Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 8086–8090, November 1988
Developmental Biology

Gene expression in the embryonic nervous system of *Xenopus laevis*

(neural induction/gastrula/neurula/subtractive cloning)

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Contributed by Igor B. Dawid, August 1, 1988

ABSTRACT **Development of the nervous system in the amphibian embryo is initiated during gastrulation by an inductive interaction between chordamesoderm and dorsal ectoderm. The induced ectoderm forms the neural plate while uninduced ectoderm generates epidermis. We screened for genes activated during gastrulation and expressed specifically in the nervous system of *Xenopus laevis* in the expectation that clones representing such genes will constitute useful markers for the study of early neurogenesis. Probes were prepared from adult brain RNA by subtraction with RNA from ovary and from different combinations of adult kidney, muscle, and skin; cDNA libraries prepared from early to late neurula embryo RNA were screened with these probes. Six clones were chosen for further study. Three of these clones are not represented in the maternal RNA population but are activated at the late gastrula stage; the other three increase from a maternal base. Expression of five of the genes is restricted to the neural plate during embryogenesis, and all six are restricted to the central nervous system in premetamorphic tadpoles and adults. One of the clones encodes an apparently neurospecific isoform of β-tubulin; the identity of the other clones is unknown. Expression of all six genes is suppressed in axis-deficient embryos that lack dorsal structures including the brain.**

Neural induction triggers the development of the nervous system, one of the first stages of tissue differentiation in the vertebrate embryo. The nature of the neural induction signal has been a challenging problem since the time of the Spemann and Mangold experiment (1) in which the phenomenon of induction was discovered. These authors transplanted the dorsal blastopore lip from one embryo into the ventral region of another and showed that this transplant was able to induce a second body axis including a second nervous system. In spite of considerable efforts since that time, comparatively little information has been gained on the nature of the inducing agent or agents and the molecular mechanisms of induction. Yet, considerable progress has been made in analyzing neural induction mechanisms at the cellular and tissue level and in describing biochemical events that accompany the earliest differentiation of the nervous system (2–7).

In the amphibian embryo, neural induction occurs during gastrulation when dorsal mesodermal cells migrating along the blastocoel roof influence the overlying ectoderm to differentiate into the neural plate (4–6). An important function during the induction process is provided by the appropriate disposition of cell-surface molecules, as has been demonstrated for fibronectin (8, 9), and by differential expression of cell adhesion molecules (CAMs) (10). One of the first detectable biochemical events in the presumptive neural plate during gastrulation is the disappearance of epidermal markers including the termination of cytokeratin expression (11–13). Genes known to be activated in the developing neural plate include those encoding neural CAM (N-CAM) (6, 14), a homeobox-containing gene (15), and somewhat later a neurofilament gene (16).

We have initiated a program aiming to provide a more extensive array of genes that are activated specifically and early during neural plate differentiation in the expectation that such markers would be useful in the molecular dissection of the inductive process. In this paper, we report the isolation and initial characterization of six clones that fulfill the criteria of early activation in the embryo of *Xenopus laevis* and specificity of expression in the nervous system.

MATERIALS AND METHODS

mRNA Preparation. RNA from whole embryos, brain, and skin was isolated by using guanidinium isothiocyanate essentially as described (17, 18) and passed over oligo(dT)-cellulose to select for poly(A)$^+$ RNA. RNA from kidney, liver, and muscle was purified over a CsCl gradient (19).

cDNA Cloning. cDNA was synthesized with reverse transcriptase from Moloney murine leukemia virus with oligo(dT) as a primer according to the manufacturer's recommendations (Bethesda Research Laboratories). The second strand was synthesized by using RNase H and DNA polymerase I (20). Double-stranded cDNA was further modified according to a standard protocol (21) and ligated with phage λgt11 arms (Promega Biotec, Madison, WI). The ligation reaction was packaged *in vitro* (Stratagene, La Jolla, CA), and the resulting libraries were amplified on Luria–Bertani (LB) agar plates. Three libraries from neurula embryos at stages 13, 17, and 24 (22) were produced.

