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Original Article

Localization of a nervous system-specific class II ß-tubulin gene in *Xenopus laevis* embryos by whole-mount *in situ* hybridization

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ABSTRACT A neural-specific ß-tubulin mRNA is expressed in the developing central nervous system shown by whole-mount *in situ* hybridization experiments. Of special interest is the fact that from the late blastula (stage 9; Nieuwkoop and Faber, 1967; Hausen and Ribesell, 1991) until the early neurula (stage 13) the signal can be found not only in the presumptive neural plate but also in the presumptive epidermis. Later in development (from stage 13) the specific mRNA becomes restricted to the presumptive brain and spinal cord area. The results are discussed in the context of predisposition and (pre)determination.

KEY WORDS: *neural induction, neural-specific ß-tubulin gene, predisposition, whole-mount in situ hybridization*

Introduction

The classic experiment of Spemann and Mangold (1924) showed that the dorsal blastopore lip (presumptive chordamesoderm) is responsible for the induction of the central nervous system (reviewed by Grunz, 1985a, 1987, 1990a; Gurdon, 1987; Dawid and Sargent, 1988; Knöchel and Tiedemann, 1989; Tiedemann, 1990). Of great interest is the information transfer of the inducing signal(s) from the blastopore lip to the ectodermal cells causing their neuralization (Grunz and Staubach, 1979a; Saxén, 1989; Grunz, 1990a; Tiedemann, 1990; Yamada, 1990). The ectodermal target cells play an important role in this process (Grunz, 1984, 1985b; Tacke and Grunz, 1986, 1988; Grunz and Tacke, 1989, 1990; Duprat *et al.*, 1990; Pituello *et al.*, 1990). One of these early events during the induction process in the ectodermal target cells is a change in gene expression, including the suppression of some genes (Jamrich *et al.*, 1987) and the activation of many others (Kintner and Melton, 1987; Sharpe *et al.* 1987; Richter *et al.*, 1988; Rosa *et al.*, 1988; Sharpe 1988). Of special interest are the genes (XlHbox6, Xhox3) that are expressed in the ectoderm prior to its interaction with the inducing chordamesoderm (Sharpe *et al.*, 1987; Ruiz i Altaba, 1990).

By differential screening it is possible to isolate cDNA clones corresponding to six neural-specific genes, which are activated during early embryogenesis (Richter *et al.*, 1988). One of them is a gene (clone 24-10) encoding a neural-specific ß-tubulin. The sequence indicates that the predicted protein is a class II ß-tubulin (Good *et al.*, 1989). This clone is homologous to clone D-8 identified by Dworkin-Rastl *et al.* (1986). Skillful microdissection combined with the Northern-blot-technique provided valuable information on the stage-specific and the gross spatial expression of this gene (Richter *et al.*, 1988).

In the present study *in situ* whole-mount analysis has been used to localize the expression of the ß-tubulin gene in the developing nervous system. *In situ* hybridization to sectioned *Xenopus* embryos is less sensitive when the Digoxigenin method is used. On the other hand, using autoradiography with ^{3}H- or ^{35}S-labeled antisense RNA, even with abundant and moderately abundant RNAs, an exposure time of one week to several months is often necessary (Jamrich *et al.*, 1987; Weeks and Melton, 1987; Ruiz i Altaba and Melton, 1989; Sato and Sargent, 1990). Furthermore in sections it is difficult and laborious to reconstruct the 3-dimensional distribution of the signal in the embryo. Therefore we used the whole-mount *in situ* hybridization technique, which allows the detection of the stage-specific and spatial expression of the neural-specific gene 24-10 within three days. Unexpected was the fact that in the late blastula and early gastrula the gene is expressed also in that part

Abbreviations used in this paper: XlHbox6,Xhox 3, homeobox genes; SSC, sodium-sodiumcitrate buffer; SSPE, sodium-sodiumphosphate-EDTA buffer; PBS, phosphobuffered saline; MOPS, morpholinopropane sulfonic acid; EGTA, ethylene glycol-bis(ß-aminoethylether)N,N,N',N'-tetraacetic acid; EDTA, ethylenediamonotetraacetic acid-Na2-salt.

