The Chick Engrailed-2 Gene: Structure, Expression and a Marker for Neural Pattern

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ABSTRACT

The Chick Engrailed-2 Gene: Sequence, Expression and a Marker for Neural Pattern

Diana Karol Darnell

This dissertation describes the isolation and sequence of genomic and cDNA clones containing the conserved coding and 3' untranslated regions of the chick *Engrailed-2* gene. Messenger RNA and cDNA analysis show that there are at least two transcripts and two poly adenylation sites and that the gene is expressed in the head/brain between stages 10 (day 2) and 45 (day 18). Its limited range of expression made this gene a useful marker for the patterning of the rostral central nervous system.

Antibody 4D9 recognizes the chick *Engrailed-2* protein. By immunolabeling with 4D9 in embryos with surgically induced cranial notochord deficit, I demonstrated that underlying notochord is not required for the rostrocaudal limitations of *Engrailed-2* expression. Notochord is required in dorsoventral patterning to suppress *Engrailed-2* expression in the floor plate of the stage 10 mesencephalon.

Embryos were transected near the level of Hensen's node. A continuous connection is not required between the rostral and caudal halves of the embryo beyond stage 3b (early primitive streak) to specify positional information for the mesencephalon. Mesencephalic neuroectoderm developed equally well in the rostral or caudal half, or in the region divided by the incision. Separation of the mesencephalic ectoderm from future notochord at stage 3c (mid streak) demonstrated that no cellular interaction between these two rudiments is required beyond this stage to induce and pattern the mesencephalic neuroectoderm.

Hensen's node was explanted to an ectopic site to determine if this "organizer" could induce non-neural ectoderm to express *Engrailed-2*. The expression of *Engrailed-2* in induced and self-differentiating neuroectoderm was observed.

Finally, embryos were treated with 0-10μM all-*trans* retinoic acid to determine whether this putative morphogen effects development of the mesencephalon. Embryos treated for four hours at stage (3b-c) failed to develop during a subsequent 24 hours in culture without retinoic acid. Embryos at stage 3d (definitive streak) treated similarly failed to express *Engrailed-2* and appeared to lack their mesencephalon. At stage 4, embryos developed with only minor rostral head abnormalities and expressed *Engrailed-2* in the normal pattern.

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INTRODUCTION

Early Development

The formation of a recognizable body plan during early embryonic development is characterized by several distinct stages. In the beginning, the fertilized ovum divides to form a multicellular ball or disc. In the chick, this disc sits on the surface of the yolk and is initially one cell layer, the epiblast. Delamination generates a second layer, the hypoblast, which interacts with the epiblast to establish the dorsoventral axis of the embryo at this early stage. The first obvious structure in the chick blastoderm is called the primitive streak, a dorsoventral thickening extending rostrally from the caudal margin of the disc. Most evidence suggests that the hypoblast, polarized by gravitational forces, induces the epiblast to form the primitive streak. This delineates the rostrocaudal and (mediolateral) axis of the embryo (for review see Nieuwkoop et al., 1985). At the distal (rostral) end of the primitive streak a structure called Hensen's node forms and is intimately involved with the next series of cell movements and differentiation called gastrulation.

During gastrulation, three germ layers are formed. Cells from the epiblast ingress through the primitive streak and migrate under the epiblast to form endoderm and mesoderm. The cells remaining in the epiblast layer become the ectoderm. A similar pattern of cell involution occurs in the amphibian embryo, in which a sheet of cells (endoderm and mesoderm) involutes from the surface of the blastula, through the region of the organizer at the dorsal blastopore lip and into the embryonic cavity, coming to lie underneath the ectoderm. The cytodifferentiation potential of each germ layer becomes restricted, and the axial pattern of the embryo is established.

During neurulation, the ectoderm overlying the dorsal mesoderm forms neuroectoderm (i.e., it undergoes commitment leading to differentiation), and also becomes regionally subdivided into forebrain, midbrain, hindbrain and spinal cord (segmentation and pattern formation). The interactions that are required for this rostrocaudal regionalization are unknown, although experimental evidence points to an influence from

the underlying mesoderm. In addition, the neural plate and neural tube are also regionalized with mediolateral and dorsoventral polarity, respectively. The mechanisms involved in this patterning are also unknown, however, several recent experiments, indicate that notochord plays a significant role (Tessier-Lavigne et al., 1988; van Straaten et al., 1988; Jessell et al., 1989; Smith and Schoenwolf, 1989; Wagner et al., 1990; Clarke et al., 1991; Hatta et al., 1991; Hirano et al., 1991; Yamada et al., 1991). The role of the notochord in the rostrocaudal and dorsoventral patterning of the neural tube is addressed in chapter 3.

The development of the neural axis has historically been the focus of much of experimental embryology. The early experiments that expanded our understanding of the basic mechanisms of development, especially for the formation of the neural axis are reviewed below.

The "Organizer"

During the end of the 19th century and beginning of the 20th, the field of experimental embryology was born. The first experiments were designed to determine whether the differentiation of any cell was due to the regulation of a complete set of nuclear instructions (epigenetic development) or whether, as cells divided, the information they contained was reduced and therefore their choices limited (genetic development). Experiments by Driesch, Endres, and Herlitzka in the late 1800s, in which two or four blastomeres of early sea urchin and salamander embryos were separated, indicated that the information in every cell was complete. Other experiments by Roux, however, in which one cell of the two-cell stage amphibian embryo was killed with a hot needle indicated that the remaining blastomere could form only half an embryo. This result was later shown to be due to an influence of the dead blastomere on the remaining living cell. These were the first experiments to indicate that each cell contains a complete set of developmental information and that the regulation of a cell's development is intimately linked to signals

from its neighbors. This early groundwork has lead the way to over 100 years of inquiry into the nature of that regulation (reviewed by Hamburger, 1988).

