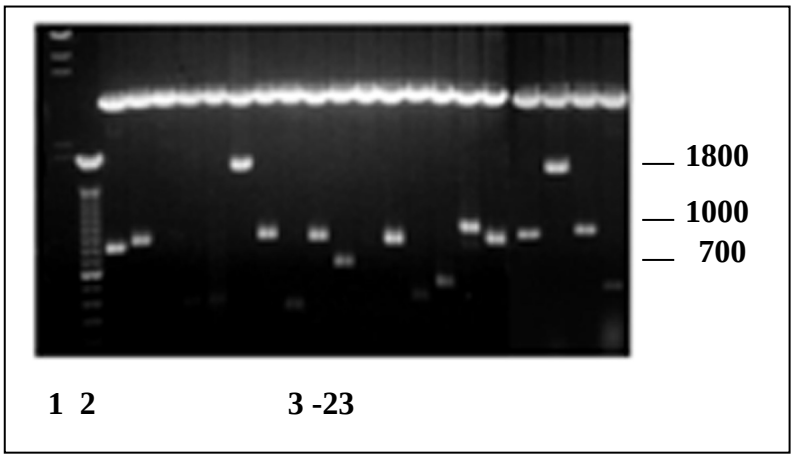


Fig.15: Determination of ligation efficiency and average cDNA insert length of the D1.1 cDNA library. Plasmids isolated from 20 single clones were digested with SalI and NotI to excise the cDNA inserts. Lane1: λDNA/HindIII marker; the sizes in kb are: 23.1; 9.4 ; 6.6; 4.6; 2.3; 2.0. Lane 2: 100 bp DNA marker; the sizes in bp are: 1500; 1000; 900; 800; 700; 600; 500; 400; 300; 200; 100. Lanes 3 - 22: cDNA inserts of individual clones. Approximate sizes of the cDNA inserts are given in bp.

For the first round of sib selection a total number of 30,000 CFU were plated, with about 3000 CFU/plate. The colonies were scraped off the plates and processed as described previously. The ten plasmid pools were linearized with AscI and RNAs were transcribed *in vitro* using SP6 polymerase. Between 4 ng and 8 ng RNA of each pool were injected into ventral vegetal blastomeres (V2.1) to assay for axis inducing activity. Injected embryos were raised at least until tail bud stages (stages 30-36) and subjected to whole mount immunostaining with the muscle specific monoclonal antibody 12/101 to visualize weak inductions of secondary paraxial tissue. Ectopic paraxial structures were detected in embryos injected with RNA from pool 1. This pool was then chosen for the second round. The results

of four rounds of sib selection are summarized in Table 3. Since none of the injected RNA pools induced obvious secondary axes, embryos were assayed for other developmental defects affecting somitic tissue that were revealed by the immunostaining with the antibody 12/101. The most prominent features detected were disruption of the segmentation of the somites and defective development of the most anterior somites. Embryos displaying the latter phenotype were shortened along the antero-posterior axis and kinked (Fig.16). Before preparing plasmid pools of the fourth round (pool 1.2.1.1 - 1.2.1.10), single colonies of each pool (30-40 colonies per pool) were picked and transferred on 24-well plates containing LB (+ ampicillin) agar with 20% glycerol. After determining the pool that induced the highest number of affected embryos (pool 1.2.1.5), the previously isolated single colonies of this pool (33) were used to grow cultures for plasmid preparations.

Table 3: The results of four rounds of sib selection are summarized. Embryos were injected with 4 ng to 8 ng of the different RNA pools into ventral vegetal blastomeres. CFU/plate specifies the number of colony forming units that each pool contained. The pool affecting somitic development in a high percentage of embryos, as determined by immunostaining with the muscle specific antibody 12/101, was selected for the next round. Only the pools that induced the most severe defects and were chosen for the following round are listed.

Sib Selection Round	Number of Pools Injected	CFU/Plate (= CFU/Pool)	Selected Pool	% Embryos with Phenotype
1	10	3000	1	15
2	5	600	1.2	26
3	6	100	1.2.1	35
4	10	30-40	1.2.1.5	94



Fig.16: Four rounds of the sib selection process are shown. Between 4 ng and 8 ng RNA of each pool was injected into ventral vegetal blastomeres to assay for induction of secondary paraxial tissue. Embryos were stained with the muscle-specific antibody 12/101. (A) Pool 1 RNA induced ectopic paraxial tissue in the first round (arrow). (B)-(D) The predominant phenotype in the following rounds was defective development of the segmented somitic tissue. These embryos were shortened along the antero-posterior axis and kinked. (B) Second round. Embryos were injected with pool 1.2 RNA. (C) Third round. Embryos were injected with pool 1.2.1 RNA. (D) Fourth round. Bottom: embryos were injected with pool 1.2.1.5 RNA; top: uninjected control embryo.

The clones from pool 1.2.1.5 were digested with SalI and NotI in order to cut out the cDNA inserts and determine their sizes. The sizes of the cDNA inserts ranged from 200 bp to 1900 bp. Plasmids containing cDNA inserts with ≥550 bp were linearized using AscI and capped RNAs were synthesized. *In vitro* transcribed RNAs from 20 clones were injected singly into ventral vegetal blastomeres to determine which clones affect the normal development of somites. However, injection of RNA from individual clones only resulted in weak defects in the development of paraxial tissue.The most common effect was a defective segmentation of the somitic tissue in that the somites did not segregate properly. The loss of the phenotype seen after injections of the whole 1.2.1.5 RNA pool suggests that the pool phenotype was the result of a synergistic effect of all RNAs in the pool. The 20 clones that had been

injected were sequenced to identify homologies to known genes and to investigate if yet unknown cDNAs had been isolated. The results of the sequence analysis are summarized in Table 4.

Table 4: Sequence analysis of 20 single clones from pool 1.2.1.5.

Clone	Sequence Homologies to
B, C, E, N,O, X, Z, a, b, d	*X. laevis* mitochondrial DNA
A, H, M, S	*X. laevis* mitotic phosphoprotein
L	*Gallus gallus* protein kinase gene
W	*X. laevis* scaffold attachment factor
F	Vector sequence
G, h, i	no matches found

Consistent with the loss of an axial phenotype when injecting RNAs from single clones, sequence analysis of these clones did not identify cDNAs that are likely to be involved in dorsal axis induction. The majority were mitochondrial or related to the cell cycle, as expected for a rapidly dividing cell. However, whole mount in situ hybridization was performed with some clones whose injections resulted in defective somite segmentation to investigate their embryonic expression pattern. Of the tested clones, transcripts of clone G and Z revealed a tissue specific spatial distribution.

4.3.3 Embryonic expression pattern of clone G

For in situ hybridization detection of gene expression the plasmid was cut with NotI to transcribe antisense RNA from the 400 bp cDNA using T7 polymerase. The earliest localized expression of clone G is seen at stage 19/20 when transcripts are detected in the head region of the neural tube (Fig.17A). By stage 23 the initial diffuse expression becomes more intense and localized to the area that gives rise to the cement gland; however, weak staining is seen throughout the head region (Fig.17B). Expression of clone G expands in the

developing head and by stage 34/35 transcripts are detected in the mandibular, hyoid, and branchial arches, in the otic vesicle (Fig.17C, D), and in the dorsal neural tube (Fig.17C, F, G). Interestingly, the differentiated cement gland is no longer positive for clone G (Fig.17D). Around stage 26/27 clone G is expressed in the trigeminal ganglia (Fig.17E), and by stage 34/35 in the VII, VIII and IX/X cranial ganglia (Fig.17D, G). A transverse section through the trunk region of a stage 34/35 embryo shows expression of clone G in Rohon Beard neurons (Fig.17F), which are derived from the neural crest, are located in the dorsal neural tube and are the embryonic equivalent of the dorsal root ganglion sensory neurons (Clarke et al., 1984).

4.3.4 Embryonic expression pattern of clone Z

For in situ hybridizations the plasmid was cut with NotI and antisense RNA was transcribed from the 950 bp cDNA using T7 polymerase. The skin of *Xenopus* contains a population of specialized ciliated cells (Fig.18A). At gastrula stages clone Z transcripts are detected in a subset of ectodermal cells (Fig.18B). This expression pattern continues into late tailbud stages and embryos appear freckled. Unstained patches in the ventral region of the tail bud stage embryos depicted in Fig.18C-E are likely a result of proteinase K treatment performed during the procedure of whole mount in situ hybridization. Proteinase K digests the epidermis and hence can destroy the cells expressing clone Z. Transverse sections demonstrated that this clone is prominently expressed in these ciliated epidermal cells (Fig.18F,G). Additionally, during development clone Z expression is detected in somites and in the mandibular, hyoid and branchial arches (Fig.18C-E).

