Original Article

Characterization and early embryonic expression of a neural specific transcription factor *xSOX3* in *Xenopus laevis*

ROLAND PENZEL[1], RALF OSCHWALD[1], YONGLONG CHEN, LOTHAR TACKE and HORST GRUNZ*

Universität GH Essen, FB 9, Department of Zoophysiology, Essen, Germany

ABSTRACT Using the powerful RDA-PCR-technique we could identify a novel *Xenopus* specific Sox-gene (*xSox3*) a transcription factor closely related to the sox sub-group B, which contains a HMG box. In normogenesis the *xSox3* gene is expressed in the presumptive central nervous system. Furthermore a maternal component is also found in oocytes and in early cleavage stages in the animal hemisphere only. By whole-mount *in situ* hybridization the first zygotic transcription activities can be detected in the late blastula in the dorsal ectoderm and the dorsal and lateral part of the marginal zone. The expression reaches the highest level at the late gastrula till the late neurula and fades after stage 30. The expression is restricted from gastrulation onwards to the presumptive brain area and the lens epithelium. Furthermore we could show that the gene is expressed in isolated Spemann organizer with adjacent neuroectoderm. The signal can be suppressed by suramin treatment, which inhibits neural development and causes a shift of dorsal to ventral mesoderm. The treatment of whole embryos with LiCl and UV results in an overexpression or an inhibition of the expression, respectively. In exogastrulae (pseudo-exogastrulae) the gene is expressed in the close vicinity to the endomesoderm only, but not in the distal most part of the ectoderm. This result indicates that it is unlikely that the gene can be activated by planar signals. The gene can also be activated in dissociated gastrula ectoderm without mesodermal or neural inducers. That means that the gene can be expressed in ectodermal cells in a cell autonomous manner.

KEY WORDS: *HMG-Box, axis formation, RDA-PCR*

Introduction

The dorsal blastopore lip (Spemann organizer) plays a key role in embryonic axis formation (Spemann and Mangold, 1924). In the classical view informative positive signals were expected to channel the naive ectodermal target cells into neural pathway of differentiation. Meanwhile several secreted proteins (noggin, chordin, follistatin, cerberus) could be identified, which are good candidates to play an important role in the neural determination (Lamb *et al.*, 1993; Hemmati-Brivanlou *et al.*, 1994; Sasai *et al.*, 1994; Bouwmeester *et al.*, 1996). However, the traditional view and expectation that positive signals are involved in neural induction must be revised on the basis of recent data (Grunz and Tacke 1989, 1990; Wilson and Hemmati-Brivanlou, 1995; Grunz, 1997; Hemmati-Brivanlou and Melton, 1997). Inconsistent with the idea of positive signals were results with disaggregated ectodermal cells, which differentiated into neural structures without inducer in a cell autonomous fashion (Grunz and Tacke, 1989; Godsave and Slack, 1991). It turned out that inhibitory signal(s) are present in the intact ectoderm, which cause their differentiation into epidermis (Grunz and Tacke 1990; Wilson and Hemmati-Brivanlou, 1995). Disintegration of the ectoderm, addition of neuralizing factors, which interact with the inhibitory molecules, or the overexpression of dominant negative mutants of BMP/activin-receptors or ligands result in the neuralization of the ectoderm, the putative ground or default state of the ectoderm (see review of Hemmati-Brivanlou and Melton, 1997). So the classical view must be interpreted in that neuralization is rather a releasing than an inducing mechanism. In this signaling pathways, several genes and their products (like BMP2/4, wnt, vent 1/2) play an important role in dorsal/ventral determination and as antagonists to factors like chordin, noggin and follistatin predominantly located in the dorsal mesoderm including Spemann organizer (Gawantka *et al.*, 1995; Kaufmann *et al.*, 1996; Miller and Moon, 1996; Onichtchouk *et al.*, 1996; Piccolo

Abbreviations used in this paper: RDA, representational difference analysis; PCR, polymerase chain reaction; EPPS, 3-[4-(2-Hydroxyethyl)-1-piperazino]-propansulfonic acid; DIG, digoxygenine; CMV, simian cytomegalovirus; HMG, high mobility group.

***Address for reprints:** Universität GH Essen, FB 9 Abteilung Zoophysiologie, Universitätsstr. 5, 45117 Essen, Germany. FAX: <49>(0)201.1833173. e-mail: h.grunz@uni-essen.de

[1]These authors contributed equally to this work.

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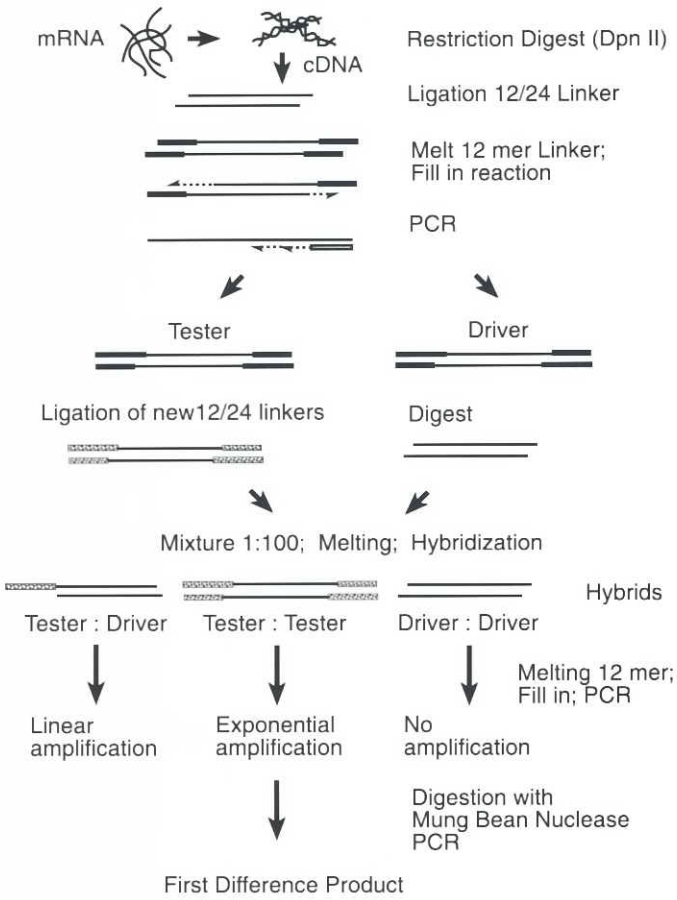

Fig. 1. Schematic diagram of the RDA-PCR method. *Solid boxes show the oligonucleotides used to generate the tester and driver representations. Hatched boxes represent the oligonucleotides used to generate the difference products. Single half-arrows symbolise fill in reactions. Double half-arrows indicate DNA amplification. To generate second and third difference products, previously obtained products are reintroduced into the process at the tester stage.*

et al., 1996; Zimmermann *et al.*, 1996). Already in early cleavage stages factors are present in the dorsal animal hemisphere, which are important for the determination of dorsal-ventral polarity (Grunz, 1977,1994; Cardellini, 1988; Li *et al.*, 1996; Larabell *et al.*, 1997).

We also started the experiments described below with the aim of finding genes, which may play an essential positive signaling role in neural determination. In contrast to earlier approaches, which need a large amount of embryonic tissues (Richter *et al.*, 1988), we used a PCR-based technique, which allows the detection of rarely expressed genes. This technique is a reasonable alternative approach to the random screening method (Gawantka *et al.*, 1995), when small and distinct parts of embryos or tissues can be isolated by time consuming sophisticated microdissection only.

We describe here a novel *Xenopus* specific gene *xSox3*, which belongs to the sox-multigene family coding for transcription factors interacting with DNA in a sequence-specific manner. The expression of *xSox3* in normogenesis and several bioassays suggest that the gene plays an important role in the neural signaling pathway.

Results

Using the Representational Difference Analysis (RDA, see method) (Figs. 1 and 2) we have isolated a neural-specific gene, which belongs to the SOX-family containing a HMG box. By this approach, starting with the neural plates of 40 embryos only, we could identify the *Xenopus* specific *xSox3*, which is expressed in a highly restricted pattern in the dorsal ectoderm and during the development of the central nervous system. However, we could also show that the gene is already expressed maternally and mRNA can be detected in the animal hemisphere of early cleavage stages. Expression of the gene in whole embryos, ectoderm and Spemann organizer after disaggregation, UV-, LiCl- and suramin-treatment suggest that *xSox3* plays an important role in the neural determination pathway.

Isolation and sequence of xSox3

We obtained a 1.660bp cDNA clone with a single open reading frame coding for a 309 amino acid protein with a calculated molecular weight of 34.039 kDa containing a HMG box characteristic of the SOX family (Fig. 3). The 79 amino acid HMG box of *X. laevis sox3* is located 38 amino acids from the first inframe methionine which is assumed to be the translation start by the high overall homology (81.8%) between the *Xenopus* and chicken *sox3* sequences. The 3'-untranslated region (UTR) comprises the putative polyadenylation signal AATAAA and a poly(A) tail with thirty adenine residues.

