Int. J. Dev. Biol. 39: 539-543 (1995)

Short Contribution

The role of vertical and planar signals during the early steps of neural induction

HORST GRUNZ[1]*, CHRISTOPH SCHÜREN[1] and KLAUS RICHTER[2]

[1]*Department of Zoophysiology, University GH Essen, Essen, Germany and*
[2]*University Salzburg, Department of Genetics and Developmental Biology, Salzburg, Austria*

ABSTRACT The classical Einsteck-test (Spemann and Mangold, *Roux Arch. Dev. Biol. 100:* 599-638, 1924) and data from total exogastrulae (Holtfreter, 1933) suggest that vertical signals are transmitted between the chordamesoderm (organizer) and reacting ectoderm in the early phase of neural induction. In contrast to these results with Axolotl (urodeles), several authors observed the expression of neural specific genes in *Xenopus* exogastrulae, isolated dorsal blastopore lip with adjacent ectoderm (open-face explants) and Keller-sandwiches. Our data with *Xenopus* (anurans) also show that the expression of neural specific genes takes place in exogastrulae. However, when we prepared open face explants and exogastrula-like structures by microdissection at very early gastrula stage, the signal of a class II ß-tubulin, characteristic of terminal neural differentiation, is not found in the ectoderm. These results suggest that planar signals transmitted from the chordamesoderm into the ectodermal part can fairly be excluded under these experimental conditions. In similar experiments with *Triturus alpestris* we could not observe either the differentiation of neural structures in the ectodermal part of exogastrulae. These results confirm earlier experiments of Holtfreter performed with *Ambystoma mexicanum* (Axoltl) embryos. On the basis of the published data of different authors and our results, we cannot exclude the existence of planar signals for early and/or transient expressed genes before the onset of gastrulation in *Xenopus,* which make the neuroectoderm susceptible for the response to vertical signals during gastrulation. On the other hand our experiments with *Triturus alpestris* suggest that planar neural signals are unlikely in this species. These differences between *Triturus* and *Xenopus* embryos are discussed in the context of the peculiarities in morphological structure, competence and speed of development of the two species.

KEY WORDS: *neural induction, species differences, exogastrulation, Xenopus laevis (anura), Triturus alpestris (urodela)*

Using a neural specific ß-tubulin as molecular marker (Richter *et al.*, 1988; Good *et al.*, 1989) we could detect signals in the ectodermal but also in the endomesodermal part of *Xenopus* exogastrulae (not shown). These data are in agreement with those of other groups also using *Xenopus*, but they are contradictory with the findings of Holtfreter, who never found neural structures in the ectoderm or endomesoderm of total exogastrulae of Axolotl embryos (Holtfreter, 1933).

Holtfreter's results suggest that neural induction takes place by vertical signals which are transmitted from the involuting chordamesoderm to the overlaying dorsal ectoderm in normogenesis. The data of Holtfreter thus demonstrated the complete absence of planar (horizontal, tangential) signals. In total exogastrulae of urodelan embryos (Axolotl) a complete segregation of endo-mesoderm from ectoderm takes place. Apparently this is not the case in *Xenopus* embryos. In contrast to urodeles (*Triturus* and Axolotl), *Xenopus* ectoderm and the area of the dorsal blastopore lip consists of two distinct cell layers (Nieuwkoop and Florschütz 1950; Asashima and Grunz, 1983; Grunz, 1985). In contrast to urodeles in *Xenopus* and another pipid frog *(Hymenochirus boettgeri)* the dorsal mesoderm is located under a superficial epithelial layer with ectodermal and endodermal character (Bolker, 1994). This epithelial layer may be the reason for the incomplete segregation of the mesoderm from the ectoderm in *Xenopus* exogastrulae and may be one of the factors leading to neuralization by the mutual interactions between mesoderm and ectoderm by planar and vertical signals. To prevent even primary steps of involution in exogastrulae, the ectoderm of very early gastrulae (stage 10) was separated from the endomesoderm by microdissection (see Experimental

Abbrevations used in this paper: MMR, Marc's Modified Ringer's Solution; HEMFA, Hepes-formaldehyde buffer.

***Address for reprints:** Universität GH Essen, FB 9 Abteilung Zoophysiologie, Universitätsstr.5, 45117 Essen, Germany. FAX: 201.183 3173.

