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Region-Specific Neural Induction of an *engrailed* Protein by Anterior Notochord in *Xenopus*

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Anterior-specific neural induction can be assayed by means of an antibody that recognizes the *Xenopus* homeobox-containing protein En-2. The En-2 antigen is an excellent early marker, since it is present as a discrete band in the anterior neural plate of neurula embryos. Regional induction was assayed by combining dorsal mesoderm with competent ectoderm. Anterior notochord from the early neurula induced En-2 frequently, while posterior notochord induced En-2 less frequently. Presumptive somitic mesoderm and presumptive head mesoderm, though they induced neural tissue, were not strong inducers of En-2. Thus, anterior notochord may be the primary mesodermal tissue responsible for the patterning of the anterior neural plate.

RECENT STUDIES ON *Xenopus* HAVE focused on general neural induction without addressing formation of an anterior-posterior pattern within the neural plate. In amphibian embryos, the presumptive neurectoderm is induced by the underlying mesoderm to become neural tissue (*1–6*). Experiments in urodeles indicate that the anterior-posterior pattern is due to induction by dorsal mesoderm (*7*). In this report, we use an anterior-specific neural marker to study the source of pattern in the neuroectoderm of *Xenopus*.

The 4D9 antibody recognizes the *Drosophila engrailed* protein as well as a *Xenopus* homolog, En-2 (*8, 9*). Early neurula (stage 12.5) *Xenopus* embryos do not express *En-2* RNA (*10*) or protein (*9*) until stage 14, when the protein is expressed in 600 cells in the anterior neural plate in a band perpendicular to the notochord (*9*). This antigen therefore provides an excellent early marker of anterior neural differentiation. The sequence of a cDNA clone confirmed that the *Xenopus engrailed* protein detected by the antibody is homologous to the murine En-2 protein, and we therefore refer to it as En-2 (*10*). Previous evidence from the examination of the distribution of En-2 in embryos where the mesoderm has been experimentally altered to be either exclusively ventral or dorsal in character suggests that dorsal mesoderm is involved in the induction of En-2 (*9*). The anterior notochord, the presumptive anterior somites, and head mesoderm underlie the En-2 expressing cells of the stage 14 embryo (*11, 12*). Any or all of these could be responsible for inducing En-2 expression.

In this study, we recombined various portions of the dorsal mesoderm of the early neurula with ectoderm to determine which mesodermal types are capable of inducing *En-2* (Fig. 1). The recombinants were cultured and subsequently assayed for expression of *En-2*. Competent ectoderm is capable of responding to neural inducing signals coming from mesoderm, but is unable to form neural structures on its own. Competent ectoderm was obtained as animal caps from stage 9 (late blastula) embryos derived from eggs irradiated with ultraviolet (UV) light during the first cell cycle. Eggs irradiated with UV light during the first cell cycle form no dorsal mesoderm and develop as ventralized embryos (*13–15*). Thus, the use of late blastula animal caps derived from eggs irradiated with UV light reduces the chance that the ectoderm is contaminated with dorsal mesoderm. Competent ectoderm almost never expressed the En-2 antigen (2 of 40 cases) when cultured in isolation to control stage 28 (tailbud) (see Fig. 2A). In contrast, ectoderm wrapped around stage 12.5 anterior notochord expressed En-2 in a high percentage of recombinants (81%) (see Figs. 2B and 3). Ectoderm wrapped around posterior notochord expressed En-2 less frequently (36% overall) and less strongly (two of five positives had fewer than 50 nuclei stained with En-2 (Fig.