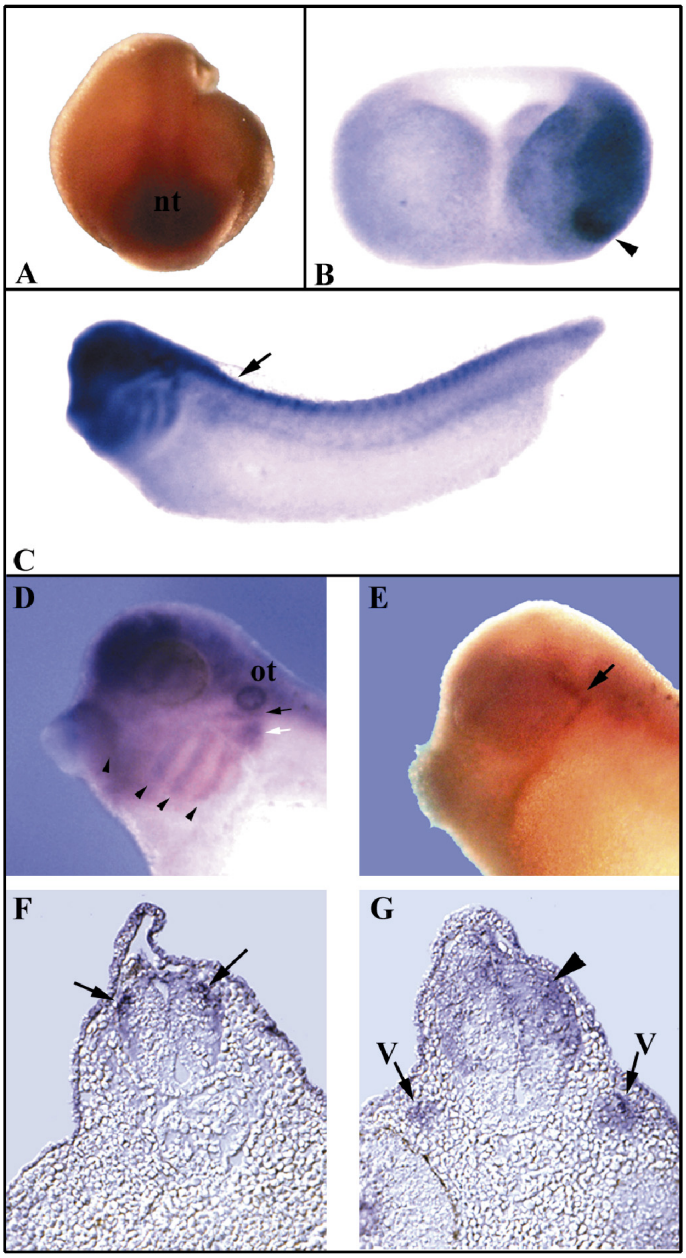


Fig.17: Embryonic expression of clone G. (A) Diffuse expression of clone G in the head region of the neural tube at stage 19/20. (B) At stage 23 clone G expression delineates the cement gland, although a diffuse expression throughout the head region is maintained. (C) and (D) By stage 34/35 the cement gland is no longer positive for clone G. Its transcripts are localized to other defined head structures such as the mandibular, hyoid and branchial arches (arrowheads), the otic vesicle (ot), cranial ganglia (VIII, black arrow; IX/X, white arrow) and dorsal neural tube (arrow in C). (E) At stages 26/27 staining for this clone is detected in the trigeminal ganglia (arrow). (F) Transverse section through the trunk region of a stage 34/35 embryo. The arrows point to Rohon Beard sensory neurons located within the dorsal neural tube. (G) Transverse section through the head region of a stage 34/35 embryo. Expression of clone G is seen in the trigeminal ganglia (V) and in the dorsal brain (arrowhead).

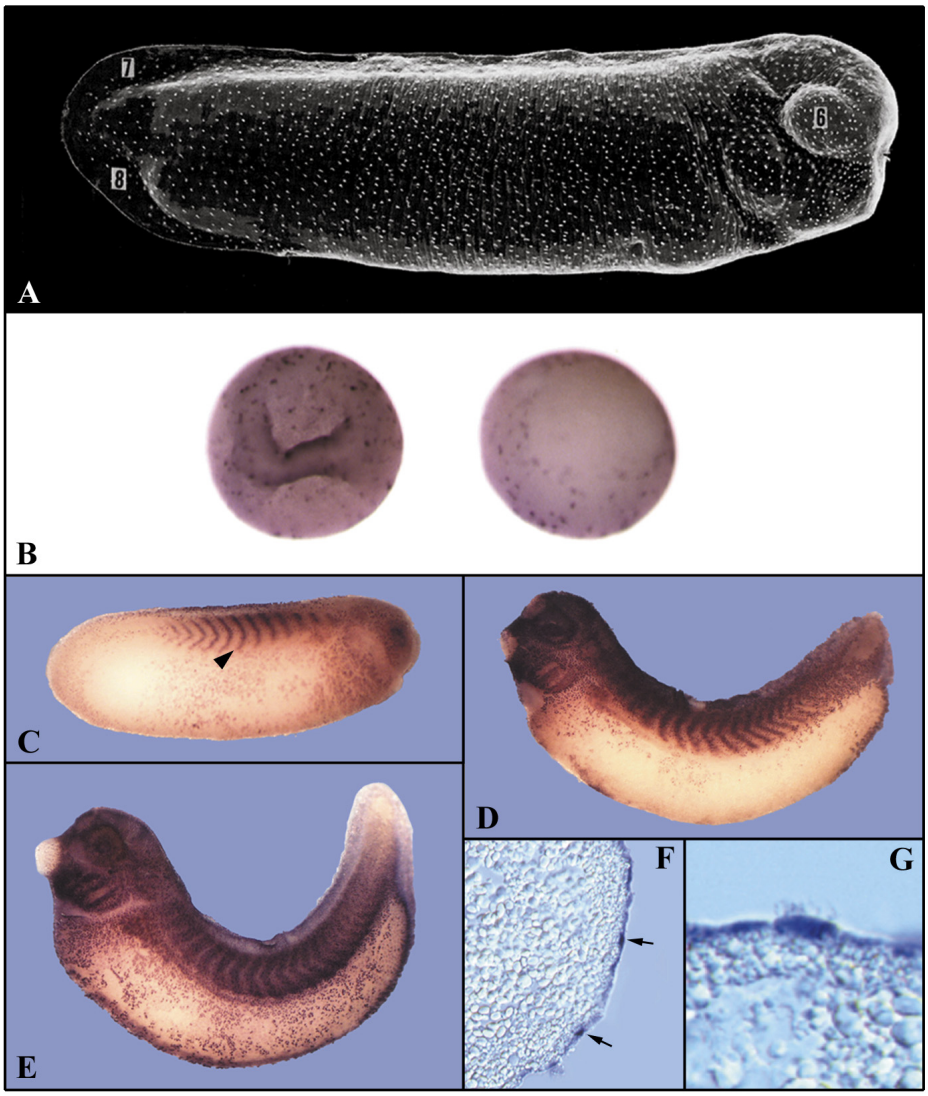


Fig.18: (A) Scanning electron microscope picture of a tailbud stage *Xenopus* embryo taken from Schoenwolf (1995). The ciliated cells in the skin are seen as white tufts. (B) - (E) Embryonic expression pattern of clone Z. (B) Transcripts are detected in a subset of ectodermal cells during gastrula stages (left: view of animal pole). They are absent in the presumptive endoderm (right: view of vegetal pole). (C) - (E) The expression in specialized ciliated cells is seen throughout tail bud stages, which gives the embryo a freckled appearance. Additionally, clone Z transcripts are present in somites (arrowhead) and the mandibular, hyoid and branchial arches. (F) - (G) Transverse sections through the trunk region of a tailbud stage embryo show the expression of clone Z in specialized ciliated cells of the skin (arrows).

Part II

4.4 Characterization of the homeobox transcription factor *XSix1*

4.4.1 Isolation of *XSix1*

A large number of homeobox-containing genes have been identified to play a role in ectodermal patterning. A distinct region in the anterior ectoderm adjacent to the neural plate and neural crest comprises the placodes, which give rise to head sensory organs and the mechanosensory lateral line system in fish and amphibians. *Six* genes constitute a relatively new subclass of homeobox transcription factors and according to their prominent expression in most anterior head structures, have been implied in their patterning.