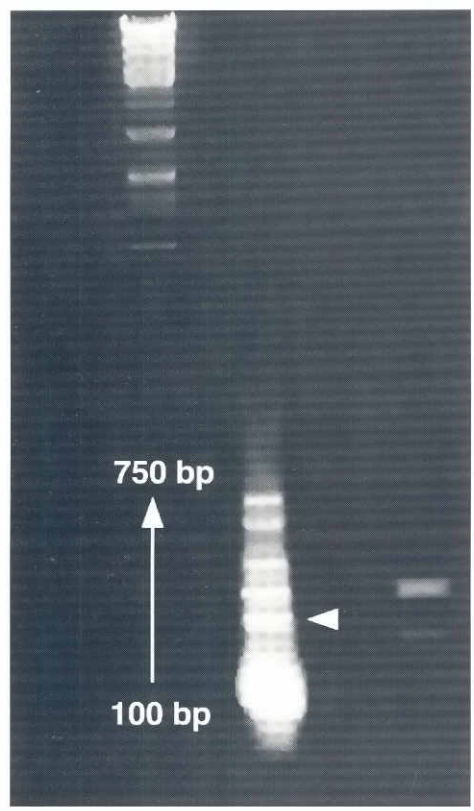

Fig. 2. Separation of the Third Difference Product (DP3) on a 2% agarose gel. *The arrow indicates the PCR fragment named N5/4 (236 bp).*

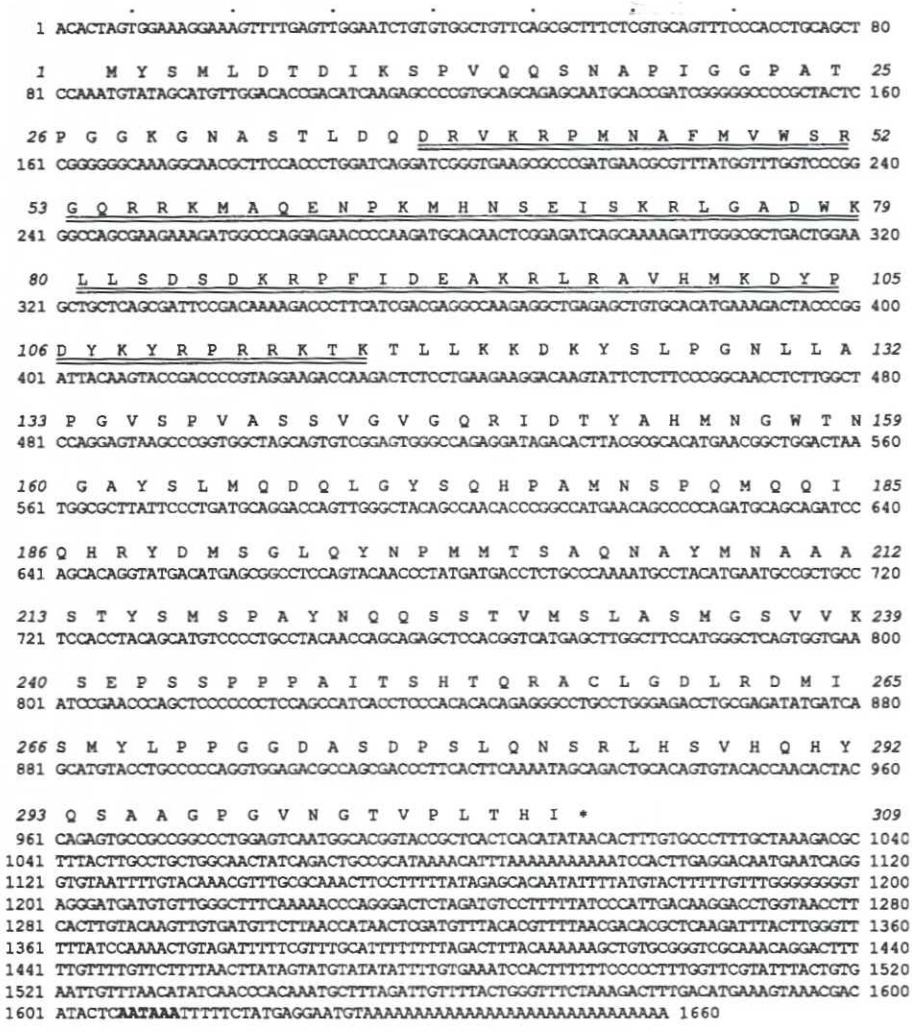

Fig. 3. Nucleotide and deduced amino acid sequence of *xSox3*. *The HMG box is double underlined. The putative polyadenylation signal is in bold and the termination codon is marked by an asterisk. The amino acid sequence is shown above the nucleotide sequence in single letter code.*

The *Sry* related HMG box-containing gene family (Sox) consists of a large number of genes with a 79-amino-acid DNA binding domain (Laudet *et al.*, 1993). The *Sox3* homologs from chicken, mice and human have been recently isolated due to their high homology within the HMG box (Stevanovic *et al.*, 1993; Uwanogho *et al.*, 1995; Collignon *et al.*, 1996). A comparison of the deduced *sox3* protein from *X. laevis* with other known SOX amino acid sequences shows a high conservation within the HMG box (not shown). The compared HMG box sequences are sharing amino acid homologies up to 96% whereas outside the DNA binding domain only the *sox3* proteins from different species share two other highly conserved regions. Forty-six amino acids downstream of the HMG box in *cSox3* and forty-seven amino acids downstream of the HMG box in *xSox3* there is a 42-44 amino acid stretch that shows high conservation between the two proteins. Twenty-five amino acids further downstream there is in *xSox3* as well as in *cSox3* a second highly conserved stretch within a Ser/Thr-rich region. Within this 38 amino acid region only four amino acids are different. However, substituted residues might not affect the properties.

Temporal and spatial transcription pattern of xSox3

The transcription activity of *xSox3* during the early development of *X. laevis* and in several tissues of adult animals was analyzed by RT-PCR. The *xSox3* transcripts are detectable in unfertilized eggs and in early blastula-stages before the zygotic transcription is turned on (Fig. 4). After mid-blastula transition there is a notable increase of the *xSox3* transcripts which continues at the same level during neurulation. From stage 25 onwards a decreasing transcription activity can be observed which leads to a low amount of *xSox3* transcripts at stage 40. In adult animals the *xSox3* RNA is present in brain and ovary whereas only very small amounts of transcripts can be detected in muscle and skin (Fig. 5). The liver lacks any *xSox3* transcription activity.

To determine the exact localization of the *xSox3* transcripts in different developmental stages whole-mount *in situ* hybridization was performed with early embryos followed after macroscopic documentation by the preparation of histological sections. Figure 6 shows the results of the whole-mount *in situ* hybridization of different developmental stages. Relatively weak signals are already found in the animal half in the uncleaved egg and early cleavage stages, which represent the maternal transcripts (Fig. 6A,C,D,E). Transcripts are found not only at the surface of the egg but also in deeper zones (Fig. 6C,D). In the late blastula and gastrula stages the signal is found in the dorsal animal and marginal zone (Fig. 6F,G,H). The *xSox3* transcripts reach down into the marginal zone (Figs. 6G,H, 7A) and in sagittal sections the signal can be frequently observed near the nuclei (Fig. 7B). However the signal is weak in the area close to the blastopore lip and is located in the inner cell layer only (Fig. 6F,G,H). In early neurula stages the signal becomes regionalized to the presumptive neural folds (Fig. 6B). Additional signals are bilaterally detectable in the zone of the prospective eye-anlagen (Figs. 7,8). Transversal sections at the level of the presumptive eye placodes reveal that the signals are localized in the inner layer of the ectoderm which in later developmental stages gives rise to the lenses (Fig. 7F,H). As it can already be seen seen in the dorsal view of the whole embryo, the neural groove lacks any *sox3* transcription activity (Fig. 7C,E). In stage 25 embryos the probe hybridizes throughout the neural structures, the otic vesicles, the branchial arches and the developing eyes (Fig. 8A,B,C). A transversal section uncovers that the signal in the eye is strictly localized in the lens epithelium and not in the fiber cells (Fig. 8H,I). The sections in the region of the pros- and mesen-

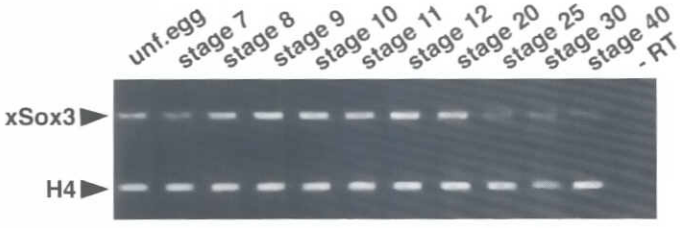

Fig. 4. Developmental profile of *xSox3* mRNA by RT-PCR and agarose gel electrophoresis. *2 µg of total RNA from different developmental stages was reverse transcribed and PCR was done with 2.5 µl of the RT reaction using pairs of primers specific for xSox3 and histon H4. The control reaction (-RT) was done with 2 µg total RNA from unfertilized eggs. The developmental stages are according to Nieuwkoop and Faber (1967), at which RNA was isolated.*

cephalon also show strong signals. The same holds true for the ear vesicles but not for the floor plate of the neural tube (Fig. 8C,F,I). Later in development another newly formed branchial arch as well as the above mentioned structures are labeled (stages 30-35; Figs. 6F, 8G). The corresponding transversal sections demonstrate in detail the distribution of *sox3* transcripts in the head area. In particular, *sox3* transcripts can be seen in the dorsolateral regions, but not in the roof of the diencephalon (Fig. 8H,I).