0214-6282/95/$03.00

Printed in Spain

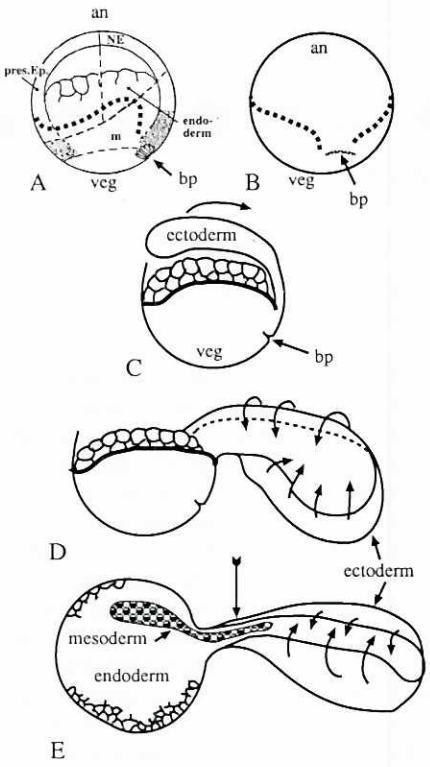

Fig. 1. Separation of ectoderm from the endomesoderm by microdissection. (A,B) *The dotted line indicates the animal area which is partially separated from the rest of the embryo.* **(C)** *Tucking up the ectodermal part.* **(D)** *Early phase of curling up of the ectoderm.* **(E)** *Pseudoexogastrula. In the intermediate zone vertical signals can be transmitted between the mesoderm and the ectoderm (arrow).*

Procedures, Fig. 1). By the neural-specific marker and standard histology we showed that neural structures have differentiated but only in the close vicinity of mesodermal structures like notochord (histology not shown) and not in the ectodermal part (Fig. 3A, arrows).

The strongest arguments that in *Xenopus* neural induction occurs by planar signalling from the mesoderm into the adjacent ectoderm are based on observations with so-called Keller sandwiches (Dixon and Kintner, 1989; Keller, 1991; Doniach *et al.*, 1992; Ruiz i Altaba, 1992). The authors found expression of neural markers in the ectodermal part of the Keller-sandwiches, which consisted of the dorsal blastopore lip with adjacent ectoderm isolated from early gastrulae (stage 10.5). Nieuwkoop (1993) pointed out that the authors (Doniach *et al.*, 1992) had performed their experiments at too late a stage, when the rolling-in of the prechordal mesoderm had already started, and that they prepared Keller-sandwiches with attached prechordal mesoderm instead of their so-called pharynx-endoderm. In these Keller-sandwiches the already involuted prechordal mesoderm may further migrate over the inner surface of the ectoderm causing neuralization by vertical signals. To exclude this possibility we isolated dorsal blastopore lip with adjacent neuroectoderm (open face explants) from very early gastrulae (stage 10, Fig. 2). By histological analysis and the use of the neural specific marker (Fig. 3C) we found neural structures in the very vicinity of notochord only (Fig. 3D). The results were quite similar in open-face explants without the superficial layer. In this way we could exclude the possibility that planar signals travel in the extracellular matrix or the intercellular space between the superficial and the deep layers of the blastopore area to the neuroectoderm (Figs. 3B, 2E). Therefore we think that under our experimental conditions signals are transmitted between mesodermal and ectodermal cells with close spatial apposition only, which results in a restricted induction of small quantities of neural tissue. It should be mentioned that other authors were also unable to detect the inducing signals that pass along the plane of ectoderm (Sharpe and Gurdon, 1990; Saint-Jeannet and Dawid, 1994). Since in contrast to *Xenopus* urodeles do not have two distinct cell layers – which may be the reason for the different outcome of the tests – we performed a large series (50 cases) of experiments with *Triturus alpestris*. Similar to Holtfreter, we did not receive exogastrulae with this species even in strong hypertonic medium. Apparently the invagination tendency of the dorsal, lateral and ventral mesoderm is higher than in Axolotl. Therefore we separated the dorsal and ventral ectoderm from the endomesoderm by microdissection (Fig. 1). A connection remained between the dorsal mesoderm (area of the blastopore lip) and the dorsal ectoderm. By this method excellent exogastrula-like structures