Synthesis of Labeled Probes. Radioactive cDNA was synthesized with reverse transcriptase by using poly(A)$^+$ RNA, $(dT)_{12-18}$, and random primer (hexamers; Pharmacia) in the presence of [α-^{32}P]dCTP (Amersham). The reaction was stopped by adding NaOH to 0.3 M, and the mixture was incubated at 65°C to hydrolyze the RNA. After purification on a Sephadex G-50 (Pharmacia) column, the single-stranded cDNA was hybridized with a 20- to 30-fold excess each of different RNA preparations (see *Results*). Finally, single-stranded DNA was separated from cDNA·RNA hybrids on a hydroxyapatite column (Bio-Rad) (23) and used to screen the libraries.

RNA Blot Analysis. RNA was separated by electrophoresis on 1.2% agarose gels containing 5 mM methylmercury(II) hydroxide (24), electroblotted onto Nytran filters (Schleicher & Schuell), and bound to the matrix by UV irradiation. The filters were hybridized as described by Church and Gilbert (25). *Eco*RI fragments from selected clones were labeled according to Feinberg and Vogelstein (26) and used to probe the blots.

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Abbreviations: CAM, cell adhesion molecule; N-CAM, neural CAM.

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RESULTS

Isolation of Brain-Specific Clones from Embryonic Libraries. Aiming to select clones that may be useful as markers for early neural development, we prepared cDNA libraries from neurula-stage embryos (stages 13, 17, and 24) and screened them with adult brain-specific probes. For the first screen, ^{32}P-labeled cDNA was synthesized from brain poly(A)$^+$ RNA and subtracted with ovary, kidney, and liver poly(A)$^+$ RNA; 36 clones each were picked from the stage-13 and stage-17 library. For the second screen, a duplicate filter set from the stage-24 library was hybridized with cDNAs synthesized from adult brain, and stage-50 to -52 tadpole brain poly(A)$^+$ RNA was subtracted with adult kidney, liver, and muscle poly(A)$^+$ RNA. A total of 48 clones positive with both brain probes were selected. In the next step, inserts of a subset of these clones were isolated, labeled, and used to probe blots of RNAs from different adult tissues (brain, kidney, liver, muscle, and skin; Fig. 1*A*). Only clones that reacted at least 100 times more intensely with brain as compared to other tissues were studied further; this step eliminated many clones that are preferentially but not exclusively expressed in brain. About half of the selected clones produced multiple bands or smears on the blots indicative of repetitive elements; these clones were not examined further. The 6 clones presented in this paper were chosen as being brain-specific in adults and late tadpoles and expressed very early during *Xenopus* development at a conveniently analyzable abundance level. These clones were analyzed by developmental gel blots (Fig. 1*B*), as discussed below for each individual gene.

Distribution of Selected RNAs in the Embryo. The data discussed above suggest that the six genes may be expressed specifically in the neural plate at the time of its differentiation. To test this suggestion, we examined the distribution of the cognate RNAs in neurulae. Two types of dissections were carried out, as indicated in Fig. 2. Stage-12 embryos were dissected into presumptive neural plate, remaining ectoderm, and a fraction that contains endoderm and mesoderm (Fig. 2*A*). At this stage, the relevant RNAs are too rare to detect in the small amount of dissected material available. Therefore the three different tissue fractions were cultured until control embryos had reached stage 17–18, assuming that genes activated before explantation would continue to be expressed in culture. RNA was then prepared and analyzed by gel blotting. In a second experiment, stage-17 embryos were dissected into closing neural tube, epidermis, and the remaining part of the embryo consisting mostly of endoderm; however, at this stage it is difficult to dissect the neural tube without contamination of underlying tissue, and the neural fraction therefore contained a contribution from notochord and somitic mesoderm. Again, RNA was isolated and analyzed. The results from the two types of dissection experiments were similar. The tested RNAs are expressed specifically in the neural fractions except for clone 24-15 (Fig. 1*C*), as is discussed further below.