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of the ectoderm (presumptive epidermis) not in contact with the inducing chordamesoderm. These results are consistent with the view that in *Xenopus* there is a neural predisposition of the ectoderm (Sharpe *et al.*, 1987, 1989; De Bernardi *et al.*, 1990; Ruiz i Altaba, 1990).

In this paper, we use the expression predisposition instead of predetermination, since the terms determination or predetermination suggest that the fate of cells is already channeled into certain pathways of differentiation and is no longer unstable or reversible.

Results

The mRNA for a neural-specific ß-tubulin is first detectable in the late blastula (stage 9) under our experimental conditions (Fig 1A). Of great interest is the fact that the mRNA is expressed not only in the dorsal ectoderm (presumptive neural plate) but also in the lateral and ventral ectoderm (presumptive epidermis). Only the endodermal area and the yolk plug failed to show any staining. This also holds true for the early and middle gastrula (stage 10 1/2 and 11 1/2) (Fig. 1B). In stage 12 (late gastrula, small yolk plug stage) the signal is found mainly in the presumptive neural plate. However, a substantial amount of ß-tubulin mRNA is also present in the lateral ectoderm (Fig. 1C). This holds true also for stage 13 (early neurula) (Fig. 1D). In the mid neurula fold stage (stage 17) the anterior part of the neural plate is now roundish. The neural folds are distinct with the exception of the mediorostral part. Now the signal is fairly limited to the presumptive spinal cord and the brain area (Fig. 1E). In stage 19 there a substantial extension of the brain can be observed and is heavily stained. The neural folds are touching each other, showing a strong signal (Fig. 1F, G). There can no longer be detected any stain in the presumptive epidermis. This holds true also for the following stages. In stage 23 (not shown) the prosencephalon has segregated into tel- and diencephalon. While there is no signal in the presumptive epidermis the neural specific ß-tubulin mRNA can be detected in the brain area and the spinal cord. In the more advanced stage 37 the signal is also restricted to the well-developed brain area and to the spinal cord (Fig. 1H, J). The signal can also be detected in the optic nerve. In stage 43 the formation of the cerebral hemispheres and the choroid plexus in the pros- and rhombencephalic roof takes place. The signal can be clearly seen in the brain area and in the neural part of the eyes (neural retina, optic nerve). A very faint staining in the spinal cord can be identified (Fig. 1K). Some embryos with partially removed animal caps (early gastrulae, stage 10 1/2) afforded a lateral view on the two ectodermal layers, which indicated that the mRNA is mainly expressed in the deep ectoderm layer. This observation was also confirmed by autoradiography using cross sections of early neurulae (stage 14), which were treated with the antisense ^{35}S-RNA of the gene 24-10 (Fig. 2). It is well known that the deep ectoderm layers form the main mass of the central nervous system, while the outer ectoderm layer differentiates into the ependymal part of the brain and the spinal cord (Asashima and Grunz, 1983; Grunz, 1985b).