For the identification of new members of the *Six* gene family in *Xenopus* degenerate primers flanking the highly conserved Six domain and the homeodomain were used to first screen a gastrula-stage library (from M. Jamrich) by PCR. Several amplicons were isolated, cloned and sequenced. Amplified cDNA fragments containing a *Six*-like sequence were used to rescreen the same library to isolate full length clones. The full length cDNA of *XSix1* in pBSKII+ was subsequently digested with XhoI/NotI to be subcloned into the expression vector pDH105 (gift from R. Harland). Both strands were sequenced and analyzed. A 1436 bp cDNA clone was obtained that contains the complete open reading frame encoding a 284 amino acid protein and the complete 3'UTR with a putative polyadenylation signal (Fig.19). A potential start site is located at nucleotide 108, favored by the base guanidine at position -3 as defined by Kozak (1981). Northern blot analyses with total RNA revealed one major transcript for this gene with a size of about 1.5 kb (Fig.20) indicating that the full length clone had been obtained. Alignments of the predicted amino acid sequence of XSix1 with human and mouse Six1 show a high homology of these proteins among the different species. The homeodomains of the three proteins are identical, as is the Six domain of human and *Xenopus* Six1 (Fig.21). The Six domain of *Xenopus* and mouse Six1 are almost identical (98% homology). Comparison of XSix1 to *Drosophila* sine oculis (so) also shows a high degree of homology in the Six domain and homeodomain, 85% and 93% respectively.

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GCACGAGTTTCCCTGCACACAGTGCCGTGGATATAGAGAGTGCGCGTGCTCCCCGTGTATGTGTTTG 67
CGTGTGCGCTGTGGGCCCCCGGCTGTGCCACACTGAGCC ATG TCT ATG CTG CCT TCC TTT 127
                                         M   S   M   L   P   S   F  7

GGC TTC ACT CAG GAG CAA GTG GCC TGT GTG TGC GAG GTG CTG CAG CAG GGA 178
 G   F   T   Q   E   Q   V   A   C   V   C   E   V   L   Q   Q   G  24

GGC AAC TTG GAG AGA CTG GGC AGA TTC CTA TGG TCC TTG CCA GCC TGC GAT 229
 G   N   L   E   R   L   G   R   F   L   W   S   L   P   A   C   D  41

CAC CTC CAC AAG AAT GAG AGC GTC CTA AAG GCA AAG GCC GTG GTG GCA TTT 280
 H   L   H   K   N   E   S   V   L   K   A   K   A   V   V   A   F  58

CAC AGG GGC AAC TTC AGA GAA CTG TAC AAG ATC CTG GAG AGC CAC CAG TTC 331
 H   R   G   N   F   R   E   L   Y   K   I   L   E   S   H   Q   F  75

TCC CCG CAC AAC CAC CCC AAA CTG CAG CAA CTC TGG CTC AAG GCT CAT TAT 382
 S   P   H   N   H   P   K   L   Q   Q   L   W   L   K   A   H   Y  92

GTG GAG GCA GAG AAA CTG AGG GGG AGA CCA CTG GGG GCA GTG GGC AAG TAC 433
 V   E   A   E   K   L   R   G   R   P   L   G   A   V   G   K   Y 109

AGG GTC AGG AGG AAA TTC CCG CTG CCC AGG ACC ATC TGG GAT GGA GAA GAG 484
 R   V   R   R   K   F   P   L   P   R   T   I   W   D  G   E   E 126

ACC AGT TAC TGC TTT AAG GAG AAG TCC CGA GGG GTG CTG AGA GAA TGG TAT 535
 T   S   Y   C   F   K   E   K   S   R   G   V   L   R   E   W   Y 143

GCC CAT AAC CCC TAC CCG TCT CCC AGG GAG AAG CGG GAG TTG GCT GAG GCC 586
 A   H   N   P   Y   P   S   P   R   E   K   R   E   L   A   E   A 160

ACT GGA CTA ACC ACC ACC CAG GTC AGC AAT TGG TTC AAG AAC AGG AGG CAA 637
 T   G   L   T   T   T   Q   V   S   N   W   F   K   N   R   R   Q 177

AGG GAC AGA GCG GCG GAG GCG AAA GAG AGG GAA AAT ACA GAA AAC AAT AAC 688
 R   D   R   A   A   E   A   K   E   R   E   N   T   E   N   N   N 194

ACA TCT ACC AAC AAA CAG AAT CAA CTG TCA CCC CTA GAC GGA GGG AAG TCG 739
 T   S   T   N   K   Q   N   Q   L   S   P   L   D   G   G   K   S 211

CTA ATG TCC AGT TCA GAA GAG GAA TTC TCG CCC CCA CAA AGC CCT GAT CAG 790
 L   M   S   S   S   E   E   E   F   S   P   P   Q   S   P   D   Q 228

AAC TCG GTG CTC CTG TTG CAG GGC AGC CTC ACC CAC CCC GGG GCC ACC TCT 841
 N   S   V   L   L   L   Q   G   S   L   T   H   P   G   A   T   S 245

TAC TCC CTG AGC GCA CTT AGC GCA TCG CAG GGC AGT CAC GGC TTA CAG GGG 892
 Y   S   L   S   A   L   S   A   S   Q   G   S   H   G   L   Q   G 262

CAC CAG CAC CAG CTG CAG GAC TCT CTG CTC GGG CCC CTC ACC TCC AGC CTG 943
 H   Q   H   Q   L   Q   D   S   L   L   G   P   L   T   S   S   L 279

GTG GAT CTG GGA TCG TAA GAGCGACCGGAACCAGCCATGGACTGCACTCTTATTGTACATA 1004
 V   D   L   G   S   *                                              284

GCAAGGCACTCATCTCATCATGGTACTTAATGTGGAAACAAACTGGGACTTTTTATACTTTTCTTCG 1071
GTACAATCAATCCCACAAAGCAGCCCCCCCTGCACCGCTAAATGCTCTTACGTTACGGCCTCCTGTG 1138
GCACACACACACCCTCTCCCTCAAAATGCTCTAACAAAATACTGTATAGGAGATACCACAAGGATAT 1205
AACTGTGTCTTATTTATGAACACCCCCTCCCCCCAAACAATGTGGTTAAATCTGGAGAGAATTGCTG 1272
TTTGCTCCCACTGTGACACCTAGAATAATTGCCCAGCTCGTTGGAGGCGCTAAGTTTACATTGCTCC 1339
CCCCCCCCCCACATTTAGTGTTGAAAGAAGATTGCAAAGTAGTTTATAATAAAAATACAGTATTAGA 1406
AAGCACAAAAAAAAAAAAAAAAAAAAAAAA                                       1436
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Fig.19: Nucleotide sequence of *XSix1*cDNA and predicted amino acid sequence. The Six domain is underlined and the homeodomain is indicated in bold. The underlined nucleotide sequence is the putative polyadenylation signal.

A higher divergence between *Xenopus* and other species exists in the C-terminal region with homologies of 82% to mouse Six1, 83% to human Six1, and only 18% to so. The tetrapeptide ETSY near the N-terminus of the homeodomain of *X*Six1, also found in mammalian Six1 and so, indicates that this protein belongs to the Six2 family, as described in Seo et al. (1999).

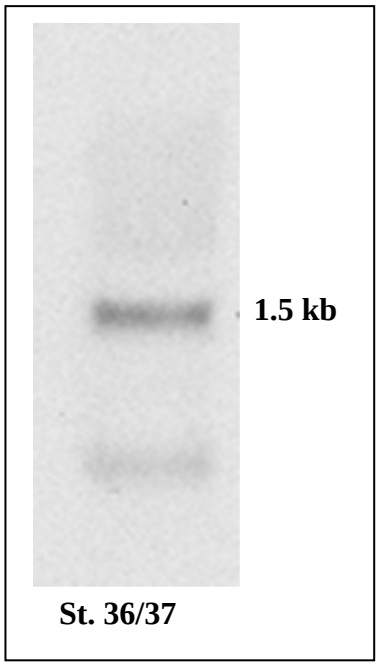


Fig.20: Northern blot analysis of *XSix1*. Total RNA was isolated from stage 36/37 embryos. The size of the detected transcript is indicated on the right and was estimated by comparison with the RNA ladder (not shown).

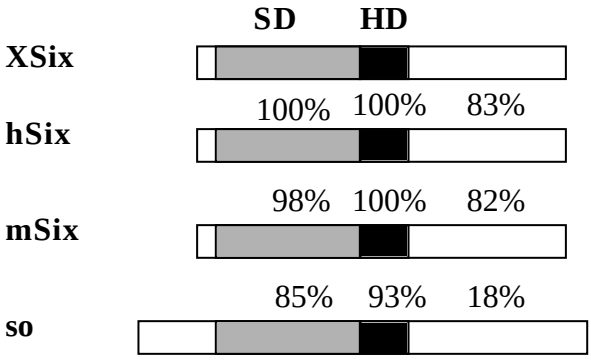


Fig.21: Comparison of the Six (SD) and homeodomain (HD), as well as the C-terminal region of XSix1 with those of murine and human Six1, and Drosophila so. Homologies are given in % with regard to XSix1.

4.4.2 Temporal expression of *XSix1*

The developmental expression of *XSix1* was determined by RT-PCR using total RNA from embryos at pre-MBT to early tadpole stages (Fig.22). No maternal transcripts were detected. Expression of *XSix1* begins at mid-gastrula and remains at a robust level through early tadpole stages. This demonstrates that *XSix1* expression is associated with both early embryogenesis and later organogenesis events, and predicts that its protein may be needed for different developmental processes.