Expression of xSox3 ectoderm after disaggregation

Disaggregation and delayed reaggregation causes neuralization of early gastrula ectoderm (Grunz and Tacke, 1989). The neural default status is prevented by BMP-4, which can be diluted out by disaggregation (Grunz and Tacke, 1990; Wilson and Hemmati-Brivanlou, 1995).

As shown in Figure 9A we find a strong expression of *xSox3* in the ectoderm after disaggregation and delayed reaggregation. On the contrary, the signal cannot be detected in ectodermal cells, which were reaggregated immediately after disaggregation (Fig. 9A). This result shows that *xSox3* can be activated in the absence of mesoderm. These data also suggest that *xSox3* is closely associated with neural determination.

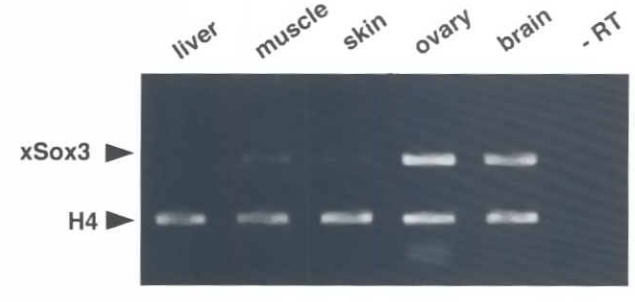

Fig. 5. Analysis of *xSox3* expression in tissues of adult animals by RT-PCR and agarose gel electrophoresis. *2 µg of total RNA from different tissues was reverse transcribed and PCR was done with 2.5 µl of the RT reaction using pairs of primers specific for xSox3 and histon H4. The control reaction (-RT) was performed with 2 µg total RNA from brain tissue without reverse transcriptase.*

Expression of xSox3 in pseudoexogastrulae

Since *xSox3* is expressed in normogenesis in the whole central nervous system, the signal should be found in all ectodermal parts of exogastrulae in the case that planar signals are migrating from the chordamesoderm to the ectoderm.

Using our pseudoexogastrulation-method (Grunz *et al.*, 1995), which in contrast to «normal» *Xenopus* exogastrulae excludes vertical neural signaling, the *xSox3* transcripts could be detected in the close vicinity of the chordamesoderm only, but not in the distal part of the ectoderm (Fig. 9B). This result suggests that long distance signaling by planar signals is unlikely.

Expression of xSox3 in Spemann organizer treated with Suramin

Suramin treatment (150 µM, 3 h) causes ventralization and the inhibition of neural structures of isolated Spemann organizer (Grunz, 1992, 1993; Oschwald *et al.*, 1993). The expression of *xSox3* is suppressed in Spemann organizer after Suramin treatment (Fig. 9C). The result is in agreement with histological analysis in an earlier paper, where no neural structures could be found after Suramin treatment of the isolated Spemann organizer (Grunz, 1992). This result indicates that the transcription of *xSox3* is an essential part of the neural signaling pathway.

Expression of xSox3 in LiCl- and UV-treated embryos

We next asked whether *xSox3* expression is correlated with the dorsoanterior axis formation.

LiCl-treatment causes a strong dorsalization of whole embryos, while UV-treatment of uncleaved embryos results in the ventralization/caudalization. By whole-mount *in situ* hybridization we observed an increased expression of *xSox3* in LiCl-treated embryos, while UV-treatment causes a dramatic decrease (Fig. 9D). Weak *xSox3* signals in UV-treatment embryos are restricted to the neural tube of the remaining tail structures. The formation of anterior (brain) structures are fully suppressed by the UV-treatment under our experimental conditions.

Discussion

In this paper we describe the isolation and characterization of a new sox-gene (*xSox3*), a member of the HMG-box containing transcription factor family, which is expressed in the presumptive central nervous system.

The primary intention of the paper was to search for genes, which are predominantly or exclusively expressed in the ectodermal, neuroectodermal, or mesodermal anlage of the late gastrula (stage $11^{3/4}$, Nieuwkoop and Faber, 1967). The aim was to detect also low abundant genes, which could not be identified in earlier approaches (Sargent and Dawid, 1983). Before PCR-techniques became available the only possibility to identify new genes was the subtraction cloning starting with relative large amounts of tissues or cells, or using whole embryos followed by time consuming skillful microdissection (Richter *et al.*, 1988; Rosa *et al.*, 1988). Another approach was the screening of cDNA-libraries with genes known from other species (Carrasco *et al.*, 1984; Nüsslein-Volhard, 1994). Astonishingly successful was recently the random screening of Niehrs and collaborators, which resulted even in the detection of a novel class of genes (Gawantka *et al.*, 1995, Onichtchouk *et al.*, 1996). However, using this technique, also a large number of intact embryos was needed as starting material. Using the traditional screening techniques recently