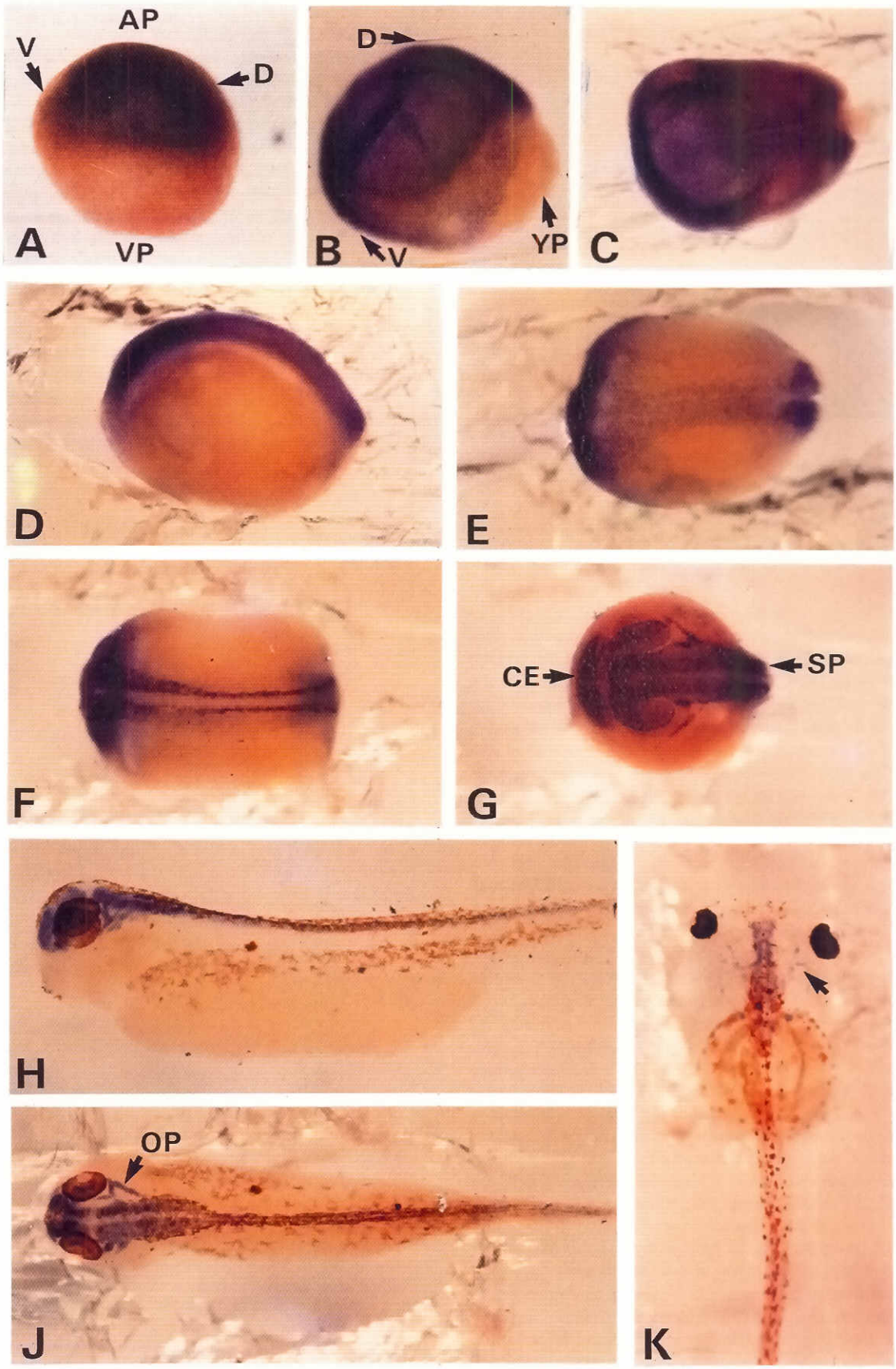
To demonstrate the specificity of the ß-tubulin signal we performed Northern-blot experiments which showed that hybridization to non-neural tissues can be excluded (Fig. 3).