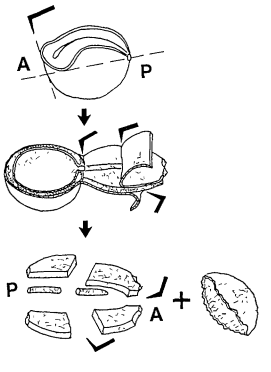

Fig. 1. Schematic of the operations used to generate different recombinants. Stage 12.5 embryos (*26*) were cut with eyebrow knives and hairloops in low calcium and magnesium modified Ringer solution (LCMR) (*15*) so that a "tongue" of dorsal mesoderm, neural plate, and archenteron roof was generated (A, anterior; P, posterior). The cut embryos were then placed in collagenase (0.4 mg/ml) (Cooper Biochemicals) in LCMR to allow the peeling away of the archenteron roof and neural plate. Isolated dorsal mesoderm was placed in 43 mM NaCl, 0.85 mM KCl, 0.37 mM $CaCl_2$, 0.19 mM $MgCl_2$, 5 mM Hepes, pH 7.2, and gentamycin (50 μg/ml) and cut into anterior notochord, posterior notochord, presumptive posterior somites, and presumptive head mesoderm with anterior somites. Each piece was then wrapped in an animal cap from a stage 9 embryo irradiated with UV light [sibling control irradiated embryos displayed ventralized phenotypes, average dorsoanterior index of less than 0.5 (*14, 15*)]. Recombinants were cultured until controls reached stage 28 then fixed and processed for immunohistochemistry (*9*). Results presented are from three independent experiments, where ectoderms from an albino spawning were combined with notochords from a pigmented embryo spawning. Each expeirment yielded the same conclusion, and the numbers from each experiment have been combined for presentation here.

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3). To ask whether recombinants that were negative for En-2 expressed other neural antigens, we also stained explants for the general neural marker, the neural cell adhesion molecule (N-CAM). When recombinants between posterior notochord and irradiated animal cap that were negative for En-2 were examined for the expression of N-CAM, all (seven out of seven) were positive (Fig. 2G). This indicates that posterior notochord is capable of inducing a general neural marker (N-CAM), in confirmation of previous work (*6*), even though it is incapable of inducing an anterior specific marker (En-2). The difference between anterior and posterior notochord in the capability to induce En-2 in *Xenopus* as seen in our experiments unifies *Xenopus* with urodeles (7). In the latter experiments, posterior dorsal mesoderm induces spinal cord, while progressively more anterior dorsal mesoderm induces brain. Our experiments demonstrate region-specific neural induction by the dorsal mesoderm in *Xenopus* and localize the strongest induction to the notochord.

Dorsal mesoderm and ectoderm of the early neurula may obtain anterior-posterior differences progressively during gastrulation (stages 10 to 12) (*16, 17*). Recent experiments suggest that anterior dorsal mesoderm from the gastrula is particularly weak in its ability to induce a general neural marker on its own (*17–20*). Its ability to induce is greatly enhanced by the use of dorsal ectoderm as the responding tissue or by inclusion of more posterior dorsal mesoderm or dorsal blastopore lip in the recombinant (*17–21*). The competent ectoderm used in the present study contained no dorsal ectoderm and would neither contain dorsal lip material nor be subjected to any signal coming from the dorsal blastopore lip, since the ectoderm was from embryos irradiated with UV light, which are radially ventral (*13–15*). The results presented in this paper suggest that by the early neurula stage (stage 12.5), anterior notochord has acquired the capability of inducing anterior neural differentiation without requiring predisposed dorsal ectoderm or signals from the blastopore lip.

Presumptive head mesoderm and anterior somites rarely induced En-2 in competent ectoderm (21%) (see Figs. 2C and 3), even though these tissues underlie approximately 70 to 80% of the region of the neural plate which expressed *En-2* (notochord underlies the other 20 to 30%) (Fig. 1) (*9*). Recombinants of anterior notochord and irradiated animal cap often showed *En-2* expressing cells far away from notochord cells (Fig. 4). The cells not directly over the notochord would have to be (i) induced by the notochord directly and then migrate away from

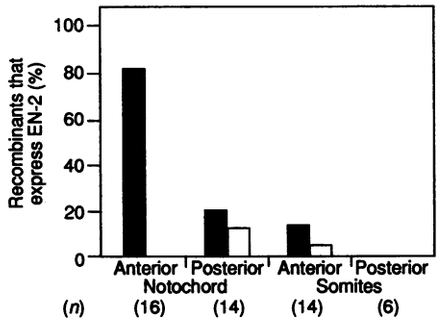

Fig. 3. Induction of En-2 in embryonic recombinants. Anterior notochord induced En-2 in competent ectoderm more frequently than posterior notochord or somitic mesoderm. Black bars, strong En-2 expression (50 to 400 nuclei); white bars, weak En-2 expression (<50 nuclei).