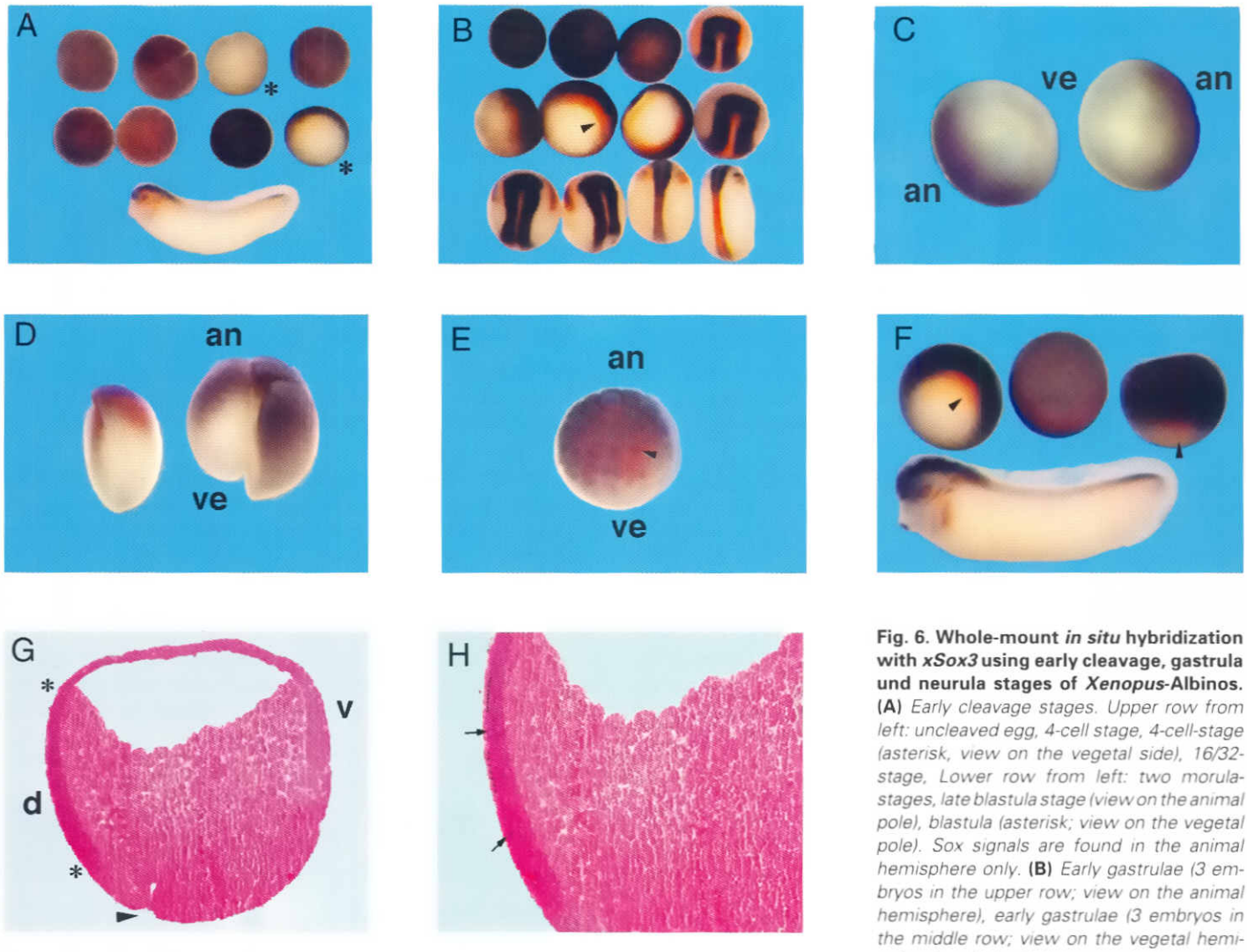

Fig. 6. Whole-mount *in situ* hybridization with *xSox3* using early cleavage, gastrula und neurula stages of *Xenopus*-Albinos. **(A)** *Early cleavage stages. Upper row from left: uncleaved egg, 4-cell stage, 4-cell-stage (asterisk, view on the vegetal side), 16/32-stage, Lower row from left: two morula-stages, late blastula stage (view on the animal pole), blastula (asterisk; view on the vegetal pole). Sox signals are found in the animal hemisphere only.* **(B)** *Early gastrulae (3 embryos in the upper row; view on the animal hemisphere), early gastrulae (3 embryos in the middle row; view on the vegetal hemisphere), middle till late neurula (lower row and the embryos on the right in the upper and middle row). Signals are found in the animal hemisphere and the neural folds.* **(C)** *The two blastomeres of a 2-cell stage were separated in the cleavage plane with fine glass-needles (next vertical cleavage starts already at the animal pole on the right). The signal can be seen like a crescent at the animal pole only.* **(D)** *One blastomere of a 4-cell stage embryo was separated in the cleavage plane by fine glass-needles. The transcripts are visible in the animal hemisphere only. The signal is not only found in the cortex of the blastomeres, but as a crescent in the deeper zones.* **(E)** *In this morula stage the signal is located close to the nuclei (Arrowhead; compare with Fig. 7B).* **(F)** *Whole-mount in situ preparation using early gastrulae (view on the vegetal side (left embryo), on the animal hemisphere (middle embryo) and side view on the dorsal blastopore lip (embro on the right). The blastopore is indicated by arrows. As control for proper staining an advanced stage (early larva) was treated under the same conditions. The signal can be seen in the brain, neural tube and lens.* **(G)** *Sagittal section of an early gastrula. The xSox signal is much stronger on the dorsal side. This especially can be seen in non-Eosin (red)-counterstained sections (not shown). The blastopore is marked by an arrowhead.* **(H)** *Same section as in G (area between the two asterisks) at higher magnification. The transcripts are mainly found in the deep layer, but not in the superficial layer. The superficial layer consists of one cell layer only (see dotted line and arrows).*

sox3 expression could be shown in *Xenopus oocytes* (Koyano *et al.*, 1997). The technique can be hardly used for the isolation of mRNA from small parts of the embryo. Therefore, we established a PCR-based method (RDA-PCR) which allows the analysis of genes starting from small amounts of tissue, in our case dissected parts of 40 gastrulae. In our first approach with DDRT-PCR (Bauer *et al.*, 1993, Liang and Pardee, 1992) we could demonstrate significant differences between the 4 different isolated tissues: neural plate, chordamesoderm, endoderm and ventral ectoderm (not shown). However, we did not get reproducible results during the following reamplification procedure. On the other hand, with the RDA-PCR-technique we could isolate in *Xenopus* a neural-specific gene, which belongs to the sox-gene-family. Meanwhile a large number of HMG-box (High mobility group)-containing proteins have been identified mainly in mammals (reviewed by Laudet *et al.*, 1993; Baxevanis *et al.*, 1995).

Some of these proteins are correlated with sex-determination. They have been named Sox for Sry-type tfMG box gene. The

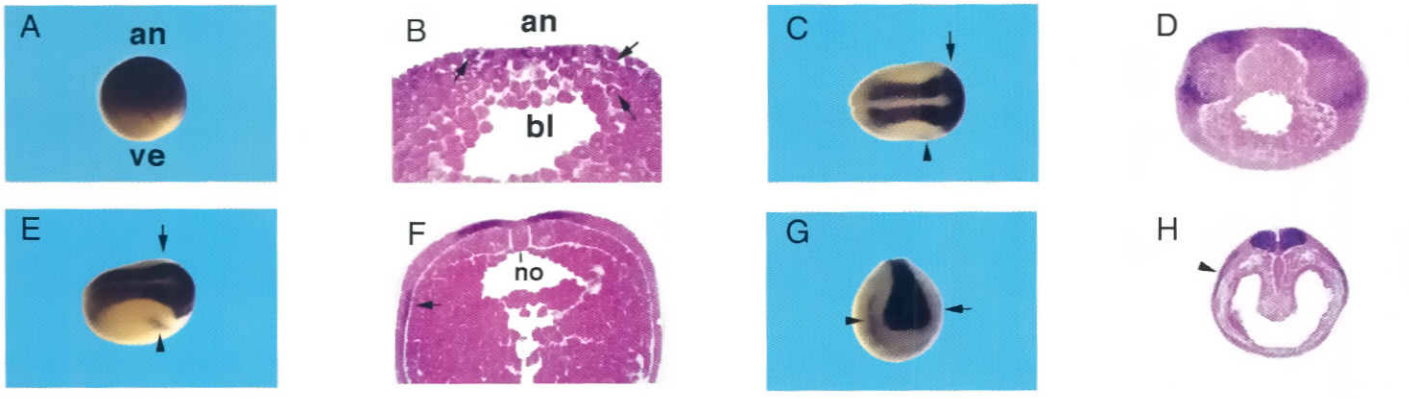

Fig. 7. Whole-mount *in situ* preparation with *xSox3* and *Xenopus*-albino-embryos. (A) *stage 9,* **(C)** *stage 15,* **(E)** *stage 17,* **(G)** *stage 19. In* **(B,D,F,H)** *the corresponding transversal sections are shown. The presumptive lens placode area is marked by arrowheads. The level of the sections is indicated my small arrows. bl blastocoel, an animal pole, ve vegetal pole. In (B) the signals can be frequently seen close to the nucleus (arrows). no notochord*

mouse genome contains about 20 Sox genes subclassed into seven groups A to G (Wright *et al.*, 1993). Genes from group B, including Sox-1, Sox-2, Sox-3, Sox-19 are thought to participate in the determination of the central nervous system (Vriz *et al.*, 1996).

All these DNA binding proteins of the HMG-type transcription factors have in common a conserved DNA binding domain, i.e. the High Mobility Group (HMG) box. It binds sequence-specific preferentially to non-B-DNA. Of great importance is the fact that LEF-1, a related HMG box-containing protein, has been shown to play an important role in tooth development (Stock *et al.*, 1996) and in dorsal mesoderm formation (Huber *et al.*, 1996).

A LEF1-like molecule is thought to form a LEF1/β-catenin complex, which enters the nucleus and binds to the promoter region of E-cadherin. This translocation is one of the last steps of the Wnt-signaling pathway leading to mesoderm dorsalization (Kühl and Wedlich, 1996; Miller and Moon, 1996, Schneider *et al.*, 1996). In further studies it should be tested if *xSox3* acts in a similar way in the regulation of neural determination, since in late blastula and early gastrula the expression is stronger on the dorsal side of the embryo.

xSox3 and its normal expression

By RT-PCR we could show that the gene is already maternally expressed. Signals can be detected during the whole early development up till stage 40. Afterwards it fades continuously. However it should be pointed out that in adult frogs signals again are found in brain and ovary.

By whole-mount *in situ* hybridization signals can be found in the future brain area. Of special interest is the fact that in late blastula and early gastrula *xSox3* message is predominantly localized at the dorsal side of the embryo.