Discussion

One of the best-known experiments in developmental biology is the organizer experiment of Spemann and Mangold (1924), which showed that the dorsal blastopore lip triggers the ectodermal target cells to form the central nervous system. This phenomenon was also called primary embryonic induction. The ectodermal cells react to an external signal transmitted from the blastopore lip or from neuralized ectoderm (Mangold and Spemann, 1927; Grunz, 1990b; Itoh and Kubota, 1991; Servetnick and Grainger, 1991b). Fractions with neuralizing activity could be isolated from *Xenopus laevis* and Triturus embryos (Janeczek *et al.*, 1984, 1986; Grunz *et al.*, 1986). However, the ectoderm forms brain structures without the interaction of the natural inducer under certain conditions. Apparently the plasma membrane is an important link in the chain of events, leading to neuralization (Grunz and Staubach, 1979a,b; Duprat *et al.*, 1982, 1985; Grunz, 1985b; Takata, 1985; Tacke and Grunz, 1986). Cell-to-cell contact between the ectodermal cells and the extracellular matrix plays a key role for the determination and differentiation of the ectodermal cells either into epidermal or into neural cells (Grunz and Tacke, 1989, 1990).

Neural induction results in the activation of neural-specific genes (Kintner and Melton, 1987; Sharpe *et al.*, 1987; Richter *et al.*, 1988) and the suppression of some others (Jamrich *et al.*, 1987). Of special interest are the genes that are already expressed as an immediate consequence of neural induction. Six different genes conforming to these criteria were described in an earlier paper (Richter *et al.*, 1988). These included a gene encoding a class II ß-tubulin (Good *et al.*, 1989). In the Northern-blot technique, it was possible to show that this gene is expressed in the early gastrula (stage 11), neurula stages, larval stages and in the brain of the adult frog. In the present approach we analyzed the spatial and temporal expression of this gene by the *in situ* whole-mount technique. Of special interest is the fact that in stage 9-11 (late blastula to large yolk-plug stage gastrula) the specific m-RNA is found not only in the presumptive neural plate area (dorsal part of the embryo) but also in decreasing quantities in the lateral and ventral ectoderm (presumptive epidermis). Earlier work showed that preneural and preepidermal ectoderm expresses cytokeratin mRNA (Jamrich *et al.*, 1987). The accumulation of these mRNAs in the presumptive neural plate is terminated after contact with the involuting chordamesoderm by mid-gastrula. This means that the epidermis-specific cytokeratins are expressed periodically also in the non-epidermal area (presumptive neural

Fig. 1. Localization of a neural-specific mRNA by whole-mount *in situ* hybridization. (A) *Late blastula (stage 9; Nieuwkoop and Faber, 1967). The signal is found in the dorsal (D) and the ventral (V) ectoderm. AP animal pole, VP vegetal pole.* **(B)** *Mid-gastrula (stage 11 1/2, large yolk plug; lateral view). The signal can be identified on the dorsal side (D) and also in the ventral (V, presumptive epidermis) and lateral parts (YP yolk plug).* **(C)** *Late gastrula (stage 12; dorsal view). The presumptive neural plate is intensively stained (left side: cranial, right side: caudal).* **(D)** *Early neurula (stage 13; lateral view). The signal is now found mainly in the neural plate.* **(E)** *Late neural fold stage (stage 17; dorsal view). Only the presumptive brain area and the presumptive spinal cord are stained.* **(F, G)** *Initial neural tube stage (stage 19; F dorsal view, G view on the future head area). The signal is restricted to the presumptive brain and spinal cord (SP) zone. Also the presumptive cement gland (CE) is stained.* **(H, J)** *Larval stage (stage 37; H lateral view, J dorsal view). The distribution of the transcript can be found in the brain and the spinal cord. Note that also the optic nerve (OP) is stained.* **(K)** *Advanced larva (stage 43, dorsal view). The staining is detected in the brain, the spinal cord and also clearly in the optic nerve (arrow).*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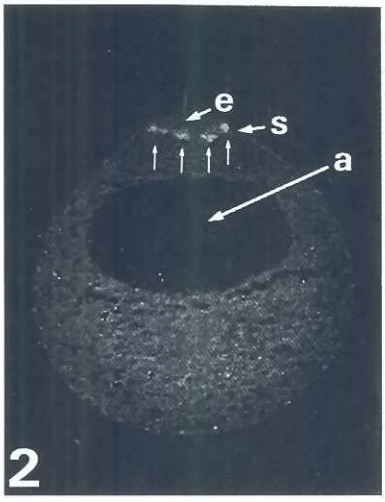