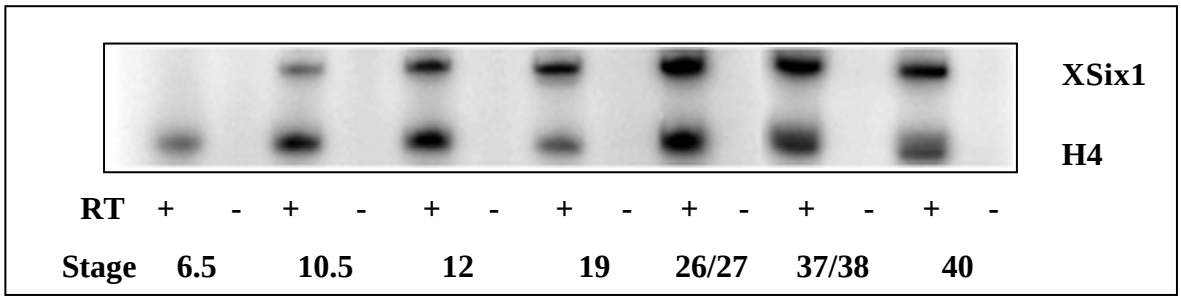


Fig.22: Developmental expression pattern of *XSix1* as determined by RT-PCR. *H4* was used as a loading control. *XSix1* transcripts are absent maternally (stage 6.5). Zygotic expression of *XSix1* starts during gastrula stages (stage 10.5) and continues into early tadpole stages (stage 40).

4.4.3 Embryonic expression of *XSix1*

During gastrula stages *XSix1* transcripts are distributed uniformly across the animal hemisphere (presumptive ectoderm) and are absent from the vegetal half (presumptive endoderm and mesoderm), as determined by whole mount in situ hybridization. A spatially restricted distribution of *XSix1* transcripts is detected during late neurula stages (stage 17/18), when expression is seen as a band in the primitive placodal thickening surrounding the cephalic neural plate and presumptive neural crest (Fig.23A). There also is fainter staining within the most anterior part of the neural plate. At stage 20/21, when the placodes of the dorso-lateral series become histologically apparent as thickenings of the sensorial layer of the epidermis (Winklbauer, 1989), *XSix1* is highly expressed in the olfactory, trigeminal and acoustico-facialis complex, which includes the preauditory lateral line, facial and auditory

placodes (Fig.23B). By stage 31/32 additional structures that express *XSix1* at high levels are the otocyst, and the epibranchial placodes of the glossopharyngeal and vagus cranial nerves (IX/X).These latter give rise to the trunk lateral lines (Winklbauer, 1989) (Fig.23C). A transverse section through the head of a stage 31/32 embryo shows the continued strong expression of *XSix1* in the ectoderm of the olfactory placodes (Fig.23F). The same expression pattern is seen in the ectodermal placodes of the trigeminal ganglion (Fig.23G) and of the glossopharyngeal and vagus ganglia (Fig.24B). Although the neuronal descendants of these placodes have begun to delaminate and coalesce as cranial ganglia (V, VII/VIII, IX/X), these differentiating derivatives do not express *XSix1.* Faintly stained cells in the mesenchyme subjacent to the placodes may be either head mesodermal cells or ganglion cells that have down-regulated this gene.

The lateral line system derives from a dorso-lateral series of placodes that extend from the posterior margin of the eye to the posterior branchial arches (reviewed in Winklbauer, 1989). The preauditory lateral line placode, part of the acoustico-facialis complex, gives rise to the head lateral lines, and a posterior cluster of placodes, part of the glossopharyngeal and the vagus (IX/X) complexes, gives rise to lateral line primordia of the trunk. Prior to stage 31, these placodes express *XSix1* at high levels and this expression is maintained in the developing lateral line primordia. Around stages 31/32 transcripts are detected in ectodermal patches posterior to the IX/X complexes at the ventral margin of the somites (Fig.23C). These may be the precursors of the ventral trunk lateral line, which is derived from the vagus complex (Winklbauer, 1989). By stage 39, the segmented cell clusters are located ventral to the pronephros (Fig.23D), which is the correct position for the elongating ventral trunk lateral line at this stage (Winklbauer, 1989). Furthermore, *XSix1* expression is prominent over the midregion of the somites, which corresponds to the dorsal/middle trunk lateral line primordium, as it elongates posteriorly. By stage 42, when individual sense organs have formed, *XSix1* delineates an anterior, ventrally pointing branch of the ventral trunk lateral line and a posterior branch that extends to the anus (Fig.23E). *XSix1* staining in the dorsal/middle trunk lateral lines has become fragmented into a series of small cell clusters, that histologically

resemble the mechanoreceptive neuromasts shown in a trunk section of a stage 37/38 embryo (Fig.23H). The preauditory lateral line placode gives rise to the supraorbital lateral line along the dorsal margin of the eye and the infraorbital lateral line along the ventral margin of the eye (Winklbauer, 1989). *XSix1* expression is found in similarly located spots in the ectoderm (Fig.24A).

XSix1 is highly expressed in the otic placode and in its derivative that gives rise to the inner ear and facial ganglion, the otocyst. Expression is strongest in the lateral wall of the otocyst, which underlies the ectoderm (Fig.24B). Weaker staining is found in the ventromedial region of the otocyst which will give rise to the acoustic ganglion and sensory epithelium of the inner ear (Norris, 1892). In the same section there also is staining visible in the dorsal brain.

Unlike murine *Six1*, *XSix1* transcripts are not abundant in mesodermal structures as determined by in situ hybridization. Very faint staining is observed in the most lateral portion of the somites (dermamyotome) underlying the ectoderm (Fig.24C).