Transcripts are found mainly in the dorsal animal cap and the marginal zone. In sections we can demonstrate that the signal is frequently located near the nuclei. The data suggest that *xSox3* is an important factor in the dorsal signaling pathway resulting in the determination of mesoderm and neural structures (Miller and Moon, 1996). It could be speculated that *xSox3* could like LEF-1

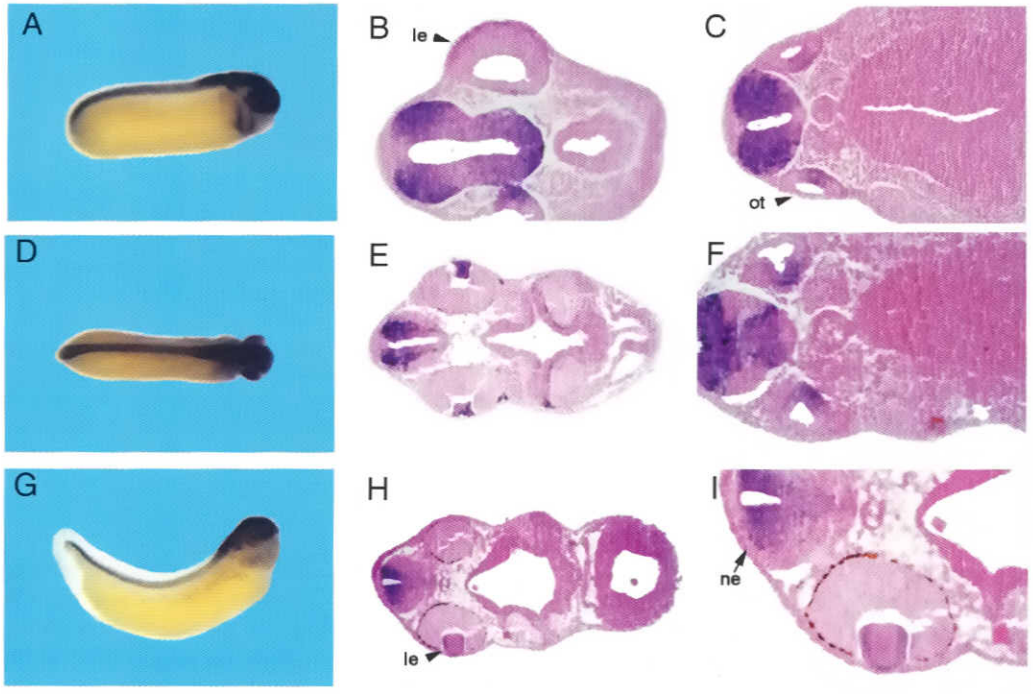

Fig. 8. Whole-mount *in situ* preparation with *xSox3* and *Xenopus*-albino-embryos. (A) *Stage 25,* **(D)** *stage 30,* **(G)** *stage 35. The corresponding transversal sections are shown in the right part of the panel.* **(B,E,H,I)** *sections in the eye region.* **(C,F)** *in the zone of the otic vesicles (see arrow in C). Note that the xSox in B is found in the deep layer of the lens placode. At higher magnification the trancripts can be identified in the lensepithelium, but not in the lens fibers (I). le lens, ne brain, ot otic placodes.*

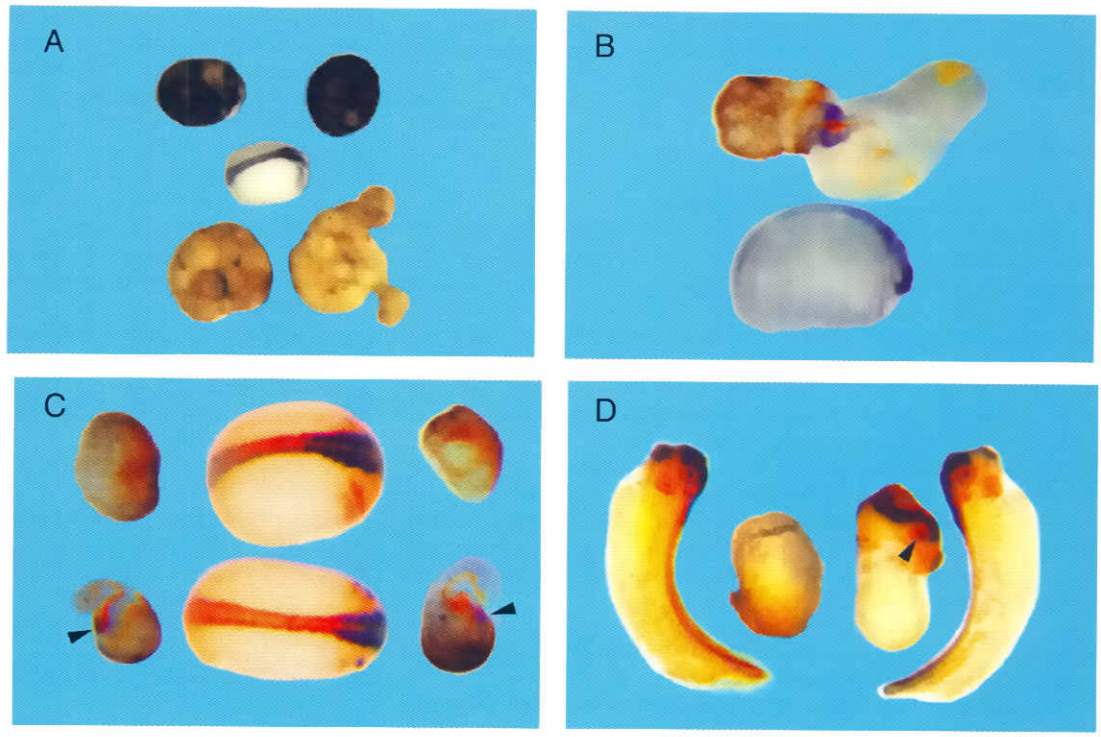

Fig. 9 Whole-mount *in situ* preparation with *xSox3* after different biological assays. (A) *Ectoderm of early gastrulae dissociated and single cells reaggregated after 4 h culture, which causes neuralization (Grunz and Tacke, 1989) show a strong xSox3 signal (upper row). Disaggregated and immediately reaggregated ectodermal cells (control series) show no expression (lower row). A late neurula (middle) is shown to indicate the large size of the reaggregated cell mass.* **(B)** *In the pseudoexogastrula xSox3 is expressed in the intermediate zone between the ectoderm (left) and the endomedoderm only, but not in the distal part of the ectoderm.* **(C)** *Dorsal blastopore lip (Spemann organizer) was treated with 150 μM Suramin for 3 h (upper row). Untreated controls and the two untreated embryos show the expression of xSox3 (arrows).* **(D)** *Treatment of uncleaved embryos with UV and 32-cell embryos with LiCl. The UV-treated embryo (second from left) without head structures shows no xSox3 expression. On the other hand the dorsalization by LiCl (second from right) causes a strong signal in the head area including the lens.*

participate in the wnt-signaling pathway at the level of the complex formation with β-catenin, which enters the nucleus for further interaction with the DNA (Huber *et al.*, 1996). Since in whole-mount *in situ* *xSox3* expression (mainly on the dorsal side) after MBT is detected slightly earlier than the neural specific β-tubulin (Richter *et al.*, 1988), characteristic for terminal neural differentiation, its expression profile fits well in the hypothetical role of a neural specific transcription factor.

Importance of zygotic spatio-temporal expression of xSox3 for neural determination

So far we do not know the role of the maternal transcripts during the early cleavage stages (Koyano *et al.*, 1997). On the other hand our UV- and LiCl experiments show that *xSox3* transcribed after MBT is only expressed, when brain formation takes place. Further experiments are needed to decide, if *xSox3* expression is a prerequisite for proper brain formation (loss of function experiments). While *xSox3* is overexpressed in the LiCl-treated embryos (defects of the posterior axis), UV-treated embryos (defects of the anterior axis) show only weak signals in the remaining posterior neural tube.

Furthermore we could show that *xSox3* is not expressed in Spemann organizer with adjacent ectoderm after treatment with Suramin (Grunz, 1992). Suramin prevents the formation of neural structures and causes a shift of dorsal to ventral mesoderm (Grunz 1992, 1993; Oschwald *et al.*, 1993). That means that *xSox3* is closely related to the determination of neural structures. Experiments with artificial exogastrulae (pseudoexogastrulae, Grunz *et al.*, 1995) indicate that *xSox3* is not activated by planar signals. *xSox3* is expressed in the intermediate zone between endomesoderm and ectoderm in the very vicinity of mesoderm only and not in the more distal part of the ectoderm. These results confirm our earlier findings that planar signals are of minor relevance for the terminal neural determination. In a further approach we could show that *xSox3* can be activated in a cell autonomous manner. Disaggregated ectodermal cells, kept as single cells for 4 h prior to reaggregation form neural structures (Grunz and Tacke, 1989; Godsave and Slack, 1991) and show a very strong *xSox3* signal. That means that the activation under these conditions does not depend on the presence of mesoderm and external neural signals like noggin, chordin, follistatin or cerberus.

In contrast to the classical view more recent data suggest that neuralization does not take place by positive signals (Grunz and Tacke 1989, 1990; Sasai *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995;). That means that neuralization can occur by inhibition of signaling by BMP-like molecules (reviewed by Hemmati-Brivanlou and Melton, 1997). This can take place by interference with the communication between cells, their positions within the animal cap (Chen and Grunz, 1997), the interaction between BMP and mesodermalizing/neuralizing molecules like activin, chordin, noggin or follistatin or by the overexpression of dominant negative BMP receptors and ligands. Taken together, the information so far available strongly suggest that inhibition of BMP signaling is sufficient for neuralization. The action of sox-transcriptions factors are expected downstream in the BMP and wnt-signaling pathway.

Further loss and gain of function experiments are needed to determine the exact role of *xSox3* in the neural determination process.