Fig. 2 Cross section of an early neurula (stage 14, caudal part). *It was incubated with antisense ^{35}S-RNA of the clone 24-10 (ß-tubulin). The signal is observed by Epi-Polarization (Grunz, 1990c) in the inner layers of the medullary plate only. e epithelial layer of the neuroectoderm, s sensorial layer of the neuroectoderm, a archenteron.*

plate). On the other hand the neural-specific ß-tubulin used in our experiments is expressed periodically in the presumptive epidermal part of the embryo and will be terminated in this part of the embryo during further development. Later in development (stage 14) the expression of the neural-specific tubulin is clearly restricted to the presumptive neural plate. This holds true also for more advanced neurulae up to the swimming tadpole. Apparently the tubulin is now only needed in this area which will participate in the final formation of the neural tube. The expression of the neural-specific gene will only be prolonged in the cells that are under direct control of the chordamesoderm.

It is known from studies with specific antibodies that the developing central nervous system expresses large quantities of ß-tubulin (Ruiz i Altaba, 1990). Cells of the lateral part of the neural plate will quickly lose their neural competence (Albers, 1987). Apparently they are now unable to express genes encoding products needed for neural differentiation. This means that the cells outside of the medullary plate can no longer react either to the inducing stimuli from the underlying chordamesoderm or signals transmitted from the neighboring cells via homoiogenetic induction (Grunz, 1990b; Itoh and Kubota, 1991; Servetnick and Grainger, 1991b). These data are in agreement with immunological studies of De Bernardi *et al.*, (1990), who showed the expression of *Xenopus* ß-tubulin by cross reaction with a chicken ß-tubulin in the lateral and ventral ectoderm of early gastrulae without any contact with the inducing chordamesoderm. These results are consistent with the view that there could exist a neural predisposition in the ectoderm (Sharpe *et al.*, 1987). Also it cannot be excluded that the neural predisposition of the ectoderm is caused by a spreading signal originating from adjacent posterior dorsal mesoderm as Ruiz i Altaba (1990) has postulated for the homeobox gene Xhox3. So far nothing is known about the molecular nature of this spreading signal. It could originate from the organizer region prior to the involution of the chordamesoderm in the early gastrula stage and could spread through the ectoderm.

Our results with disaggregated ectoderm, which forms neural structures after delayed reaggregation, show that the ectoderm can easily be shifted into the neural pathway of differentiation without inducing chordamesoderm (Grunz and Tacke, 1989, 1990). Other authors have observed an «autoneuralization» of single ectodermal cells (Godsave and Slack, 1989; Saint-Jeannet *et al.*, 1990). Furthermore an overexpression of certain homeobox genes can be observed in uncommitted embryonic cells (Cho *et al.*, 1991).

The periodic expression of neural-specific genes in the presumptive epidermal part of the ectoderm in the form of a gradient from the dorsal to the ventral sides of the embryo could be of developmental significance. After defects of parts of the future neural plate the remaining part (presumptive epidermis) could quickly restore the neural pattern, since these cells also express the neural-specific tubulin to a certain extent and presumably also other neural-specific genes. This view is supported by the fact that we observed in the microdissection experiments of an earlier article a nearly complete rescue of the neural plate in the remaining embryo after the removal of a substantial part of the neural plate (Richter