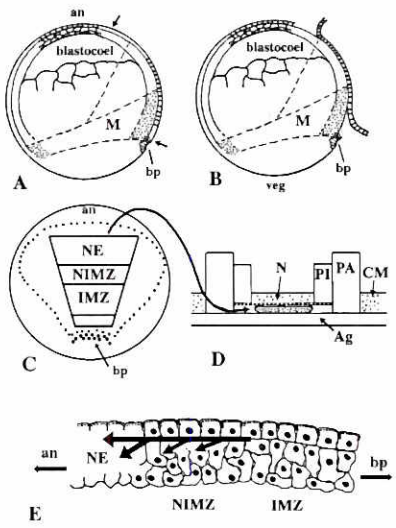

Fig. 2. Isolation of open-face explants. (A) *Sagittal section of an early gastrula (stage 10). The removed area is indicated by arrows (dotted line in C).* **(B)** *Separation of the superficial layer by fine glass- needles (series of open-face explants without the superficial layer).* **(C)** *Isolation of the dorsal mesoderm with adjacent neuroectoderm.* **(D)** *Nuclepore®-Filter-chamber, which prevents curling up of the explant.* **(E)** *Sagittal section of the area between the two arrows at higher magnification shown in panel A. The large arrows indicate the migration of hypothetical planar signals. M, marginal zone; bp, blastopore; NE, neuro-ectoderm; NIMZ, non involuting marginal zone; IMZ, involuting marginal zone; N, Nuclepore® filter; PI, inner plastic ring; PA, outer plastic ring; CM, culture medium; Ag, agar-coated Petri dish; an, animal pole; veg, vegetal pole.*