Localization of RNAs was also studied in tadpoles at the onset of metamorphosis (stage 50). Brains and eyes were dissected, and tails were cut off, leaving behind the carcass. RNA was prepared from brains and carcasses and compared with RNA prepared from whole embryos. As can be seen in Fig. 1*D*, the tested RNAs are strongly enriched in the brain.

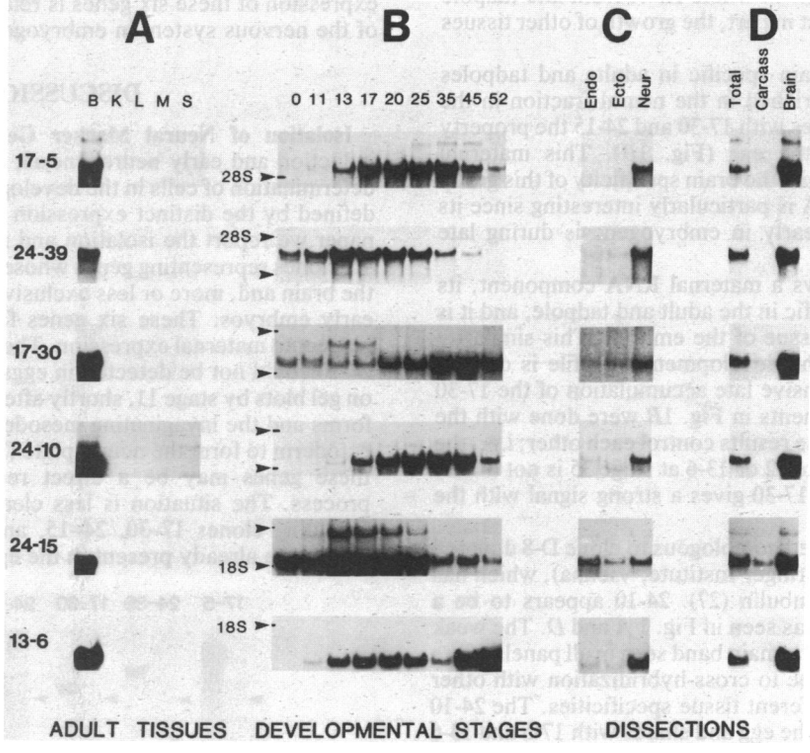

FIG. 1. Expression patterns of six genes. cDNA clones were selected as described in the text, and inserts were used in RNA gel blots. The name of each clone is given at the left and refers to all gels in *A–D*. The position of 18S and 28S rRNA is indicated. (*A*) Comparison of RNA preparations from different adult tissues. B, brain; K, kidney; L, liver; M, skeletal muscle; S, skin. Exposures: 17-5, 5 days; 24-15, 5 hr; all others, 1 day. (*B*) Developmental profile. Stages (22) are given at the top of each lane. Exposures: 24-39, 2 days; all others, 1 day. (*C*) RNA distribution in neurula embryos. Embryos were dissected as shown in Fig. 2 and described in the text. Embryos dissected at stage 12 and cultured until siblings reached stage 17 were used, except in the experiment with clone 17-30, which used embryos dissected at stage 17. Endo, endoderm plus mesoderm; ecto, ventral ectoderm; neur, neural plate or closing neural tube. Exposures: 17-5, 24-39, and 13-6, 5 days; 17-30, 10 days; 24-10 and 24-15, 2 days. (*D*) RNA distribution in stage-50 tadpoles. Exposures: 17-5 and 24-39, 5 days; all others, 1 day. In *A*, *B*, and *D*, 2 μg of RNA selected once on oligo(dT)-cellulose was applied on each lane; in *C*, RNA isolated from fragments of 12 embryos was used in each lane.