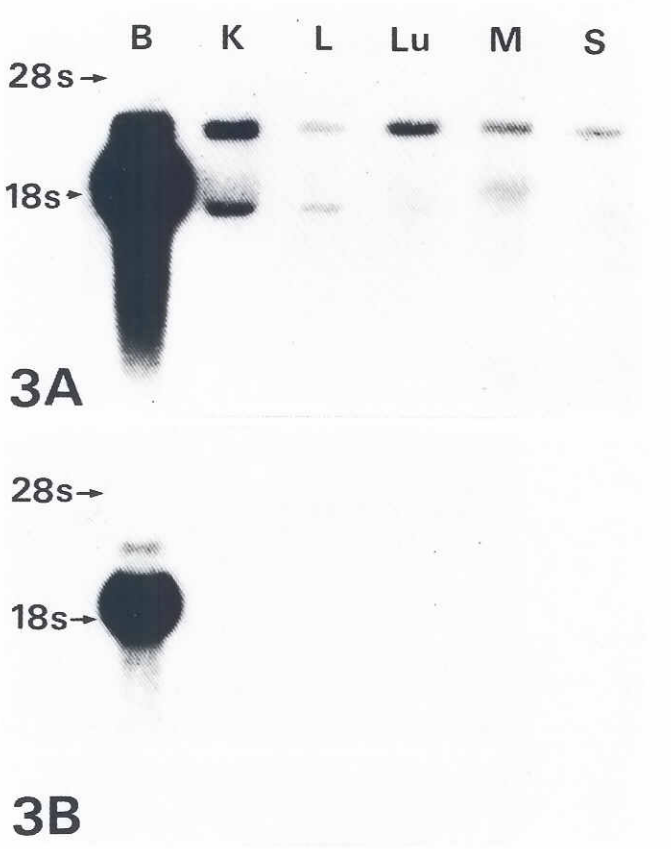

Fig. 3. Demonstration of the neural-specific expression of the ß-tubulin gene (clone 24-10). *RNA preparations from different adult tissues were compared. 10 µg of RNA was applied on each lane.* **(A)** *The NYTRAN®-membrane was washed 2 times with 2x SSPE at 65°C. The film was exposed for 2 days.* **(B)** *The same membrane as in (A) was washed again with 0.2 x SSPE (high stringency) at 60°C for 20 min followed by a film exposure for 3 days. The stringency in Fig. 3B corresponds to that used in the whole-mount* in situ *preparations. B, brain; K, kidney; L, liver; Lu, lung; M, muscle; S, skin.*

et al., 1988; preliminary experiments, not mentioned in this paper). The formation of brain structures was only prevented when we removed the presumptive neural plate together with some adjacent parts of the presumptive epidermis (outside the border of the presumptive neural plate). On the other hand it is unlikely that remaining uncommitted ectoderm will be induced by the underlying chordamesoderm, since neural competence is lost at stage 12 in *Xenopus* embryos (results of several laboratories, cited by Servetnick and Grainger, 1991a).

Also during mesoderm formation first an ubiquitous transcription of MyoD takes place in the early embryo, which is restricted to the muscle precursor cells during further development (Rupp and Weintraub, 1991).

However, it should be pointed out that a possible neural predisposition is unstable and reversible. Isolated ectoderm (presumptive neural plate as well as presumptive epidermis) will develop into ciliated epidermis, so-called »atypical epidermis« (Grunz *et al.*, 1975). Only under the influence of a *directive* induction (Saxén, 1977) will the ectoderm be neuralized. Furthermore, the determination of the ectoderm of late blastula and early gastrula is not restricted to forming epidermis and neural derivatives only. It will differentiate also in endodermal and mesodermal derivatives (Grunz, 1983; Grunz and Tacke, 1986; Knöchel *et al.*, 1987; Slack *et al.*, 1987; Smith, 1987; Grunz *et al.*, 1988, 1989). Of special interest is the fact that the ectoderm can be channeled into endodermal-derived tissues either by bone marrow, vegetalizing factor, XTC-MIF or FGFs (Takata and Yamada, 1960; Yamada and Takata, 1961; Minuth and Grunz, 1980; Grunz, 1987; Green *et al.*, 1990).

Traditionally the phenomenon of restoration and rescue of deleted parts of the embryo was called regulation, *i.e.* cells can change their prospective determination under certain conditions. The periodical expression of neural-specific genes in future epidermal cells supports this view at the molecular level.