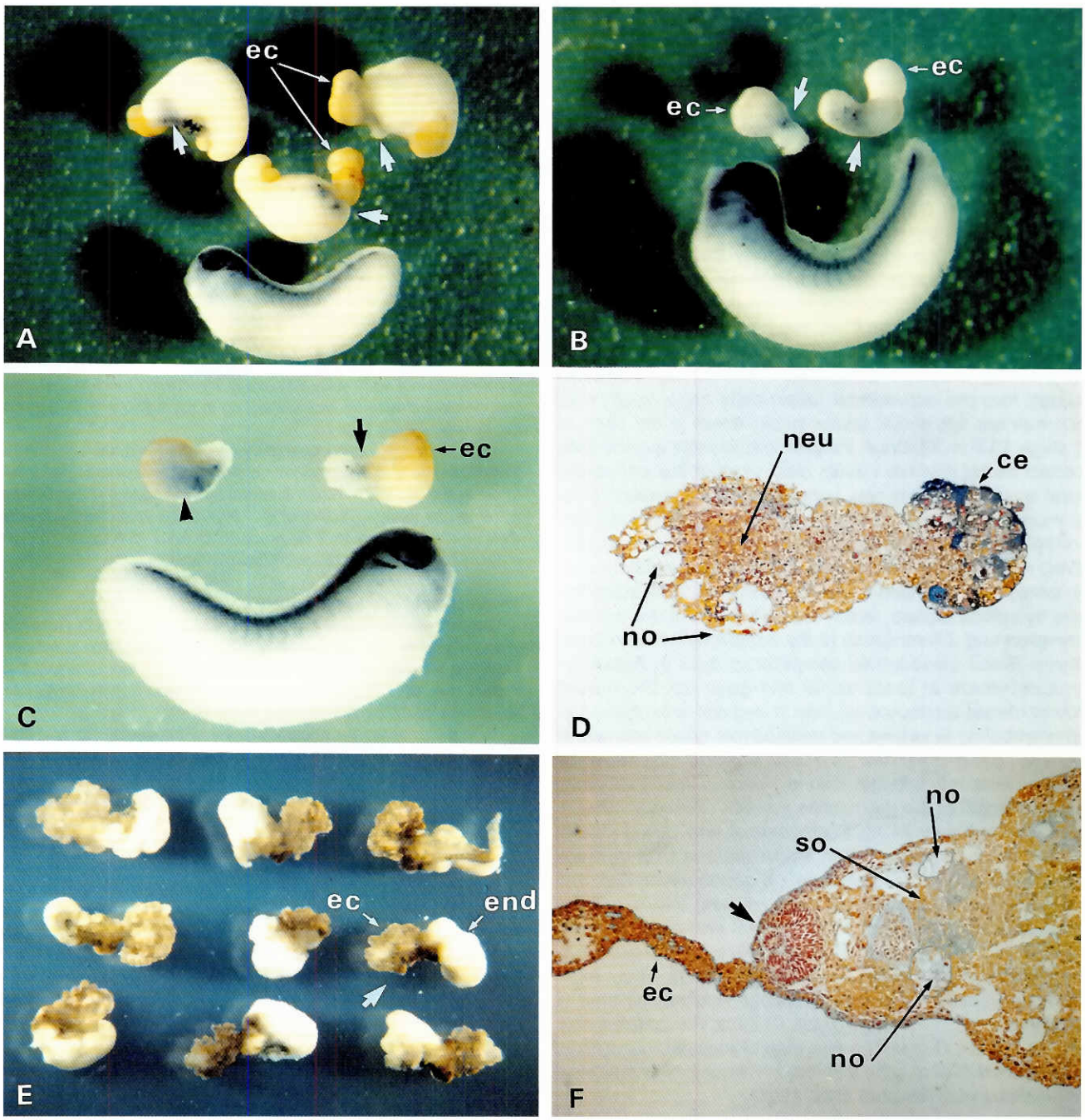

Fig. 3. Neural signals and differentiation in exogastrulae and open-face explants. **(A)** *Pseudoexogastrulae (Xenopus) prepared by microdissection described in Fig. 1. After 24 h the exogastrulae were fixed for whole-mount in situ hybridization with a neural specific ß-tubulin. The signal (neural specific tubulin) is seen only in the intermediate zone between the ectoderm and the endomesoderm (arrows).* **(B)** *Open-face explant without the superficial layer prepared by the technique described in Fig. 2. The signal of the neural-specific ß-tubulin is seen in the intermediate zone between chordamesoderm and ectoderm (ec) only (arrows).* **(C)** *Open-face explant with superficial and inner layers prepared by the technique described in Fig. 2. The signal of the neural-specific ß-tubulin is expressed in the intermediate zone between chordamesoderm and ectoderm (ec) only (arrow). The other explant on the left side also shows faint staining in the middle part of the ectoderm (arrowhead).* **(D)** *Histological section of an open-face explant contains a few neural cells (neu) in the vicinity of notochord (no) only. ce, cement gland in the ectodermal part of the explant. The blue color in this micrograph represents anilin-blue (normal histology), which has stained the mucous material of the cement gland.* **(E)** *A selection of pseudoexogastrulae (Triturus alpestris) prepared by microdissection described under Fig. 1. After 10 days culture at 20°C the ectoderm has separated from the endomesoderm (end) and has differentiated into atypical (ciliated) epidermis (ec).* **(F)** *Histological section of the explant of panel E (arrow) shows that archencephalic brain structures (eye with lens, arrow) have differentiated in the intermediate zone between atypical epidermis(ec) and the endomesodermal area only. Most of the ectodermal part (only one third is shown) has differentiated into atypical epidermis (ciliated) epidermis only. notochord (no), somites(so).*

were received (Fig. 3E). Since the neural structures are found in the intermediate zone between the endomesoderm and the ectoderm only (Fig. 3F), but not in the middle or the distal most part of the ectoderm, the transfer of planar signals over long distance is highly unlikely.

Of special importance is the fact that planar neural signals are postulated to take place in *Xenopus*, but not in Axolotl, *Rana* or *Triturus*. The question is why in contrast to Axolotl or *Triturus* in *Xenopus* neuralizing signals should be tangentially transmitted from the dorsal blastopore lip into the neighboring neuroectoderm. It would be more likely that mesodermal homoiogenetic signals are transmitted between dorsal mesoderm and neighboring neuroectoderm. Assuming that planar neuralizing signals are transmitted, one has to postulate a sharp border between dorsal mesodermal cells (Spemann's organizer) and neighboring neuroectodermal cells. In this concept it must be postulated that the ectodermal target cells have nearly lost mesodermal but not neural competence, which is the case at about stage 10.5 in *Xenopus*. Furthermore in most experiments with certain neural markers it is not clear whether the expression of neural specific genes is only transient. If the expression lasts only a short period further vertical signals must follow to establish a terminal differentiation into histotypic neural structures. However, in *Xenopus* planar signals and transient expression of certain genes may be important for "conditioning" of the ectoderm followed by vertical signals, which are necessary for the terminal determination and differentiation of the neuroectoderm into brain structures. Since mesodermal competence lasts in Axolotl or *Triturus* ectoderm at least up till mid-gastrula, the dorsal mesoderm (dorsal blastopore lip) may *in vivo* and in exogastrulae induce neighboring ectoderm into mesodermal rather into neural pathway of differentiation. In *Rana pipiens* mesodermal competence lasts much longer than in *Xenopus* in correlation to the different length of the gastrulation process (*Xenopus* about 4 h, *Rana* approximately 24 h). Saint-Jeannet and Dawid (1994) concluded from their data with *Rana pipiens* that vertical induction predominates in initiating neural development. Both the absence of the external epithelial sheet covering the mesoderm and the longer presence of mesodermal competence may prevent the shift of large parts of ectoderm into neural pathways of differentiation in Axolotl and *Triturus* exogastrulae. There exist significant differences in the basic morphogenetic mechanisms of gastrulation between urodeles (Axolotl, *Triturus*, *Pleurodeles*) and anurans *(Xenopus, Rana)*. The fate map of mesodermal cells in the early gastrula is also quite different in *Xenopus, Rana pipiens* and *Pleurodeles waltl* (Delarue *et al.*, 1994).