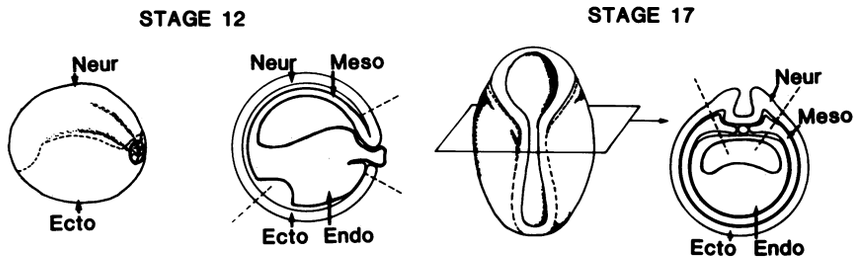

FIG. 2. Dissection of embryos. The dashed lines indicate the incisions made with glass needles. At stage 12 to 12½, the neural plate was dissected without underlying mesoderm (meso); ventral ectoderm (ecto) and endoderm together with mesoderm (labeled endo) were also collected and cultured for 5 hr at 24°C in modified Barth's solution (88 mM NaCl/1 mM KCl/0.8 mM $MgCl_2$/0.8 mM $CaCl_2$/2.4 mM $NaHCO_3$/7.5 mM Tris, pH 7.6) before RNA extraction. At stage 17–19, the closing neural tube was dissected together with the underlying chordamesoderm (neur). Ectoderm (ecto) was peeled off; the lateral plate mesoderm was collected together with the endoderm (labeled endo).

Developmental Behavior of Individual Genes. The genes represented by the six clones studied have in common that they are brain specific in the adult and already expressed in early embryogenesis, but there are various differences in their developmental behavior.

17-5. This gene is expressed in a neurospecific manner at all stages tested (Fig. 1 *A*, *C*, and *D*). The 17-5 RNA shares with 24-10 and 13-6 the property of being undetectable in the egg but accumulates from the gastrula stage onward. A signal is visible at stage 11 (Fig. 1*B*), which raises the interesting possibility that brain-specific products may be synthesized immediately after neural induction has begun to act and before a morphologically discernible neural plate is present. The 17-5 RNA then accumulates to a peak at mid to late neurula and subsequently declines. Nevertheless 17-5 RNA is moderately abundant in late tadpole and adult brain (Fig. 1 *A* and *D*), and its decline in whole RNA from late tadpole stages may reflect, at least in part, the growth of other tissues relative to the brain.

24-39. This gene is brain specific in adults and tadpoles (Fig. 1 *A* and *D*) and enriched in the neural fraction in the embryo (Fig. 1*C*); it shares with 17-30 and 24-15 the property of being expressed in the egg (Fig. 1*B*). This maternal component obviously limits the brain specificity of this group of genes. The 24-39 RNA is particularly interesting since its expression peaks very early in embryogenesis during late gastrula (stage 13).

17-30. This gene shows a maternal RNA component, its expression is brain specific in the adult and tadpole, and it is enriched in the neural tissue of the embryo. This similarity with 24-39 ends when the developmental profile is considered, which shows extensive late accumulation of the 17-30 RNA. (Since all experiments in Fig. 1*B* were done with the same RNA samples, these results control each other; i.e., the low level of 24-39 at stage 52 or 13-6 at stage 35 is not due to RNA degradation since 17-30 gives a strong signal with the same RNA).

24-10. This clone proved homologous to clone D-8 donated by Mark Dworkin (Boehringer Institute, Vienna), which has been identified as a β-tubulin (27). 24-10 appears to be a brain-specific β-tubulin; as seen in Fig. 1 *A* and *D*. The weak bands above and below the main band seen in all panels of the figure are likely to be due to cross-hybridization with other β-tubulin mRNAs of different tissue specificities. The 24-10 RNA is undetectable in the egg and shares with 17-5 and 13-6 the property of expression in early gastrula.