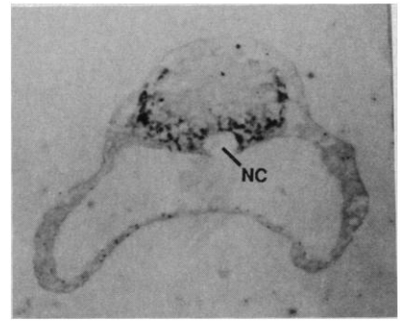

Fig. 4. Transverse sections of an anterior notochord–UVAC recombinant explant. The same anterior notochord–UVAC recombinant shown in Fig. 2B was embedded in Paraplast and sectioned. (NC, notochord). Note En-2–positive cells both close to and far from the vacuolated notochord.

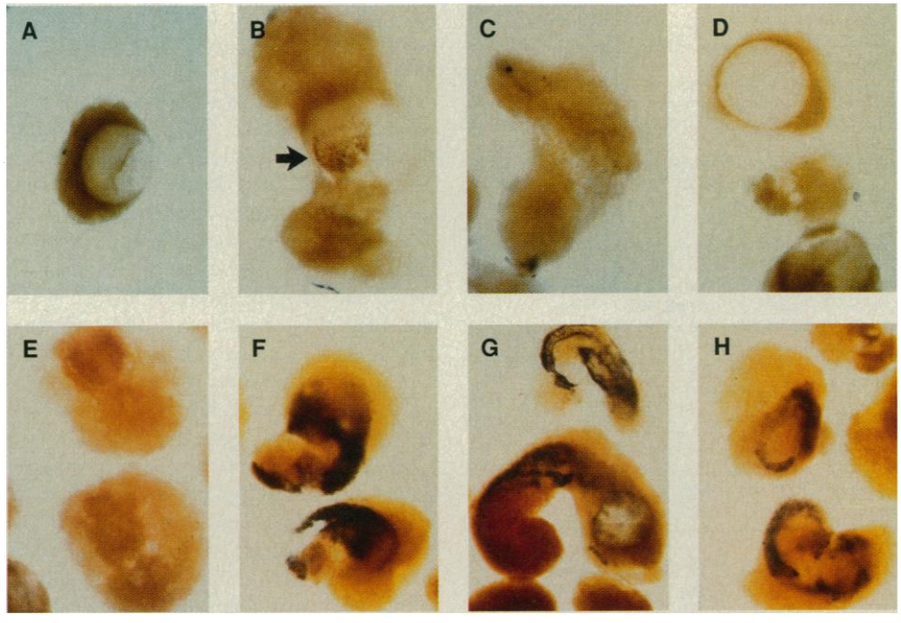

Fig. 2. Recombinants stained for En-2 (A to D) or N-CAM (E to H) cultured until control embryos reached stage 28. (**A** and **E**) Animal cap from a stage 9 embryo irradiated with UV light (UVAC) was isolated and cultured to control stage 28 and stained. (**B** and **F**) Anterior notochord–UVAC recombinant at control stage 28. The arrow points to En-2–stained nuclei. Examination of a number of recombinants and sections indicates that the staining is often in the shape of a disk, with the center of the disk surrounding the notochord, and extending many cell diameters from the notochord (see Fig. 4). (**C** and **G**) Posterior notochord–UVAC recombinant at control stage 28. Although vacuolated notochord is visible and the recombinant is elongated, no En-2 staining is detected in the recombinant shown. Posterior notochord–UVAC recombinants that were negative for En-2 were positive (seven out of seven cases) when stained for the presence of N-CAM antigen, indicating that these recombinants were capable of expressing a general neural marker (N-CAM). N-CAM staining follows the notochord in both posterior notochord–UVAC and anterior notochord–UVAC recombinants. (**D** and **H**) Presumptive anterior somite with presumptive head mesoderm combined with UVAC at control stage 28. No En-2 staining is detected in the recombinants shown. En-2 staining was seen in 21% (3/14) (see Fig. 3). These same 14 recombinants were stained for the presence of N-CAM, which was seen in 6 of the 14 (including the 3 that expressed En-2) (see Fig. 3). As expected, all En-2–positive recombinants were always positive for N-CAM. However, as the above results indicate, N-CAM–positive recombinants did not always express En-2. Immunohistochemistry with the N-CAM antibody was done under conditions where major quantitative differences in the expression of N-CAM were detectable. The intensity of N-CAM staining obtained with ecotderm wrapped around anterior notochord was quantitatively similar to the intensity we observed with ectoderm wrapped around posterior notochord [compare (F) and (G)].