Experimental procedures

Xenopus embryos

Xenopus laevis eggs were obtained by injecting female frogs with 1000 IU human chorion gonadotropin (Schering AG, Berlin, Germany) prior to *in vitro* 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 till stage 10 according to Nieuwkoop and Faber (1956). The jelly coat was removed by treatment with 3.5% cysteiniumchloride (pH 7.4) for 5 to 7 min depending on the temperature of the solution. The embryos were rinsed several times in Holtfreter solution to which penicillin/streptomycin had been added. The vitelline membrane was removed mechanically with fine watchmakers' forceps.

Triturus alpestris embryos

Triturus alpestris embryos were received by natural spawning. The embryos were kept in tap water until they reached the desired stage. The jelly coat was opened by an iridectomy knife. The vitelline membrane was removed by fine watchmakers' forceps. Since *Triturus* develops much more slowly than *Xenopus*, embryos and explants were raised for up to 10 days in Holtfreter solution at 20-21°C.

Preparation of Xenopus and Triturus exogastrulae by microdissection

At the very beginning of gastrulation (stage 10, first indication of the blastopore by pigment concentration only) the whole ectoderm was separated from the endomesoderm. A bridge remained between the dorsal mesoderm and the ectoderm. The embryos were transferred to hypertonic culture medium (0.95x MMR, Newport and Kirschner, 1982) for several hours to prevent the reunification of the ectodermal with the endomesodermal part. Since in contrast to Axolotl *Triturus alpestris* embryos do not form exogastrulae in hypertonic medium, we prepared pseudoexogastrulae by microdissection in the same way as described for the *Xenopus* series (Fig. 1).

Several of those pseudoexogastrulae were fixed after 24 h in HEMFA for whole-mount *in situ* preparation described elsewhere (Harland, 1991; Oschwald *et al.*, 1991, 1993). A neural specific class II ß-tubulin was used as molecular marker (Richter *et al.*, 1988; Good *et al.*, 1989). Another part was cultured for up to 3 days at 20°C prior to fixation followed by histological standard techniques (Grunz, 1983).

Preparation of open-face explants

Dorsal mesoderm and adjacent neuroectoderm of very early gastrulae were isolated by fine glass needles. The mesodermal part consisted of the involuting mesodermal (marginal) zone (IMZ) and the non-involuting zone (NIMZ) (Fig. 2). For details concerning the gastrulation movements and the exact explanation of terms see Keller (1991).

In a further series the superficial layer of the animal cap and the dorsal blastopore area was removed prior to dissection of the deep layers (Fig. 2 B).

Explants of both series were transferred to agar-coated plastic dishes filled with Barth solution and were covered with a Nuclepore®-Filter-chamber to prevent curling up. The advantage of this device is the moderate pressure on the flattened cell sheet because the distance between filter and agar is fixed. Thus a shortage of oxygen and damage of the cells, which could cause autoneuralization, can be excluded. After 8 h (control embryos have reached stage 14, middle neurula) the explants were transferred to Holtfreter solution. The explants were cultured for further 8 h. Several cases were fixed in HEMFA for whole-mount *in situ* preparation. The remaining explants were cultured for further 48 h at 20°C prior fixation and processing for standard histology described above.