24-15. Although brain specific in the adult, this RNA was found in all regions of the embryo that were tested (Fig. 1*C*) and also in the carcass fraction of the tadpole (Fig. 1*D*). The non-neural representation in the early embryo could be the result of persistence of the very large maternal component (Fig. 1*B*).

13-6. The distribution of this RNA is highly brain specific in adult and tadpole and enriched in the embryo (Fig. 1 *A*, *C*, and *D*). The RNA has no maternal component, accumulates rapidly in the gastrula and peaks during neurula (stage 20), then declines, and eventually accumulates again to become quite abundant in the late tadpole (Fig. 1*B*).

Dependence of Gene Expression on the Formation of the Dorsal Axis. Amphibian eggs irradiated with ultraviolet light on their vegetal surface prior to first cleavage are deficient in the formation of dorsal axial structures (28). In the extreme case, called grade 5 (29), the embryos are acephalic and fail to form any neural structures, notochord, or somites. We asked whether the accumulation of the six candidate neurospecific RNAs did occur in axis-deficient embryos. As shown in Fig. 3, the levels of the six RNAs were greatly reduced in grade-5 embryos. The least affected gene is 24-15, which has a large maternal component and is not neurospecific in the embryo (Fig. 1 *B* and *C*). These results support the view that expression of these six genes is related to the differentiation of the nervous system in embryogenesis.

DISCUSSION

Isolation of Neural Marker Genes. In studying neural induction and early neurogenesis, it should be useful if the determination of cells in the developing neural plate could be defined by the distinct expression of specific genes. In this paper,we report the isolation and initial characterization of six clones representing genes whose expression is confined to the brain and, more or less exclusively, to the neural plate of early embryos. These six genes fall into two classes with respect to maternal expression. The RNAs for 13-6, 17-5, and 24-10 could not be detected in eggs but were already visible on gel blots by stage 11, shortly after the dorsal blastopore lip forms and the invaginating mesoderm induces the overlying ectoderm to form the neural plate (22, 30). The expression of these genes may be a direct response to the induction process. The situation is less clear with the second class, including clones 17–30, 24–15, and 24–39, whose cognate RNAs are already present in the egg. This maternal compo-

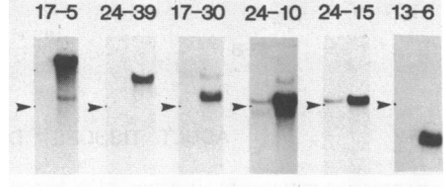

FIG. 3. Gene expression in axis-deficient embryos. Embryos treated with ultraviolet irradiation before first cleavage were cultured until untreated siblings reached stage 25, and grade-5 (29) embryos were selected. RNA was analyzed by gel blotting. Each lane was loaded with 2 μg of RNA enriched as described in the legend to Fig. 1. In each pair, the left lane contains RNA from grade-5 embryos and the right lane contains RNA from normal embryos. Arrowheads indicate the position of 18S rRNA.

nent may contribute to the presence of these RNAs in non-neural tissues of the embryo, especially in the case of 24-15 (see Fig. 1). The effective suppression of activation of all six genes in axis-deficient embryos (Fig. 3) supports the view that these sequences will be useful markers in the study of early neural development.

Markers for the Study of Neural Induction in Amphibians. During the past decade, approaches to the study of early neural development have focused on the use of molecular markers that could supplement morphological criteria of differentiation. Beyond the suppression of cytokeratins and other epidermal markers (11–13), the expression of N-CAM is an early positive index of neural plate formation and has been a very useful marker (6, 14). Except for the presence of maternal RNA (14), N-CAM expression is effectively neurospecific in the frog embryo and tadpole (14, 31, 32); during metamorphosis N-CAM is expressed strongly on skeletal muscle membranes (32).