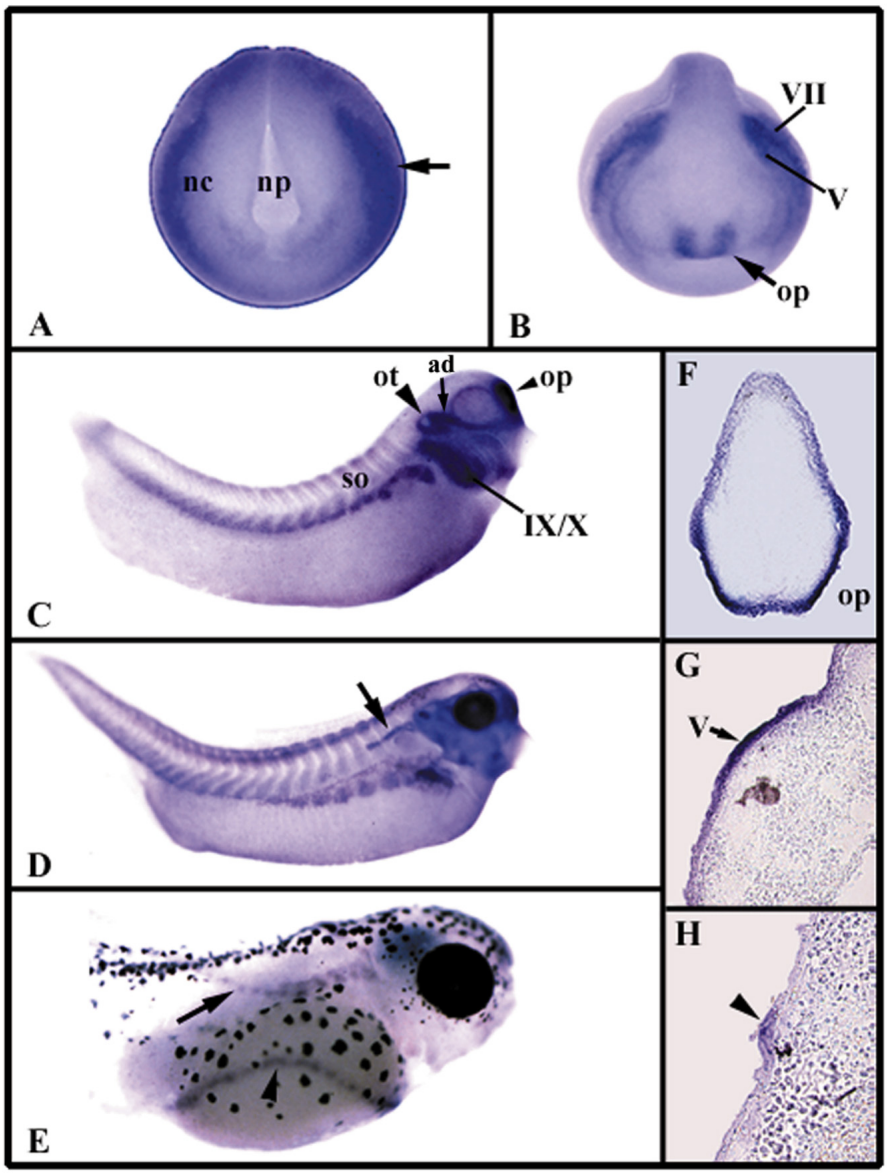


Fig.23: Embryonic expression pattern of *XSix1*. (A) Localized expression of *XSix1* is first visible during late neurula stages (stage 18) (arrow). Transcripts are detected in the region adjacent to the neural plate (np) and neural crest (nc), which will give rise to head placodal derivatives. (B) By stage 20/21 high levels of *XSix1* expression are seen in the olfactory placodes (op), in the trigeminal placode (V), and in the acoustico-facialis complex of placodes including the preauditory lateral line placode (VII) and the auditory placode. (C) High levels of expression of *XSix1* in the olfactory placodes (op) continues into tailbud stages. In addition, *XSix1* is detected in the otocyst (ot), the anterodorsal lateral line placode (ad) and glossopharyngeal/vagus placode (IX/X). *XSix1* also is expressed in cell clusters ventral to the somites (so) which may be precursors of the developing ventral trunk lateral line system. (D) By stage 39 *XSix1* transcripts also are detected in the dorsal/middle trunk lateral line system which is elongating posteriorly (arrow). (E) At early tadpole stages (stage 42) *XSix1* expression is predominantly seen in the lateral line system (arrow: dorsal/middle trunk lateral lines; arrow head: ventral trunk lateral line).
Transverse sections of a stage 30/31 embryo reveal the predominantly ectodermal expression of *XSix1* in the olfactory placodes (F), and in the trigeminal (V) placode (G). H shows *XSix1* expression in a neuromast (arrowhead) of the lateral line system.

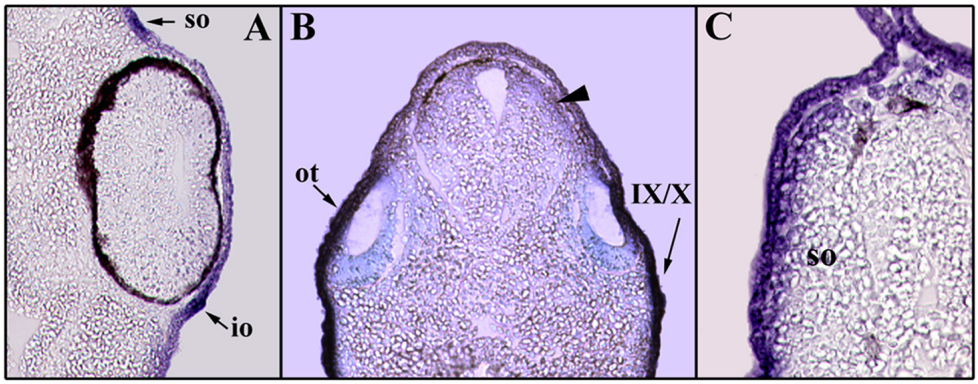


Fig.24: *XSix1* expression in the head of a stage 30/31 embryo (A, B) and in the trunk region of a stage 34/35 embryo (C). (A) *XSix1* expression is intense in the supra- and infraorbital lateral line primordium (so, io) in the head. (B) Transcripts of *XSix1* are detected in the dorsal brain (arrow), the lateral wall of the otocyst (ot) underlying the ectoderm, and the glossopharyngeal/vagus placode (IX/X). (C) A low level of *XSix1* expression can be detected in the dermamyotome of the somites (so).

4.4.4 Animal cap explants

To identify genes that regulate the initial expression of *XSix1* the ability of selected genes to induce *XSix1* in ectodermal animal cap explants was investigated. Embryos were injected bilaterally at the 2-cell stage in the animal pole with 20 pg of either *BMP4*, *noggin* or *cerberus* mRNA or 100 pg *pax6* mRNA. Small animal caps from injected and control, uninjected, embryos were dissected at stage 8 and cultured until sibling embryos reached stage 23/24, at which time they were harvested for analysis by RT-PCR. Animal caps taken from *cerberus* or *noggin* mRNA injected embryos expressed *XSix1* (Fig.25). This result is in accordance to *XSix1*'s expression in placodes that will give rise to neural structures and dorsal brain. Consistent with these results, BMP4, a member of the TGF-β family that directly induces epidermis and antagonizes neuralization, does not induce *XSix1* expression. Thus, the ectodermal regions that express *XSix1* are likely the neurogenic fields that give rise to the various peripheral neural structures in the head. Also, overexpression of *pax6*, a paired-box homeobox transcription factor present in a subset of head placodes, induces *XSix1.* Furthermore, *XSix1* expression is expanded in the olfactory placode of embryos that were injected with 100-150 pg *pax6* RNA unilateral into a dorsal lateral blastomere at the 16-cell stage (D1.2) (Fig.26).

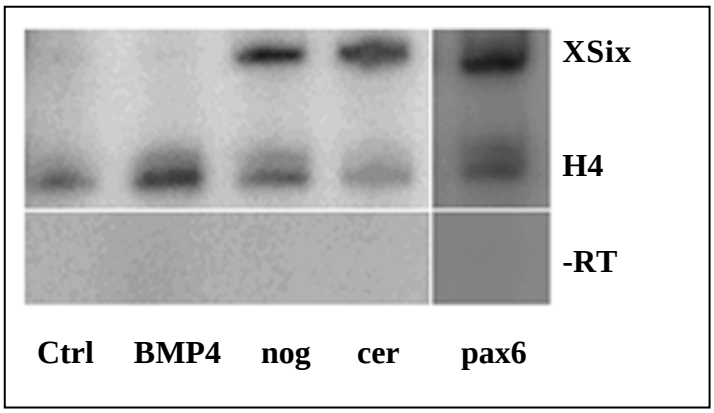


Fig.25: Animal cap explants to determine upstream regulators of *XSix1*. Animal caps overexpressing the neural inducers Noggin (nog) or Cerberus (cer) express *XSix1*. *XSix1* transcripts are not detected in caps that overexpress the antineurogenic factor BMP4. Overexpression of the head placodal marker pax6 also induces *XSix1*.

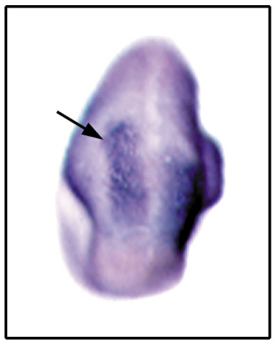


Fig.26: Face-on view of a tailbud stage embryo. Overexpression of pax6 in a dorsal lateral blastomere at the 16-cell stage (D1.2) results in an expansion of *XSix1* expression in the olfactory placode on the injected side (arrow).

However, the expression of *XSix1* is not confined to ectodermal derivatives. *XSix1* transcripts can be detected by RT-PCR in embryonic somitic tissue and adult skeletal muscle (Fig.27A). Recent reports also suggest that *Six* genes are involved in myogenesis (Spitz et al., 1998; Ohto et al., 1999). To test whether *XSix1* also might have a later role in myogenesis, we examined its ability to induce myogenin, a bHLH transcription factor, that is involved in secondary myogenesis in *Xenopus* (Nicolas et al., 1998). Overexpression of *XSix1* in animal

caps led to the induction of myogenin (Fig.27B). Thus, *XSix1* likely has a second role in muscle formation later in development.

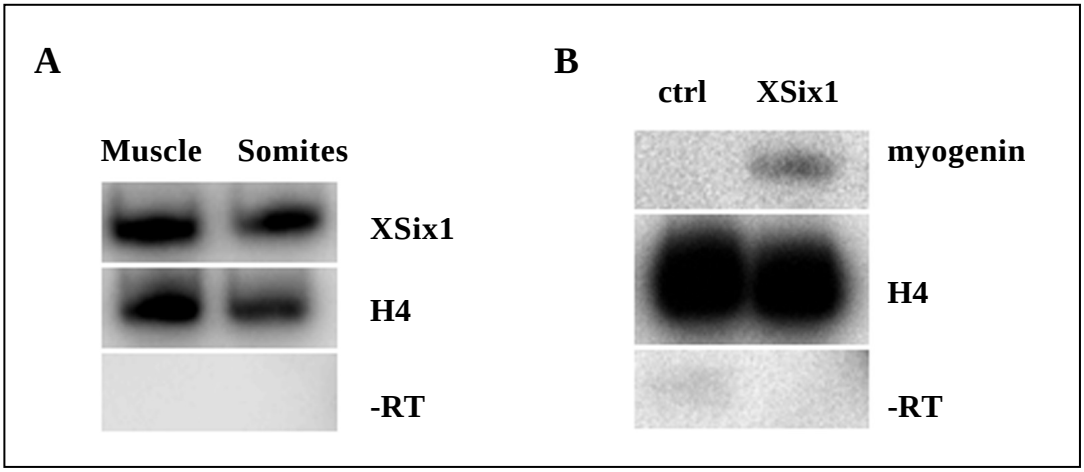


Fig.27: (A) Total RNA was extracted from adult muscle and somites from stage 27/28 embryos. RT-PCR showed that XSix1 transcripts are present in both tissues. (B) Animal cap assay. Overexpressing XSix1 induces *myogenin.*