Materials and Methods

The *in situ* hybridization technique was adapted from Tautz and Pfeifle (1989) and Hemmati-Brivanlou *et al.* (1990).

Embryos

Xenopus laevis eggs were obtained by injection of albino females and males (wild-type) with 500 IU of human chorion gonadotropin (Schering AG, Berlin) prior to the *in vitro* fertilization. Developmental stages were determined according to Nieuwkoop and Faber (1967). The embryos were raised in a mixture of 750 ml Steinberg solution (58.18 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.8 mM $MgSO_4$) and 500 ml chloride-free tap-water at a pH of 7.4. The embryos were kept in this solution until stage 10 (early gastrula). The jelly coat was removed by treatment with 2.4% cysteinium chloride pH 7.35 for four to six min. The embryos were rinsed several times and were cultured in Steinberg-solution with added penicillin/streptomycin until the desired stage. The vitelline membrane was removed with sterile forceps. Selected embryos of the desired stage were transferred to a sterile 20 ml scintillation-vial filled with DEPC (diethylpyrocarbonate)-treated sterile aqua bidest prior to the fixation in MEMFA (100 mM MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) for at least 1.5 h at room temperature. The fixation was terminated by replacement of MEMFA with 5 ml methanol. After a few min of equilibration the methanol was replaced by 15 ml fresh methanol. The embryos were stored at -20°C.

Probes

The DNA-template of clone 24-10 (Richter *et al.* 1988; Good *et al.* 1989) was linearized with Bam H I. Dig-labeled RNA was synthesized in an *in vitro* transcription assay in the presence of digoxygenin 11-UTP with T3 RNA sense polymerase (Boehringer, Mannheim). Linearized template DNA (1 µg) was transcribed in a 20 µl-assay consisting of transcription buffer, 2 mM rATP, 2 mM rCTP, 2 mM rGTP, 1.3 mM rUTP, 0.7 mM rDigUTP, 20 u RNase-inhibitor, 20 u T3 RNA-polymerase for 2 h. After treatment with DNase I for 15 min at 37°C the reaction was stopped by addition of 2 µl 0.5M EDTA (pH 8.0), 2.4 µl 4M LiCl and 75 µl prechilled (-20°C) ethanol. The RNA was precipitated at -70°C overnight and recovered by centrifugation at 11000 rpm in a Biofuge (Heraeus) at 4°C. The pellet was washed with 70% ethanol with added 0.5% NaCl and again centrifuged for 5 min. The RNA was dried for one minute in a Speedvac and resuspended in 100 µl DEPC-H_2O. A sample of 5 µl was taken for analysis on a 1% agarose gel. Different transcription assays yielded between 2-4 µg of labeled RNA.