Acknowledgements

H.G. was financially supported by the Deutsche Forschungsgemeinschaft (Gr 439/5-3) and in part by the Forschungspool of the Universität GH Essen. K.R. was supported by a grant of the Austrian Fonds zur Förderung der wissenschaftlichen Forschung (8240). H.G. discussed with Prof. Pieter Nieuwkoop the difficulties to get total exogastrulae with Triturus in hypertonic medium during the International Xenopus meeting in the Netherlands in spring 1994. Many thanks for his suggestions to prepare exogastrulae by microdissection. We thank Sabine Effenberger for skilful technical assistance in the histological preparations.

References

ASASHIMA, M. and GRUNZ, H. (1983). Effects of inducers on inner and outer gastrula ectoderm layers of *Xenopus laevis*. *Differentiation 23:* 157-159.

BOLKER, J.A. (1994). Comparison of gastrulation in frogs and fish. *Am. Zool. 34:* 313-322.

DELARUE, M., JOHNSON, K.E. and BOUCAUT, J-C. (1994). Superficial cells in the early gastrula of *Rana pipiens* contribute to mesodermal derivatives. *Dev. Biol. 165:* 702-715.

DIXON, J.E. and KINTNER, C.R. (1989). Cellular contacts required for neural induction in *Xenopus* embryos: evidence for two signals. *Development 106:* 749-757.

DONIACH, T., PHILLIPS, C.R. and GERHART, J.C. (1992). Planar induction of anteroposterior pattern in developing central nervous system of *Xenopus laevis*. *Science 257:* 542-545.

GOOD, P.J., RICHTER, K. and DAWID, I.B. (1989). The sequence of a nervous system-specific, class II ß-tubulin gene from *Xenopus laevis*. *Nucleic Acids Res. 17:* 8000.

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. (1985). Effect of concanavalin A and vegetalizing factor on the outer and inner ectoderm layers of early gastrulae of *Xenopus laevis* after treatment with cytochalasin B. *Cell Differ. 16:* 83-92

HARLAND, R.M. (1991). *In situ* hybridization: an improved whole-mount method for *Xenopus* embryos. In *Methods in Cell Biology*, Vol. 36 (Ed. B.K. Kay and H.B. Peng). Academic Press Inc., San Diego, pp. 685-695.

HOLTFRETER, J. (1933). Die totale Exogastrulation, eine Selbstablösung des Ektoderms von Entomesoderm. Entwicklung und funktionelles Verhalten nervenloser Organe. *W. Roux Arch. Entw.Mech. Org. 129:* 669-793.

KELLER, R. (1991). Early embryonic development of *Xenopus laevis*. In *Methods in Cell Biology*, Vol. 36 (Ed. B.K. Kay and H.B. Peng). Academic Press Inc., San Diego, pp. 61-113.

NEWPORT, J. and KIRSCHNER, M. (1982). A major developmental transition in early *Xenopus* embryos. II. Control of onset of transcription. *Cell 30:* 687-696.

NIEUWKOOP, P.D. (1993). Vertical and planar induction in early amphibian development during meso-endoderm formation and neural plate development. *Acta Biol. Exp. Sinica 26:* 307-315.

NIEUWKOOP, P. and FLORSCHÜTZ, P. (1950). Quelques characteres speciaux de la gastrulation et de la neurulation de l'oeuf de *Xenopus laevis*, Daud. et de quelques autres Anoures. *Arch. Biol. 61:* 113-150.

NIEUWKOOP, P.D. and FABER, J. (1956). *Normal Table of Xenopus laevis (Daudin).* North Holland, Amsterdam.

OSCHWALD, R., CLEMENT, J.H., KNÖCHEL, W. 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 a nervous system-specific class II ß-tubulin gene in *Xenopus laevis* embryos by whole-mount *in situ* hybridization. *Int. J. Dev. Biol. 35:* 399-405

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

RUIZ i ALTABA, A. (1992). Planar and vertical signals in the induction and patterning of the *Xenopus* nervous system. *Development 115:* 67-80.

SAINT-JEANNET, J-P. and DAWID, I.B. (1994). Vertical versus planar neural induction in *Rana pipiens* embryos. *Proc. Natl. Acad. Sci. USA 91:* 3049-3053.

SHARPE, C.R. and GURDON, J.B. (1990). The induction of anterior and posterior neural genes in *Xenopus laevis*. *Development 109:* 765-774.

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

Accepted for publication: April 1995