5 Discussion

5.1 **Part I:** Identification of localized maternal RNAs

An initial RT-PCR based screen was performed to test if known maternal RNAs with dorsal axis inducing activity are differentially localized in either dorsal (D1.1) or ventral (V1.1) animal midline blastomeres at the 16-cell stage. Interestingly, among the tested molecules *XWnt-8b*, a maternal member of the Wnt family of signaling peptides, was found to be enriched in V1.1 blastomeres. This is striking because *XWnt-8b* belongs to the *Wnt1* class, whose members (*XWnt1, XWnt3a, XWnt8*) induce a complete secondary axis when overexpressed in ventral vegetal blastomeres before the onset of zygotic transcription (MBT) (reviewed in Moon and Kimelman, 1998). Despite their ability to induce a dorsal axis when ectopically expressed, it can not simply be concluded that these *Wnt* genes play a role in establishing the dorsal axis *in vivo*. Depletion studies showed that the key component of the Wnt signaling pathway essential for dorsal axis formation is the multifunctional protein β-catenin (Heasman et al., 1994). Overexpression of dominant-negative constructs of other members that are upstream of β-catenin, *XWnt8* and *dishevelled* (Hoppler et al., 1996; Sokol, 1996) do not block the formation of the endogenous axis, suggesting that a maternal *XWnt* is an unlikely candidate to elicit this signaling cascade to direct dorsal axis formation. Nevertheless, it is intriguing that endogenous, ventrally-localized *XWnt8b* does not initiate a dorsal program in ventral animal blastomeres (pre-MBT) since it could activate the Wnt signaling pathway, resulting in the activation of dorsalizing target genes such as *siamois*, *Xtwn* or *Xnr-3*. It is possible that other factors necessary for the activation of the Wnt-signaling cascade are not coexpressed with *XWnt8b*, most importantly, an appropriate Wnt receptor (frizzled). Of the five known *Xenopus frizzled* (*Xfz*) only two are maternally expressed. *Xfz-3* however, is only expressed at low levels and does not become localized before neurula stages (Shi et al., 1998) and *Xfz-7* is expressed on the dorsal side of the embryo at gastrula stages

(Wheeler and Hoppler, 1999). These data do not strongly support a role for these receptors as likely candidates to mediate maternal signaling by *XWnt8b.*

Another *Wnt* gene closely related to *XWnt8b* is the zygotically expressed *XWnt8* whose transcripts are distributed in the ventrolateral marginal zone of gastrula stage embryos (Christian et al., 1991). Although *XWnt8* RNA, as indicated above, induces a secondary axis when ectopically expressed before MBT, after MBT, XWnt8 has no axis inducing activity and functions in patterning the ventrolateral mesoderm *in vivo* (Christian et al., 1991; Christian and Moon, 1993). It thereby regulates the expression of ventral and lateral marker genes, such as *Xpo* (Sato and Sargent, 1991), *Evx1* (Ruiz i Altaba and Melton, 1989) and *XMyoD* (Hopwood et al., 1989). The differences in early (pre-MBT) and late (post-MBT) *Wnt* signaling also were demonstrated for *XWnt8b* by overexpressing a construct, CSKA-*XWnt8b,* that is controlled by the cytoskeletal actin promoter to drive expression after MBT (Cui et al., 1991). In contrast to *XWnt8b* RNA, which is translated before MBT, injections of CSKA-*XWnt8b* DNA into the ventral marginal zone did not result in the formation of a secondary axis (Cui et al., 1991), suggesting that *XWnt8b,* like *XWnt8,* functions in DV axis specification by establishing a ventral rather than a dorsal bias in the cells in which it is expressed. The interesting observation presented in this work that maternal *XWnt8b* transcripts are localized to ventral animal midline blastomeres at the 16-cell stage raises the question of whether pre-MBT *XWnt8b* signaling also is involved in determining ventral fates and already does so during early cleavage stages.

As described previously D1.1 blastomeres are biased towards a dorsal fate and autonomously elongate in culture (Gallagher et al., 1991). If *XWnt8b* acts as a ventralizing factor, it should counteract this dorsal bias and inhibit elongation. Indeed, this was demonstrated by D1.1 explants overexpressing *XWnt8b.* The same effect was seen in cocultures of V1.1 with D1.1 blastomeres, suggesting that endogenous *XWnt8b* signaling from ventral animal blastomeres can impair the dorsalizing properties of D1.1 blastomeres. It can be excluded that other maternal factors are involved in causing these effects since overexpression of *XFrzb,* which specifically blocks *XWnt8b* function, restores elongation. Phenotypes of the blastomere

explants were most obvious when sibling embryos reached stage 23/24 and were then scored. Considering the late developmental stage when explants were scored it could be argued that the inhibition of explant elongation is caused by the zygotically expressed *XWnt8*. However, this is unlikely since Ding et al. (1998) showed that *XWnt8* is not expressed in animal blastomere explants when dissected at the 16-cell stage, which was confirmed during this work. Still, the inhibitory function of *XWnt8b* could be a late (post-MBT) effect, which would be similar to *XWnt8*. Therefore, it needs to be determined if *XWnt8b* is being translated before MBT and can account for the effects. Polysomal and non-polysomal RNA isolated from 16- to 32-cell stage embryos was tested for the presence of *XWnt8b* transcripts. *XWnt8b* was not found to be associated with polysomes at the investigated stages and hence we can not confirm that it is translated at that time point. However, this finding does not exclude that *XWnt8b* might be translated earlier. To clarify this issue it will be necessary to test polysomal and non-polysomal RNA isolated from different early cleavage stages for the presence of *XWnt8b* transcripts. Also, sequence analysis of the 3'UTR of *XWnt8b* could reveal sequence motifs that control its polyadenylation status. Such a motif was identified in the 3'UTR of *XWnt8* that directs the de-adenylation of this transcript (Tian et al., 1999). This temporally regulated translation prevents the inappropriate differentiation of cells and tissues in which *XWnt8* is expressed. A similar situation could be hypothesized for *XWnt8b*.

With the above described RT-PCR screen only known maternal dorsalizing genes could be tested for differential expression along the DV axis in animal blastomeres. Another goal was to identify possible novel dorsalizing factors that are localized to dorsal animal midline blastomeres (D1.1) that become activated on a post-transcriptional level between the 8- and 16-cell stages. Supporting data for this assumption comes from findings that show that (1) D1.1 blastomeres differentiate autonomously into dorsal mesodermal structures when cultured in a simple salt medium (Gallagher et al., 1991) and (2) total RNA isolated from D1.1 blastomeres at the 16-cell stage has axis inducing activity whereas total RNA isolated from D1 blastomeres at the 8-cell stage does not induce secondary axial structures (Hainski

and Moody, 1992; 1996). Of the several post-transcriptional activation mechanisms known, cytoplasmic polyadenylation was the most likely mechanism to render these dorsal-specific RNAs active; isolated RNA pools from D1 blastomeres are protein-free and hence could elicit the induction of a secondary axis when ectopically expressed if associated proteins inhibited their translation *in vivo*. Indeed, after the *in vitro* elongation of the poly(A) tails of poly(A-) RNAs isolated from D1 blastomeres, this RNA pool was able to induce partial secondary axes. In contrast to previous results (Hainski and Moody, 1992; 1996) in which total RNA was used, the present study shows that D1 mRNA weakly elicited the formation of secondary axial structures. This suggests that the activation of these dorsalizing RNAs does not occur at once in a 'switch-like' fashion between the 8- and 16-cell stages, but instead seems to be a gradual process that begins at least by the 8-cell stage and produces an effective concentration at the 16-cell stage. Therefore, RNA from D1.1 blastomeres was used to construct an expression cDNA library in order to isolate dorsalizing RNAs through sib selection techniques.