Whole-mount in situ *hybridization*

The following steps were carried out by placing between three and six embryos (depending on the stage) in an autoclaved and heat sterilized (8 h, 121°C) round-bottom 2 ml Eppendorf tube with a RNase-free pasteur-pipette. Each batch (different stages of embryos) was handled with a different pipette to exclude any crosscontamination. During the change of solutions embryos remained always covered by a small amount of liquid to avoid any damage by pipetting. For hybridization the embryos were rehydrated by incubation with 3 different concentrations of ME (90% methanol, 10% 500 mM EGTA) and PTw (1 x PBS. 0.1% Tween 20), *i.e.* 75% ME + 25% PTw, 50% ME + 50% PTw, 25% ME + 75% PTw. The embryos were then rinsed four times in PTw for 5 min each. We found it necessary to refix embryos prior to stage 20 with PFA (4% paraformaldehyde in 1 x PBS pH 7.5) immediately after rehydration. After incubation for 15 min in 10 µg/ml proteinase K in PTw at room temperature and rinsing (two times, 5 min each) in PTw the embryos were refixed with PFA. The refix was followed by five washing steps in PTw, five min each. In the last step PTw was replaced by hybridization solution (50% formamide, 5 x SSC, 0.1 x Tween 20, 50 µg/ml Heparin, 5% blocking reagents (Boehringer, Mannheim), 500 µg/ ml yeast RNA (Boehringer, Mannheim). We found that the presence of blocking reagents in the hybridization solution reduces background. After settling of the embryos the hybridization buffer was changed prior to an incubation for 180 min at 50°C. The prehybridization solution was replaced by 200 µl hybridization solution with added 200 ng of the probe. Hybridization was done for 40 hours at 50°C. Sense strand hybridization was used as a background control. The solution was replaced by a mixture of hybridization buffer and 2 x SSC at a ratio of 4:1, 2:1, 1:4 (10 min each at 37°C) prior to two washes (20 min each) with 2 x SSC at the same temperature. Single-stranded RNA was digested with 20 µg/ml RNAse A in 2 x SSC at 37°C for 30 min prior to a high stringency wash twice with 0.2 x SSC at 55° C for one hour. To block nonspecific antibody-binding the embryos were treated as follows: three washes with 0.2 x SSC and P1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl) at a ratio of 4:1, 2:1, 1:4 10 min each, followed by 10 min P1 and 60 min P2 (0.5% blocking reagent in P1). The latter step was done on a rocking platform at low speed of 2 rotations per 5 seconds. The solution was replaced with P2 and PTw 1+1 for 10 min. The embryos were washed in PTw for 10 min and then in PBT (PBT is 1 x PBS + 2 mg/ml BSA + 0.1% Triton X-100) for another 10 min prior to the incubation in PBT + 10% Normal Sheep Serum (Gibco) for one hour. This solution was replaced by <DIG>AP (anti-digoxygenin alkaline phosphatase conjugate; Boehringer, Mannheim). The <DIG>AP stock solution was diluted 1:2000 in PBT + 10% normal sheep serum and preabsorbed for 60 min against fixed embryos omitting the hybridization treatment (Tautz and Pfeifle, 1989) and incubated overnight on a rocking platform at 4°C. The embryos were washed 4 times, 60 min each, in PBT at room temperature to remove excess antibody. An equilibration step with P3 (100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20, 1 mM Levamisole) made freshly from stock solutions (except Levamisole) 3 times 5 min followed. The embryos were incubated for 30 - 180 min at 37°C in tiny petri dishes filled with P3 + 3.5 µl/ml X-phosphate (50 mg/ml 5-brom-4-chlor-3-indolylphosphate toluidin salt in dimethylformamide) + 4.5 µl/ml NBT (75 mg/ml nitroblue tetrozolium salt in 70% dimethylformamide). The color-reaction was stopped by 3 washes, 5 min each, in PBS. The embryos were dehydrated in methanol for 5 min and then mounted in benzyl benzoate/benzylalcohol (BB/BA) mixed in the ratio 3:1 to clear them. The signal can easily be detected under a stereo-microscope. The embryos were photo-

graphed after one week in the upper BB/BA-solution. We have used albino embryos in our experiments although the blue stain can also be detected in the pigmented wild-type embryos. We have also tried with good success to bleach stained embryos (Dent *et al.*, 1989). When changed at least 8 times in 2 days the hydrogen peroxide bleaches the embryo nearly completely. The blue stain is resistant to this bleaching procedure. Since we have also introduced a special autoradiographic detection method (Epi-polarization) for silver grains in sections of wild type embryos (Grunz, 1990c), the use of albino embryos is no longer a prerequisite for good results with these techniques.

In situ *hybridization (histological sections)*

Histological sections of early neurulae (stage 14) were incubated with sense and antisense ^{35}S-RNA of the clone 24-10 (ß-tubulin). The histological and autoradiographic techniques were essentially the same as described elsewhere (Jamrich *et al.*, 1987; Grunz, 1990c).

Northern-blots

The RNA isolation and Northern-blotting were performed as described previously (Richter *et al.*, 1988).

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