the notochord, (ii) induced by the notochord at a distance (implying that the inducer is diffusible), or (iii) secondarily induced by the notoplate by means of a propagating (homeogenetic) induction (*22, 23*). The last possibility has been called into question recently, since evidence for homeogenetic induction in *Xenopus* has not been obtained (*6*). All three of the above possibilities could explain the areas of expression extending away from the notochord in our recombinants (Fig. 4). However, none of the three possibilities explains the rectangular shape of the stage 14 patch of expression of En-2 in the normal embryo (*9*). It is possible that a gradient within the neuroectoderm in the anterior-posterior direction is responsible for controlling the shape of the patch by modulating the response to the inducer. Thus, signals passing through the plane of the neurectoderm may not be essential for initiating *En-2* expression, but they might be important for fine-tuning the area of *En-2* expression in normal embryos. Alternatively, dorsal mesoderm may induce other region-specific genes in the neuroectoderm and local interactions between these gene products may sharpen and further elaborate anterior-posterior pattern in the neural plate, a situation similar to the establishment of anterior-posterior pattern in *Drosophila* (*24, 25*).

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Specification of Jaw Muscle Identity in Zebrafish: Correlation with *engrailed*-Homeoprotein Expression

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Molecules that determine the specific features of individual muscles in vertebrates are unknown. Antibody labeling studies described here revealed a molecular difference among muscles in the zebrafish head, in that two functionally related jaw muscles (the levator arcus palatini and the dilator operculi), and not other head muscles, expressed *engrailed*-homeodomain proteins. Expression began in mesoderm-derived muscle-precursor cells in the paraxial mesenchyme and continued during muscle morphogenesis and differentiation. Growth cones of trigeminal motoneurons that innervate these muscles associated with the precursors within a few hours of the time they began to express *engrailed*. It is proposed that the *engrailed* proteins in these cells establish muscle identity and neuromuscular target recognition.

EACH MUSCLE HAS A CHARACTERISTIC location, relationship to supporting structures, and association with selectively innervating motoneurons. These features are signatures of individual muscle "identities," but molecules that specify muscle identities are unknown (*1–3*). Homeodomain proteins and other DNA-binding proteins are likely candidates, since they specify cell fates and pattern formation in invertebrates, and accumulating evidence suggests that they have similar functions in vertebrates, particularly within the central nervous system (*4*). Here we report the expression of *engrailed*-type homeodomain proteins (Eng) in cells that develop to form two specific jaw muscles in the zebrafish.

We used the monoclonal antibody 4D9 that was originally produced against *invected*, one of the two Eng proteins in *Drosophila*, and that also recognizes Eng in diverse animals (*5–8*). Homeobox gene expression patterns are generally conserved among vertebrates. For example, homologs of the murine *engrailed*-type gene *En-2* (*9, 10*) are expressed as a stripe in the neural tube at the border of the midbrain and hindbrain of other tetrapods (*6*) and of zebrafish (*8*).

The time course of Eng expression during zebrafish jaw muscle development is shown in Fig. 1. The first cranial expression outside of the central nervous system was observed in a scattered group of about 15 mesenchymal cells, caudal to the eyes and lateral to the hindbrain, at 24 to 26 h (*11*). The number of labeled cells increased to approximately 30 to 40 on each side of the head at 28 h, at which stage the cells are only loosely associated (*12*). By 36 h the Eng-positive cells moved rostrally, aggregated, and subsequently compacted to form a single cluster located in the mandibular arch by 48 h. At 72 h (when many embryos have hatched), Eng was expressed in two jaw muscles that develop at the same location as the earlier labeled mesenchyme. These muscles are the levator arcus palatini (LAP) and the dilator operculi (DO). LAP and DO are closely apposed and cooperate functionally (*13*). Eng was expressed in the nuclei of every cell in these two muscles, and expression persisted for at least 3 weeks while the number of labeled nuclei increased to a few thousand. Other head and gill arch muscles expressed little or no Eng (*14*). Expression is transient; we could find no muscles in adult fish (at 5 months) that expressed Eng.

The head mesenchymal cells that express Eng might originate from either paraxial mesoderm or the neural crest: although some (possibly all) head muscles in the zebrafish and other vertebrates derive from mesoderm (*15, 16*), the earliest Eng expression is in neuroectoderm (*5*), from which the neural crest originates. Thus, the mesenchyme that expresses Eng might be derived from either or both sources (*17*). We used lineage tracing to clarify the origins of LAP and DO, and showed directly that the muscle precursor cells were already in the head paraxial mesenchyme at a stage before neural

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