Materials and methods

Embryological manipulation and cell treatments

Xenopus laevis embryos were obtained by injection of female frogs with 1000 IU of human chorion gonadotropin (Schering AG, Berlin, Germany) and artificial fertilization. The embryos were raised in Steinberg solution (58.18 mM NaCL, 0.67 mM $MnCL_2$, 0.34 mM $Ca(NO_3)_2$, 0.8 mM $MgSO_4$: pH 7.4) up to stage $11^{3/4}$ according to Nieuwkoop and Faber (1967). The jelly coat was removed by treating the embryos for 8 min at 20°C with 3.5% cysteiniumchloride. The embryos were rinsed several times in Holtfreter solution with penicillin/streptomycin and the vitelline membrane was removed mechanically with fine watchmakers' forceps.

The presumptive neural plate, the chordamesoderm and the ventral ectoderm were dissected from 40 embryos (stage $11^{3/4}$) by using fine glass needles as described elsewhere (Richter *et al.*, 1988; Rosa *et al.*, 1988). Total RNA from these tissues was isolated by the guanidinium isothiocyanate/phenol method (Cox, 1968) with a following LiCl precipitation. The isolated RNAs were stored at -70 °C.

Preparation of Xenopus pseudoexogastrulae and treatment of Spemann organizer with suramin

Exogastrula-like structures were prepared from very early gastrulae (stage 10) by microdissection as described (Grunz *et al.*, 1995). Pseudoexogastrulae were cultured until sibling control embryos reached stage 20, followed by fixation for 2 h in PEMFA for whole-mount *in situ* hybridization.

Isolated Spemann organizer with adjacent ectoderm was treated with suramin (150 µM, 3 h), which results in ventralization of dorsal mesodermal structures and in an inhibition of neural structures (Grunz, 1992). The explants with or without suramin treatment were fixed in PEMFA, when normal sibling embryos reached stage 20.

Preparation of reaggregates from Xenopus animal caps

The experiments were performed essentially as described previously (Grunz, 1969; Minuth and Grunz 1980; Grunz and Tacke, 1989, 1990). Briefly, forty animal caps in each series were isolated from stage 9 embryos by using fine glass needles. The explants were disaggregated in Ca^{2+}/Mg^{2+}-free Barth's solution supplemented with 1/9 vol. of isethionate buffer (Newport and Kirschner, 1982). In one series the cells were kept dispersed for 3 h prior to reaggregation, in another series the dispersed animal cap cells were immediately reaggregated. Both series were fixed for whole-mount *in situ* hybridization, when normal embryos of the same batch have reached stage 22.

UV and Lithium chloride treatments

UV treatment was performed 30 min after fertilization for 4 min. Embryos were placed in several Petriperm™- Petri dishes (Fa. Hereus; Grunz and Tacke, 1986) with a UV-transparent-bottom (membrane) on a Transilluminator-Screen (Fa. Bachhofer; wavelength 302 nm), which allows an UV-treatment with exact timing (Scharf and Gerhart, 1983).

LiCl treatment was carried out in 0,3 M LiCl in Holtfreter solution for 10 min starting at the 32-cell stage (Kao and Elinson, 1988).

Representational Difference Analysis (RDA)

The Representational Difference Analysis (RDA) is a PCR-coupled subtractive hybridization technique primarily used for the identification of differentially expressed genes in two complex genomes (Lisitsyn *et al.*, 1993). The modified method for cDNAs (Hubank and Schatz, 1994) allows the identification of differential expressed genes requiring only small amounts of tissue RNA (Fig. 1). First so called cDNA-representations from neural plates, chordamesoderm and ventral ectoderm from 40 late gastrulae (st. 12) were synthesized. Therefore the RNAs from the mentioned tissues were reverse transcripted, the primary ds cDNAs were digested with a four cutting restriction enzyme (DpnII) and specific oligonucleotides were ligated to the 3' and 5'-ends of the cDNA- fragments. After a «fill in»-reaction the same specific oligonucleotides were used to perform PCR to amplify the primary ds cDNAs. The first set of PCR-linkers was removed from the three cDNA representations by DpnII digestion. Only the cDNA representation which was used as the tester (neural plate) was ligated to a new set of oligonucleotides. The two other representations (chordamesoderm and ventral ectoderm) were pooled and used as the driver without specific sequences for PCR. After the first hybridization and the fill in reaction only the tester/tester hybrids were amplifiable in an exponential manner. Two repeat of both steps (hybridization with increasing driver concentrations and PCR) leads to an enrichment of several neural-specific PCR-fragments which were separated on an agarose gel. The eluted fragments were cloned into the Bam HI site of the pBluescript KS + II vector (Stratagene; further details see below).

Oligonucleotides

Sequences of oligonucleotides used in the cDNA RDA:
R-Bgl-12: 5'-GATCTGCGGTGA-3'
R-Bgl-24: 5'-AGCACTCTCCAGCCTCTCACCGCA-3
J-Bgl-12: 5'-GATCTGTTCATG-3'
J-Bgl-24: 5'-ACCGACGTCGACTATCCATGAACA-3'
N-Bgl-12: 5'-GATCTTCCCTCG-3'
N-Bgl-24: 5'-AGGCAACTGTGCTATCCGAGGGAA-3'

cDNA synthesis and generation of representations for cDNA RDA

The PCR-coupled subtractive process of representational difference analysis (RDA) was performed according to Hubank and Schatz (1994). Double stranded cDNA from the above mentioned tissues RNA was generated by using the RiboClone® cDNA Synthesis System AMV RT with $Oligo(dT)_{15}$ Primer (Promega). 2 µg of double stranded cDNA was digested with DpnII (New England Biolabs), phenol extracted, ethanol precipitated and resuspended in TE. About 1.2 µg of cut cDNA was ligated to the R-Bgl-12/24 adapter in the following mixture: 2 mg/ml desalted R-Bgl-24 oligo, 1 mg/ml desalted R-Bgl-12 oligo. The ligation reaction was heated to 50°C in a PCR cycler to anneal the oligonucleotides to each other and to the cDNA, then cooled to 10°C over a period of 1 h, and the ligation was performed by adding 3 µl of T4 DNA ligase (400 U/µl), and incubating for 16 h at 14°C. The ligations were diluted to 6 µg/ml and several PCR reactions were performed as follows to generate the initial representations. A 200 µl reaction contained 2 µl of diluted ligation and 20 µl 10 x reaction buffer (MBI Fermentas), 4 mM $MgCl_2$, 100 µg/ml BSA, dATP, dCTP, dGTP and dTTP (all 0.3 mM) and 2 µg R-Bgl-24 primer. The 12 mer oligo was melted away (3 min 72°C) and the 3' ends were filled in with 4 U of Taq DNA polymerase (MBI Fermentas) (5 min 72°C). Finally twenty cycles of PCR amplification (1 min 95°C; 3 min 72°C) were performed and the PCR products were combined, phenol extracted, ethanol precipitated and resuspended in TE at 0.5 mg/ml. The representations were digested with DpnII, phenol extracted and ethanol precipitated to remove the R-adapters. The representation to form the driver was prepared at this point. A portion (20 µg) of the representation to form the tester was gel-purified on a 1.2% TAE agarose gel and eluted using the Qiaquick Gel Extraction Kit (Qiagen). 2 µg of the tester were ligated to the J-Bgl-12/24 adapter in the same way as described above.

Hybridization and selective amplification

The first subtractive hybridization was carried out with 0.4 µg J-ligated tester and 40 µg driver. The mixture was phenol extracted, ethanol precipitated, and resuspended in 4 µl 3xEE buffer [30 mM EPPS (Sigma), pH 8.0 at 20°C; 3 mM EDTA]. The sample was overlaid with mineral oil and the DNA denatured (6 min 98°C). The salt concentration was adjusted with 1 µl 5 M NaCl and the hybridization was performed for 20 h at 67°C. The hybridized DNA was diluted with 8 µl TE containing 5 µg/µl yeast RNA, and thoroughly resuspended in 400 µl TE. Four PCR reactions were

set up with 20 µl of the diluted hybridization mixture, but without the primer. The J-Bgl-12 oligo was melted away, ends were filled in with 5 U of Taq DNA polymerase, and 2 µg J-Bgl-24 primer added. After ten cycles of amplification (1 min 95°C; 3 min 70°C) the four reactions were combined, and phenol extracted, isopropanol precipitated and resuspended in 40 µl 0.2xTE. A mung bean nuclease (New England Biolabs) digestion was performed, to remove unannealed ssDNA. Four amplification reactions were set up with 20 µl of the digested product and 2 µl J-Bgl-24 primer (1 mg/ml). 5 U of Taq DNA polymerase were added at 80°C and 18 cycles (1 min 95°C; 3 min 70°C) performed. The products were combined and phenol extracted, isopropanol precipitated and resuspended to 0.5 µg/µl, to get the first difference product (DP1). The J-adapters of DP1 were changed to the N-Bgl-12/24 oligos as described above, and the procedure of subtractive hybridization and selective amplification were repeated to give the DP2. In the second hybridization step 50 ng tester was mixed with 40 µg driver, and amplifications, annealing and extension were performed at 72°C. The third difference product (DP3) was generated with 100 pg J-ligated tester and 40 µg driver, and 22 cycles in the final amplification (Fig. 2).