Much attention has been focused on the neural-specific members of the large family of the intermediate filaments. Specific antibodies allowed the visualization of intermediate filaments in the nervous system of the late neurula (33). A detailed immunocytochemical study revealed that midsize neurofilament (NF-M) expression in the neural tube begins between stages 22 and 24, whereas immunoreactivity related to glial fibrillar acidic protein could be detected at stage 24 (34). The *Xenopus* homolog of porcine NF-M is expressed from mid-neurula on (16). The onset of neurofilament expression agrees well with the beginning of neurite extension (35).

Two homeobox-containing genes are expressed in the nervous system of *Xenopus* embryos. The XlHbox 6 gene is restricted to the posterior part of the neural tube, which provides an interesting regional specificity (15). This RNA can be detected at stage 13 and rises to a fairly high level but disappears before the tadpole starts feeding. Gene Xeb1 transcripts are expressed in the neural tube but are not strictly neurospecific (36).

Neurospecific Gene Expression in Embryogenesis. Although the experiments described in this paper appear to have generated additional markers for early neurogenesis in *Xenopus*, only in the case of 24-10 do we know the nature of the gene product, β-tubulin. The importance of microtubules in neurons is well known, and tissue-specific expression of different tubulin isoforms has been reported previously (37). An approach to functional characterization of developmental genes is possible through genetics, as applied effectively to neurogenesis in *Drosophila*. Several regulatory genes, including homeotic and segmentation genes, exhibit their highest level of expression in the embryonic nervous system (reviewed in ref. 38). Furthermore it was shown that the segmentation genes fushi tarazu and evenskipped are not only expressed in a striped pattern at the blastoderm stage but also in a subset of neuronal precursor cells and in glial cells in the developing central nervous system (39, 40). Genes in vertebrate animals isolated by homology to *Drosophila* genes have proved of interest in the analysis of neural differentiation. Homologs to Krüppel and engrailed are expressed specifically in the embryonic and adult nervous system (41, 42). The homeobox gene-containing gene *Hox-1.5* in the mouse is first expressed at gastrula in different regions, but later in development it is restricted to the posterior part of the central nervous system (43, 44). The protooncogene *int-1*, which shows a neural-specific expression pattern in the developing mouse embryo (45, 46), is homologous to the segmentation gene wingless in *Drosophila* (47, 48). In the fly, however, wingless expression is not specific to the nervous system (47, 49).

A different approach to neurospecific gene expression starts from a consideration of factors required for differentiation of the nervous system; this question leads to a consideration of growth factors. Nerve growth factor was discovered mostly due to its influence on events occurring fairly late in neural development (50). As additional growth factors were discovered, it became apparent that several such factors play important roles in different periods of neural development. Brain-derived neurotrophic factor supplied by the neural tube is essential for survival and differentiation of neural crest-derived sensory neurons in chicken embryos (51); platelet-derived growth factor is involved in the differentiation of glial cells in the central nervous system of the rat (52); and insulin-like growth factors exert a specific mitogenic activity on mouse neuroblasts (53). A relevant finding connecting growth factors with neural development is the homology of the neurogenic locus Notch in *Drosophila* to epidermal growth factor (54, 55).

The earliest stage of neural development in the frog, induction of the neural plate during gastrulation, may well involve growth factor-like substances. Such factors, specifically transforming growth factor β2 and fibroblast growth factor, have already been implicated in the induction of mesoderm, the earliest known cell interaction in the amphibian embryo (56–60). The activation of the genes described in this paper may be a direct consequence of the action of neural inducers and may therefore provide useful test systems for their identification.

We thank Sheryl Sato for a gift of grade-5 embryo RNA and Tom Sargent and Alison Snape for comments on the manuscript. K.R. was supported in part by a fellowship from the Max Kade Foundation.

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