Sib selection is a widely used method that has led to the identification of several important dorsalizing genes, such as *noggin* (Smith and Harland, 1992) and *siamois* (Lemaire et al., 1995), that play a role in DV axis specification. The potential feasibility of this method was demonstrated in the first round when some of the injected D1.1 RNA pools induced partial secondary axes. Unfortunately, due to a contamination it was not possible to continue the experiment with this particular D1.1 cDNA library. A new library was constructed and the experiment repeated. However, this time the induction of ectopic axial structures by different RNA pools was not as strong. This can be explained by a relatively large amount of partial length cDNAs contained in this library, which was shown by the determination of the average insert length (≈ 850 bp). An oligo(dT) primer was used for first strand cDNA synthesis and it appears that not many full length cDNAs were obtained. Synthesized cDNAs are therefore likely to consist primarily of sequences localized in or near the 3'UTR of the corresponding RNA. Additionally, the subsequent PCR step probably resulted in an overproportional amplification of short cDNAs. As a consequence, the *in vitro* transcribed

RNAs from these truncated cDNAs were most likely overrepresented in the injected RNA pools. Nevertheless, ectopic somitic tissue was detected in some embryos after the first round of sib selection. Yet another phenotype, which affected normal somite development, was more prominent during the following rounds. Although injections of RNA pools resulted in an obvious malformation of anterior somites, the effect became more subtle and could not have been detected without immunostaining using a somite-specific antibody when only single RNAs were injected in the last round. Somites develop in a characteristic segmented pattern through subdivision of the paraxial mesoderm, which in *Xenopus* is unique in that a group of myotomal cells (somitomeres) segregates, rotates 90°, and orients parallel to the antero-posterior axis (Hamilton, 1969). Injection of single RNAs obtained through sib selection led to a disruption of this symmetrically arranged tissue. Most common was the fusion of adjacent somites or a general disorganization of the somitic blocks. Fusion of somites also can be caused by heat shock treatment, which was detected by an altered pattern of the extracellular matrix proteins fibronectin and laminin (Danker et al., 1992). A molecular mechanism for the orderly segmentation of somites was provided with the Notch signaling pathway (Jen et al., 1997; Jen et al., 1999). The Notch receptors are large transmembrane proteins that are bound and activated by a family of related ligands, the Delta-Serrate-Lag2 (DSL) proteins (reviewed in Artavanis-Tsakonas et al., 1995). Somite defects have been reported when the Notch signaling pathway was either blocked by dominant-negative forms of X-Delta-2 or inappropriately activated by misexpression of Delta homologues (Jen et al., 1997). It was further demonstrated that proper segmentation of somites requires the periodic repression of the Notch signaling pathway (Jen et al., 1999). It could be hypothesized that some of the isolated cDNA clones through sib selection code for genes that might interfere with this signaling pathway and hence disturb the orderly formation of somitic segmentation. However, sequence analyses of candidate cDNA clones did not identify homologies to genes that could be involved in Notch signaling.

Nevertheless, the embryonic expression of some clones was determined in whole mount in situ hybridizations. Two clones displayed interesting expression patterns. Clone Z is

expressed in specialized ciliated cells in the epidermis. Interestingly, the evenly spaced pattern that is characteristic for the distribution of these cells is under the control of lateral inhibition, which is regulated by the Notch signaling pathway (Deblandre et al., 1999). Furthermore, this clone also is expressed in somites and marks their development starting rostral and moving caudal. Sequence analyses however, indicated high homologies of this clone to *Xenopus* mitochondrial DNA. This is in accordance to its expression pattern, since ciliated cells and muscle tissue possess a relatively high energy turnover. Moreover, the isolation of mitochondrial DNA during this screen is not surprising, since Yost et al. (1995) showed that mitochondrial organelles are asymmetrically distributed along the DV axis in animal blastomeres with an enrichment on the dorsal side.

Clone G is expressed predominantly in neural structures during later development, such as the cranial ganglia and Rohon-Beard neurons. The latter are primary sensory cells that, in amphibia, are functionally replaced by dorsal root ganglia after metamorphosis. Sequence analyses of the partial cDNA clone did not reveal any homologies to known genes and therefore, clone G could be a novel factor involved in neural development. Again, the identification of a cDNA clone whose endogenous product seems to function during neurogenesis is in accordance with the embryonic region from which it was isolated since dorsal animal midline blastomeres are the major precursors of the nervous system in *Xenopus* (Moody, 1987a,b).

5.2 **Part II:** Characterization of a novel *Xenopus Six* gene and its involvement in sensory organ formation

Members of the *Six* gene family are characterized by two highly conserved domains, the homeodomain and the Six domain. Sequence comparisons on the amino acid level revealed that the homeodomains of *Xenopus,* murine, and human Six1 are identical, as are the Six domains of *Xenopus* and human Six1. Therefore, this clone is referred to as *XSix1.* The Six domain of the murine Six1 only differs from that of XSix1 in two amino acid residues near the

N-terminus. Since the C-terminal half of the Six domain and the homeodomain are required for sequence-specific DNA binding (Kawakami et al., 1996a), small differences near the N-terminus may not be significant. Despite the extremely high sequence homology between *Xenopus* and mouse Six1, *XSix1* does not seem to be functionally identical to its murine counterpart, as there are significant differences in the embryonic expression patterns of these two genes during early developmental stages. Expression of *XSix1* is prominently ectodermal whereas murine *Six1* is predominantly mesodermal (Oliver et al., 1995a). There are several potential explanations for these differences in expression patterns. These two genes may be flanked by different regulatory genomic sequences, or may contain different regulatory regions in the 5' and/or 3' UTRs of the mRNAs. There may be different splice forms of these genes that are responsible for the developmental expression differences as well. For example, murine *Six1* is characterized by two major transcripts with approximate sizes of 2.3 kb and 2.9 kb (Oliver et al., 1995a), whereas Northern hybridization analyses for XSix1 revealed only one major transcript of approximately 1.5 kb. In addition, the less homologous C-terminal region of the murine and *Xenopus* Six1 proteins contains a potential activation domain that is critical for an optimal activation of target genes in cooperation with specific Eya proteins (Ohto et al., 1999). Eya proteins, which are the vertebrate homologues of the *Drosophila eyes absent* gene, and Six proteins interact by forming a complex via their evolutionary conserved Six and Eya domains (Pignoni et al., 1997; Ohto et al., 1999). In this context it is important to understand the distribution of *eya* gene expression to elucidate *Six* gene function in the distinct tissues in which these genes might be coexpressed. For example, murine Eya1 and Eya2 are in part differentially expressed in the cranial placodes. *Eya1* transcripts are present in the olfactory and lens placodes, whereas *Eya2* transcripts are absent from the lens placode (Xu et al., 1997). The trigeminal placode and ganglion only express *Eya2*, whereas only *Eya1* transcripts are detected in the otic vesicle (Xu et al., 1997). The expression pattern of zebrafish *eya1* is most similar to the expression of *XSix1*, in that it is expressed throughout the placodal region and later in all lateral line placodes and sensory cells (neuromasts) (Sahly et al., 1999). A possible *Xenopus* homologue of zebrafish *eya1* may

also be localized to these placodes and hence a likely cooperation of these two genes in the development of the lateral line system is suggested.

Since the embryonic expression pattern of *XSix1* suggests a function for this gene in the establishment of cranial placodes, it is of interest to elucidate *XSix1*'s position in the molecular hierarchy of placode development. It should be noted that despite the strong mesodermal expression of murine *Six1*, its transcripts also are detected in the nasal placodes and in structures derived from head placodes, such as the otic vesicle and the ectoderm-derived Rathke's pouch, which gives rise to the adenohypophysis (Oliver et al., 1995b). The differentiation of a broad field of placode-competent ectoderm into discrete anlagen of the head sensory organs must involve differential signaling leading to the differential expression of a number of transcription factors. The characterization of genes that are only expressed in a subset of placodes, such as *eya*, should shed more light on the mechanisms by which these sensory organs are specified. There are several transcription factors whose expression domains overlap within different subsets of presumptive head sensory tissues (reviewed in Torres and Giraldez, 1998). Members of the Hox gene complex are likely to play a role in the regulation of placode identity (Northcutt, 1996; Metscher et al., 1997). Three genes, *otx2*, *pax6*, and *sox3*, are expressed in presumptive olfactory and lens ectoderm (Walther and Gruss, 1991; Grindley et al., 1995; Zygar et al., 1998). The homeobox transcription factor *X-dll3* is expressed in the olfactory and otic placodes but is absent from the lens placode (Papalopulu and Kintner, 1993). In contrast, *Xlens1*, which belongs to the fork head family of transcription factors, is expressed in the ectoderm of the lens placode exclusively (Kenyon et al., 1999). *Pax3* is expressed exclusively in the trigeminal placode in chicken (Baker et al., 1999). Undoubtedly, there are several more transcription factors involved in placode specification (e.g. see list for otic placode in Torres and Giraldez, 1998). However, *XSix1* seems to be unique by being expressed in all neurogenic placodes and differentiating lateral lines.

Recent data indicates that *pax* genes are upstream of *Six* genes and may regulate a new signaling cascade (Heanue et al., 1999). This can be confirmed by the initial studies reported herein that demonstrate *XSix1* to be downstream of *pax6.* However, factors other than Pax6 are likely to activate *XSix1* outside *pax6*'s expression domain. Furthermore, it is likely that different combinations of Pax, Eya and Six genes play a role in determining individual placodal identity.