The resulting products were digested with DpnII and separated on a 1.2% TAE agarose gel. The visible fragments were isolated and cloned into the BamHI site of the pBluescript KS^+ II vector (Stratagene). Plasmid DNA was prepared by using the JET prep Plasmid Miniprep Kit (Genomed), and sequenced by an ALF® DNA Sequencer (Pharmacia). The sequence of clone N5/4 with a length of 236 bp was analyzed in the EMBL database, Heidelberg, Germany, with the FASTA program.

Screening of a cDNA libraries

A ZAP EXPRESS™ cDNA library from *X. laevis* stage 29/31 (courtesy of Thomas Hollemann, Tomas Pieler, Göttingen) was screened with primers complementary to the 5'- and 3' ends of clone N5/4 (5'-AGCGCAGGTATGACATGAGCG-3'; 5'-TATCTCGCAGGTCTCCC-AGGC-3'). The corresponding cDNA was isolated from the positive fraction by using a PCR-coupled strategy and the manufactures protocol (Stratagene) for the *in vivo* excision. The obtained 1.660bp cDNA was sequenced and analysed as described above. DNA and protein sequence comparison and alignment were carried out with the MacMolly™ Tetra program, version 1.0 (Soft Gene, Berlin). The nucleotide sequence of *xSox3* has been deposited in the EMBL Data Library with the accession number Y07542.

RT-PCR

Total RNA from tissues and different developmental stages was digested with 20 U deoxyribonuclease I (Boehringer Mannheim) for 30 min at 37°C to avoid genomic DNA contamination. RNA (2 µg) was reverse transcripted with Superscript reverse transcriptase for 1 h at 37°C using oligo$(dT)_{15}$ primer. RT-PCR was performed in a 22.5 µl reaction with 2.5 µl of the RT reaction (1x95°C, 3 min; 58°C, 1 min; 72°C, 1 min; 30x94°C, 1 min; 58°C, 45 sec; 72°C, 1 min) with the following primers: 3'(L) sox3 primer 5'-AGCGCAGGTATGACATGAGCG-3', 3'(U) primer 5'-TATCTCGCAGGTCTCCCAGGC-3'. The histone H4 primers were provided by Thomas Hollemann and Tomas Pieler, Göttingen.

Whole-mount in situ hybridization

Xenopus laevis eggs for the whole-mount *in situ* hybridization were obtained as described above and cultured in Steinberg solution with added penicillin/streptomycin until the desired stage. The vitelline membrane of embryos up to gastrula stages were penetrated by a Proteinase K (10 µg/ml) incubation. The vitelline membrane of elder stages were removed with watchmakers forceps. All embryos were fixed for 2 h in HEMFA and transferred to ethanol before storage at -20°C.

To generate DIG-labeled antisense RNA probes from the *xSox3* cDNA clone the pBK-CMV phagemid containing the insert was linearized with EcoRI. The *in vitro* transcription was performed according to the Boehringer Mannheim standard protocol. *in situ* hybridization was essentially carried out as described elsewhere (Oschwald *et al.*, 1991, 1993).

For sections stained embryos were dehydrated through a series of 25, 50, 75 and 100% ethanol in distilled water and stored at -20°C. For embedding (modified after Grunz, 1970, 1983), the ethanol was exchanged for an ethanol/xylol solution (1:1), 100% xylol (both 1 h at room temperature), Paraffin (Merck) for 6 h at 48°C, Jung-Histowax™ (Cambridge Instruments) overnight at 60°C and finally embedded in freshly prepared Jung-Histowax™. After sectioning at 12.5 µm, slides were dried overnight at 37°C and treated with Rotihistol (Roth; 3x15 min), isopropanol (5 min), 96, 85, 70 and 50% ethanol (6 min each) and finally distilled water. After staining in 0.1% eosin solution for 15 min, the slides were rinsed in distilled water, differentiated in 80% ethanol, followed by 96% ethanol (3 min), isopropanol (3 min), Rotihistol (15 min) and mounted in Canada balsam.

Photography of embryos was performed under a Zeiss STEMI SV8 dissecting microscope using a Kodak Ektachrome 200x professional film. Sections were photographed under a Leitz-Orthoplan-microscope using a Kodak Ektachrom 64T professional film. Slides were scanned with a Nikon LS-1000 35 mm-film scanner. Images were imported into Adobe Photoshop 4.0, processed and printed with a KODAK 8650PS Color Printer (hard copies) and simultaneously submitted as CMYK-TIFF files on a writable CD.

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References

BAUER, D., MÜLLER, H., REICH, J., RIEDEL, H., AHRENKIEL, V., WARTHOE, P. and STRAUSS, M. (1993). Identification od differentially expressed mRNA species by an improved display technique (DDRT-PCR). *Nucleic Acids Res. 21:* 4272-4280.

BAXEVANIS, A.D. and LANDSMAN, D. (1995). The HMG-1 box protein family: classification and functional relationships. *Nucleic Acids Res. 23:* 1604-1613.

BOUWMEESTER, T., KIM, S.H., SASAI, Y., LU, B. and DE ROBERTIS, E.M. (1996). *cerberus* is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature 382:* 595-601.

CARDELLINI, P. (1988). Reversal of dorsoventral polarity in *Xenopu laevis* embryos by 180° rotation of the animal micromeres at the eight-cell stage. *Dev.Biol.128:* 428-434.

CARRASCO, A.E., McGINNES, W., GEHRING, W.J. and DE ROBERTIS, E.M. (1984). Cloning of an *X. laevis* gene expressed during early embryogenesis coding for a peptide region homologous to *Drosophila* homeotic genes. *Cell 37:* 409-414.

CHEN, Y. and GRUNZ, H. (1997). The final determination of *Xenopus* ectoderm depends on intrinsic and external positional information. *Int. J. Dev. Biol. 41:* 525-528.

COLLIGNON, J., SOCKANATHAN, S., HACKER, A., COHEN-TANNOUDJI, M., NORRIS, D., RASTAN, S., STEVANOVIC, M., GOODFELLOW, P.N. and LOVELL-BADGE, R. (1996). A comparison of the properties of *Sox-3* with *Sry* and two related genes, *Sox-1* and *Sox-2. Development 122:* 509-520.

COX, R.A. (1968). The use of guanidinium chloride in the isolation of nucleicacids. *Methods Enzymol12b:* 120-129.

GAWANTKA, V., DELIUS, H., HIRSCHFELD, K., BLUMENSTOCK, C. and NIEHRS, C. (1995). Antagonizing the Spermann organizer: role of the homeobox gene X*vent-1* . *EMBO J.14:* 6268-6279.

GODSAVE, S.F. and SLACK, J.M.W. (1991). Single cell analysis of mesoderm formation in the *Xenopus* embryo. *Development 111:* 523-530.

GRUNZ, H. (1969). Hemmung der Reaggregation dissoziierter Amphibienzellen durch Inhibitoren der RNS- und Proteinsynthese. *Roux Arch. Dev. Biol.163:* 184-196.

GRUNZ, H. (1970). Abhängigkeit der Kompetenz des Amphibien-Ektoderms von der Proteinsynthese. *Roux Arch. Dev. Biol. 165:* 91-102.

GRUNZ, H. (1977). The differentiation of the four animal and the four vegetal blastomeres of the eight-cell-stage of *Triturus alpestris. Roux Arch. Dev. Biol. 181:* 267-277.

GRUNZ, H. (1983). Change in the differentiation pattern of *Xenopus laevis* ectoderm by variation of the incubation time and concentration of vegetalizing factor. *Roux Arch. Dev. Biol. 192:* 130-137.

GRUNZ, H. (1992). *Suramin* changes the Fate of Spemann's Organizer and prevents neural induction in *Xenopus laevis. Mech. Dev. 38:* 133-142.

GRUNZ, H. (1993). The dorsalization of Spemann's organizer takes place during gastrulation in *Xenopus laevis* embryos. *Dev. Growth Differ. 35:* 21-28.

GRUNZ, H. (1994). The four animal blastomeres of the eight-cell stage of *Xenopus laevis* are intrinsically capable of differentiating into dorsal mesodermal derivatives. *Int. J. Dev. Biol.38:* 69-76.

GRUNZ, H. (1997). Neural induction in amphibians. In *Current Topics in Developmental Biology,* (Eds. R. Petersen and G. Shatten). Academic Press, San Diego, CA pp 191-228

GRUNZ, H. and TACKE, L. (1986). The inducing capacity of the presumptive endoderm of *Xenopus laevis* studied by transfilter experiments. *Roux Arch. Dev. Biol. 195:* 467-473

GRUNZ, H. and TACKE, L. (1989). Neural differentiation of *Xenopus laevis* ectoderm takes place after disaggregation and delayed reaggregation without inducer. *Cell Differ. Dev. 28:* 211-218.

GRUNZ, H. and TACKE, L. (1990). Extracellular matrix components prevent neural differentiation of disaggregated *Xenopus* ectoderm cells. *Cell Differ. Dev. 32:* 117-124.

GRUNZ, H., SCHÜREN, C. and RICHTER, K. (1995). The role of vertical and planar signals during the early steps of neural induction. *Int. J. Dev. Biol. 39:* 539-543

HEMMATI-BRIVANLOU, A. and MELTON, D. (1997). Vertebrate embryonic cells will become nerve cells unless told otherwise. *Cell 88:* 13-17.H 345

HEMMATI-BRIVANLOU, A., KELLY, O.G. and MELTON, D.A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell 77:* 283-295.

HUBANK, M. and SCHATZ, D.G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res. 22:* 5640-5648.

HUBER, O., KORN, R., MCLAUGHLIN, J., OHSUGI, M., HERRMANN, B.G. and KEMLER, R. (1996). Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. *Mech. Dev.59:* 3-10.

KAO, K.R. and ELINSON, R.P. (1988). The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. *Dev. Biol. 127:* 64-77.

KAUFMANN, E., PAUL, H., FRIEDLE, H., METZ, A., SCHEUCHER, M., CLEMENT, J.H. and KNOCHEL, W. (1996). Antagonistic actions of activin A and BMP-2/4 control dorsal lip-specific activation of the early response gene XFD-1' in *Xenopus laevis* embryos. *EMBO J.15:* 6739-6749.

KOYANO,S., ITO, M., TAKAMATSU, N., TAKIGUCHI,S. and SHIBA, T. (1997). The *Xenopus* Sox3 gene expressed in oocytes of early stages. *Gene 188:* 101-107.

KÜHL, M. and WEDLICH, D. (1996). *Xenopus* cadherins: Sorting out types and functions in embryogenesis. *Dev. Dynamics 207:* 121-134.

LAMB, T.M., KNECHT, A.K., SMITH, W.C., STACHEL, S.E., ECONOMIDES, A.N., STAHL, N., YANCOPOLOUS, G.D. and HARLAND, R.M. (1993). Neural Induction by the Secreted Polypeptide Noggin. *Science 262:* 713-718.

LARABELL, C.A., TORRES, M., ROWNING, B.A., YOST, C., MILLER, J.R., WU, M., KIMELMAN, D. and MOON, R.T. (1997). Establishment of the dorso-ventral axis in *Xenopus* embryos is presaged by early asymmetries in beta-catenin that are modulated by the Wnt signaling pathway. *J. Cell Biol.136:* 1123-1136.

LAUDET, V., STEHELIN, D. and CLEVERS (1993). Ancestry and diversity of the HMG box superfamily. *Nucleic Acids Res. 21:* 2493-2501.

LI, S.H., MAO, Z.R., YAN, S.Y., and GRUNZ, H. (1996). Isolated dorsal animal blastomeres of *Xenopus laevis* are capable to form mesodermal derivatives, while the ventral animal blastomeres differentiate into ciliated epidermis only. *Zool. Sci.13:* 125-131.

LIANG, P. and PARDEE, A.B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science 257:* 967-971.

LISITSYN, N., LISITSYN, N. and WIGLER, M. (1993). Cloning the difference between two coplex genomes. *Science 259:* 946-951.

MILLER, J.R. and MOON, R.T. (1996). Signal transduction through beta-catenin and specification of cell fate during embryogenesis. *Genes Dev.10:* 2527-2539.

MINUTH, M. and GRUNZ, H. (1980). The formation of mesodermal derivates after induction with vegetalizing factor depends on secondary cell interactions. *Cell Differ. 3:* 229-238.

NEWPORT, J. and KIRSCHNER, M. (1982). A major developmental transition in early *Xenopus* embryos. I. Characterization and timing of cellular changes at the mid-blastula transition. *Cell 30:* 675-686

NIEUWKOOP, P.D. and FABER, J. (1967). *Normal Table of Xenopus laevis* (Daudin), 2nd ed. North Holland Publ. Co., Amsterdam.

NÜSSLEIN-VOLHARD, C. (1994). Of flies and fishes. *Science 266:* 572-574.

ONICHTCHOUK, D., GAWANTKA, V., DOSCH, R., DELIUS, H., HIRSCHFELD, K., BLUMENSTOCK, C. and NIEHRS, C. (1996). The *Xvent-2* homeobox gene is part of the *BMP-4* signalling pathway controlling dorsoventral patterning of *Xenopus* mesoderm. *Development 122:* 3045-3053.

OSCHWALD, R., CLEMENT, J.H., KNÖCHEL, W. K. and GRUNZ, H. (1993). Suramin prevents transcription of dorsal marker genes in whole *Xenopus laevis* embryos, isolated dorsal blastopore lips and activin A induced animal caps. *Mech. Dev. 43:* 121-133.

OSCHWALD, R., RICHTER, K. and GRUNZ, H. (1991). Localization of nervous system specific class II β-tubulin gene in *Xenopus laevis* by whole-mount *in situ* hybridization. *Int. J. Dev. Biol. 35:* 399-405.

PICCOLO, S., SASAI, Y., LU, B., and DE ROBERTIS, E. (1996). Dorsoventral patterning in *Xenopus:* Inhibition of ventral signals by direct binding of *chordin* to *BMP-4. Cell 86:* 1-20.

RICHTER K, GRUNZ H, and DAWID I B (1988). Gene expression in the embryonic nervous system of *Xenopus laevis. Proc. Natl. Acad. Sci. USA 85:* 8086-8090.

ROSA, F., SARGENT, T. D., REBBERT, M. L., MICHAELS, G. S., JAMRICH, M.M., GRUNZ H., JONAS, E., WINKLES, J. A., and DAWID, I. B. (1988). Accumulation and decay of DG42 gene products follow a gradient pattern during *Xenopus* embryogenesis. *Dev. Biol.129:* 114-123.

SARGENT, T.D. and DAWID, I.B. (1983). Differential gene expression in the gastrula of *Xenopus laevis. Science 222:* 135-139.

SASAI, Y., LU, B., STEINBEISSER, H. and DE ROBERTIS, E.M. (1995). Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus. Nature 376:* 333-336.

SASAI, Y., LU, B., STEINBEISSER, H., GEISSERT, D., GONT, L.K. and DE ROBERTIS, E.M. (1994). *Xenopus chordin*: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell 79:* 779-790.

SCHARF, S.R. and GERHART, J.C. (1983). Axis determination in eggs of *Xenopus laevis*: a critical period before first cleavage, identified by the common effects of cold, pressure and ultraviolet irradiation. *Dev. Biol. 99:* 75-87.

SCHNEIDER, S., STEINBEISSER, H., WARGA, R.M. and HAUSEN, P. (1996). beta-Catenin translocation into nuclei demarcates the dorsalizing centers in frog and fish embryos. *Mech. Dev. 57:* 191-198.

SPEMANN, H. and MANGOLD, H. (1924). Über Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. *Roux Arch. Dev. Biol. 100:* 599-638.

STEVANOVIC, M., LOVELL-BADGE, R., COLLIGNON, J. and GOODFELLOW, P.N. (1993). SOX3 is an X-linked gene related to SRY. *Hum. Mol. Genet 2:* 2007-2012.

STOCK, D.W., BUCHANAN, A.V., ZHAO, Z.Y. and WEISS, K.M. (1996). Numerous members of the sox family of HMG box-containing genes are expressed in developing mouse teeth. *Genomics 37:* 234-237.S 745.

UWANOGHO, D., REX, M., CARTWRIGHT, E.J., PEARL, G., HEALY, C., SCOTTING, P.J. and SHARPE, P.T. (1995). Embryonic expression of the chicken *Sox2, Sox3* and *Sox11* genes suggests an interactive role in neuronal development. *Mech. Dev. 49:* 23-36.

VRIZ, S., JOLY, C., BOULEKBACKE, H. and CONDAMINE, H. (1996). Zygoytic expression of the zebrafish Sox-19, an HMG box-containing gene, suggests an involvement in central nervous system development. *Mol. Brain Res.40:* 221-228.

WILSON, P.A. and HEMMATI-BRIVANLOU, A. (1995). Induction of epidermis and inhibition of neural fate by *BMP-4* . *Nature 376*: 331-333.

WRIGHT, E.M., SNOPEK, B. and KOOPMAN, P. (1993). Seven new members of the Sox gene family expressed during mouse development. *Nucleic Acids Res. 21:* 744.

ZIMMERMANN, L.B., DE JESUS-ESCOBAR, J.M. and HARLAND, R.M. (1996). The Spemann Organizer signal noggin binds and inactives bone morphogenetic protein 4. *Cell 86:* 599-606.

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