According to the early expression pattern of *XSix1,* it likely functions in the development of the lateral line. The fact that mammals lack a lateral line system might explain part of the differences observed in the expression of murine and *Xenopus Six1.* However, there is an evolutionary link, since murine *Six1* is detected in the otic vesicle, which in part evolved from the lateral line. At stage 20/21 *XSix1* transcripts are detected in the preauditory lateral line placode, which gives rise to the supraorbital, infraorbital and hyomandibular lateral line primordium, and in the vagus placode, which gives rise to the dorsal, middle, and ventral body lateral lines (Winklbauer, 1989). *XSix1* is not expressed in the ganglion forming parts of the lateral line placodes. Instead, at later developmental stages it identifies primary lateral line organs, which form by the segregation of small groups of cells from the trailing edge of a migrating lateral line primordium. Moreover, expression of *XSix1* also demonstrates the temporal differences regarding the onset of migration of distinct lateral line primordia. For example, at stage 31/32 staining for *XSix1* is already seen in the primary organs of the ventral body lateral line, whereas the segregation of primary organs in the dorsal and middle lateral lines has not begun yet. Heterochronic transplantations of body line primordia in *Ambystoma* revealed that the start of migration is apparently an autonomous property of a given primordium (Stone, 1937) with the ventral primordium always commencing migration first.
A recent investigation of the cause for the loss of the lateral line system in the direct developing frog *E.coqui* (Schlosser et al., 1999) raises the intriguing question of what function *XSix1* may have in this animal. Transplantation experiments demonstrated that the absence of the lateral line system in *E.coqui* is due to the loss of ectodermal competence. If *XSix1* is a

factor that modifies ectoderm to make it competent to respond to lateral line inducing signals, it may be absent or mutated in this frog. The authors suggest that this loss of competence could be due to altered BMP gradients in *E.coqui.* The expression pattern of *XSix1* and its induction by BMP antagonists (Noggin and Cerberus) is consistent with the idea that *XSix1* may not be expressed in *E.coqui.* However, competence for lateral line placode formation can be dissociated from the competence to form other types of placodes, leading the authors to hypothesize that different signaling pathways are involved in the induction of the individual placodes. Since *XSix1* is expressed in additional neurogenic placodes, it can not be the only candidate for the etiology of this evolutionary change in *E.coqui.* A similar case was reported for the loss of the cement gland in *E.coqui,* which was shown to correlate with the missing expression of *otx2* in the cement gland primordium (Fang and Elinson, 1999).

It is interesting that despite the extremely high sequence homology between murine and *Xenopus* Six1 their embryonic expression pattern differs during early developmental stages. Murine *Six1* is predominantly expressed in mesodermal tissues, and it becomes restricted to the muscles, tendons and ligaments of the limbs (Oliver et al., 1995a). Because of their preeminent expression in mesodermal tissues, several murine *Six* genes have been tested for their ability to activate the transcription of *myogenin,* a member of the MyoD family of basic helix-loop-helix proteins. Because of the strong mesodermal expression of murine *Six1,* the presence of *XSix1* in embryonic and adult muscle was investigated. Its presence in the embryonic somite required PCR amplification for detection. Nonetheless, its robust expression in adult muscle and its ability to induce myogenin in explants suggest that *XSix1* functions in muscle development at later stages, just as its mammalian homologues. In *Xenopus* the accumulation of endogenous myogenin transcripts is restricted to secondary myogenesis (Nicolas et al., 1998), which takes place during metamorphosis (Nishikawa and Hayashi, 1994). Thus, the expression of *XSix1* in muscle precursors during early developmental stages may be unnecessary. This difference in mesodermal expression may be due to the different temporal onset of myogenesis in these two species.

6 Summary

Part I of the dissertation focused on (1) the identification of maternal dorsalizing RNAs that are localized within the animal hemisphere along the dorsal-ventral axis at the 16-cell stage and (2) the determination of their post-transcriptional activation mechanism.

An RT-PCR screen to investigate the differential expression of known maternal dorsalizing factors revealed the localized accumulation of *XWnt8b* transcripts in ventral animal midline blastomeres. This finding is interesting since *XWnts* have been shown to induce secondary axis formation when ectopically expressed on the ventral side before the onset of zygotic transcription (MBT). Overexpression of *XWnt8b* in dorsal animal midline blastomeres inhibited their ability to autonomously elongate, implicating that *XWnt8b* counteracts an intrinsic dorsal program of these blastomeres. However, the mere localization of *XWnt8b* transcripts does not predict protein distribution and therefore, the time point at which these transcripts are in the translatable RNA pool needs to be determined carefully.

In addition, cytoplasmic polyadenylation was demonstrated to be the post-transcriptional activation mechanism of dorsalizing RNAs located in dorsal animal midline blastomeres at the 8- to 16-cell stages. For the identification of possible novel dorsalizing RNAs that are localized to dorsal animal midline blastomeres (D1.1), a D1.1 plasmid cDNA library was constructed and dorsalizing messages were screened by sib selection. This process led to the isolation and identification of two partial cDNA clones that displayed an interesting embryonic expression pattern. Clone Z is expressed in specialized ciliated cells in the epidermis and marks the development of somites. Sequence analyses however, indicated high homologies to *Xenopus* mitochondrial DNA. Zygotic expression of clone G is detected in the cement gland during early tail bud stages and becomes restricted to neural structures such as cranial ganglia and Rohon-Beard sensory neurons at later developmental stages. Sequence analyses of the partial clone did not reveal any homologies to known genes and therefore, clone G could be a novel factor involved in neural development.

Part II of the dissertation focused on the characterization of the previously unknown *Xenopus* homeobox transcription factor *XSix1*, whose embryonic expression pattern implicates a role for this gene in the establishment of the 'embryonic field' that gives rise to head sensory organs and the lateral line system in fish and amphibians. It shares a high degree of sequence homology with mouse and human Six1 but, unlike these genes, *XSix1* is prominently expressed in ectodermal tissues during early developmental stages. *XSix1* is highly expressed in the neurogenic cephalic placodes: olfactory placodes, dorso-lateral and epibranchial placodes consisting of the auditory and trigeminal placodes and the preauditory, glossopharyngeal and vagus placodes and their associated lateral lines. *XSix1* expression is upregulated by the neural inducers Noggin and Cerberus, and by the head placodal transcription factor Pax6. During early development *XSix1* is barely detectable in mesoderm, in contrast to its mammalian counterparts. However, transcripts of *XSix1* can be detected in embryonic and adult skeletal muscle by RT-PCR, and it can induce the expression of myogenin. Thus, the function of *Six1* in *Xenopus* has an early component that has not been well-elucidated in mammals, and although it shares high sequence identity with its mammalian homologues, its common activity in muscle development seems to be a later developmental event.

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8 Appendix

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Publications and Poster Presentations

Poster Presentations

7th International *Xenopus* Molecular Development Conference (1998):

Petra D. Pandur, Steven A. Sullivan, and Sally A. Moody

'Maternal dorsal specific mRNAs become activated by polyadenylation during early cleavage stages'

39th American Society for Cell Biology Annual Meeting (1999):

Petra D. Pandur and Sally A. Moody

'Characterization of a *Six* homeobox transcription factor in *Xenopus* and its role in the development of anterior head structures'

Publications

Pandur, P. D. and Moody, S. A. (1999)

Characterization of a *Six* homeobox transcription factor in *Xenopus* and its role in the development of anterior head structures

Mol. Biol. Cell. 10 (Supplement): 596

Pandur, P. D., Brugmann, S. A., and Moody, S. A. (2000)

Xenopus Six1 gene is expressed in neurogenic cranial placodes and maintained in the differentiating lateral lines

(Mech. Dev. in press)

Curriculum Vitae

Petra Daniela Pandur, *27 January 1971 in Heidenheim

Education:	1981-1990	Werkgymnasium Heidenheim, graduated with Allgemeiner Hochschulreife
	1990-1996	University of Ulm, graduated with diploma in biology
		Oral exams for the degree in: Zoology, Microbiology, Biochemistry and Genetics/Molecular Biology
		Research project for diploma: **Identification of novel, zygotic cadherins in *Xenopus laevis*** (Department of Biochemistry, advisor Prof. Dr. D. Wedlich)
	1996-2000	George Washington University, Washington, D.C., USA
		Research project for dissertation: **Specification of the Orthogonal Axes in *Xenopus laevis*: Maternal and Zygotic Genes Involved in Patterning the Dorsal Axis** (Department of Anatomy and Cell Biology, advisor Prof. Dr. S. A. Moody)
	June 2000	Dissertation defense at the University of Ulm; obtained the degree Dr. biol.hum.
Teaching experience:	1994	Teaching assistant in the course 'Medical Microbiology and Virology'
	1995	Teaching assistant in the course 'Biochemistry'
	2000	Teaching assistant in the International, postgraduate course 'Cell and Developmental Biology of *Xenopus*' in Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA
Honors:	1996-1998	Fellowship from the German Academic Exchange Service (DAAD): Doktorandenstipendienprogramm HSPIII