In 1901, Hans Spemann constricted the eggs of the common European salamander, *Triturus taeniatus*, with a baby hair to ascertain how amphibian embryo halves were capable of regulative development. By constricting embryos in different planes and observing the orientation of the early salamander gastrula, Spemann was able to deduce that the blastopore (the primitive streak equivalent) was required for the development of the dorsal structures and provided them with an axis of orientation. During gastrulation, the involution of the mesoderm through the blastopore was documented, as was the fact that the first involuting mesoderm would underlie the head ectoderm, and the later mesoderm the trunk and tail. These observations lead to the experimental investigation of the regulation of differentiation and patterning and the dependence of the development of different tissues, especially the neural axis, on cell-cell interactions with the mesoderm.

Transplant experiments were undertaken in which the inductive and self-differentiating capacity of different tissues could be ascertained. In 1916, Hilde Mangold, (née Proescholdt) did the first experiment in which the dorsal lip of the blastopore was transplanted to the flank of a host gastrula and induced the formation of an entirely new axis at the ectopic site. Spemann introduced the term "organizer" at that time to describe a structure that "creates an 'organization field' of a certain (axial) orientation and extent, in the indifferent material in which it is normally located or to which it is transplanted." (Spemann, 1921) as reviewed by (Hamburger, 1988).

In the definitive "organizer" experiment, a piece of the upper lip of the blastopore was transplanted from an unpigmented newt egg into the flank of a pigmented newt egg resulting in a secondary embryo initiated by the transplant and composed of both host and graft organs. The presence of pigmentation served as a marker to ascertain which tissues were induces and which were differentiated. The neural tubes of such secondary embryos formed exclusively or almost exclusively from host tissue, whereas the notochord was

(almost always) entirely from graft cells. These results lead Spemann to the interpretation that the organizer was self-differentiating with respect to invagination and mesoderm differentiation (especially the notochord), that the neuroectoderm is induced and that mesoderm and ectoderm interact to regulate the size and distribution of various tissues. Induction, self-differentiation and regulation are all important in the formation of an embryo, ectopic or natural. An understanding of the interactions of these forces in the generation of the normal embryo is key to unlocking the secrets of differentiation, segmentation and pattern formation.

Models for Development: differentiation, segmentation, patterning

Many models of early development have been proposed, and most contain some mechanism to explain the three obvious components of the evolving body plan: differentiation, segmentation and patterning. Models may use different subdivisions and terminology for these mechanisms, such as morphogenesis and regionalization, but some combination of the mechanisms is important for the formation of individual tissues and regions, and all three are required for the establishment of the neural axis.

Differentiation is characterized by the accumulation of abundant or unusual proteins within a cell. In order to give rise to cellular and therefore tissue differentiation (eg. neural, muscle, cartilage, adipose), the activation of master regulatory genes have been proposed via lineage prespecification and asymmetrical division, sequential inductive signals (planar and vertical) or other mechanisms (for review see Gurdon, 1992). Within a tissue rudiment, these master regulatory genes in turn presumably control the activation and repression of a cascade of genes for both regulatory and structural proteins specific for that particular cell type (for example see Lassar et al., 1986; Davis et al., 1987; Pinney et al., 1988). The expression of these genes in a coordinated fashion results in appropriate *cytod*ifferentiation. Primary inductive signals from mesoderm appear to be sufficient for

the cytodifferentiation of neuroectoderm, however, other mechanisms including planar induction have also been demonstrated. The specific signals involved in neuro-cytodifferentiation have been elusive.

In addition to having multiple differentiated tissue types, many animals are segmented or otherwise require some mechanism (e.g., a wave prepattern) to generate repetitive structures (e.g., parasegments, somites, digits, teeth; for example see MacWilliams, 1978). Although the hindbrain and spinal cord seem to be segmented as evidenced by their rhombomeres and spinal nerves, respectively, the segmentation of the rostral neuroectoderm is still a matter of controversy.

In addition to cytodifferentiation and segmentation, developmental models have had to account for positional differences along the rostrocaudal, mediolateral and dorsoventral axes. To this end, morphogenetic fields and gradients of developmental morphogens have been invoked. Particular threshold concentrations of such morphogens might establish "coordinates" of positional identity to which other regulatory genes or gene families could respond. These genes would then be activated and in turn regulate the activation and repression of genes that would regionally specify or pattern tissues (for review see Wolpert, 1989).

In the general model then, some mechanism is invoked to identify tissue type, another to establish the metameric nature of the tissue and a third to give a particular region a positional identity. Significant effort has been spent during the past 100 years trying to characterize the signals and cell interactions responsible for these three developmental components, especially for the developing neural axis. This dissertation represents a continuation of these efforts; specifically, the elucidation of the tissue interactions and signals responsible for the patterning of the cerebellar rudiment, a component of the rostral neural tube in the chick.

NEURAL DIFFERENTIATION

The question of whether the signal for neural induction (primary induction) came from the underlying endomesoderm or from a (planar) signal from organizer within the grafts has been a continuous point of controversy (reviewed by Hamburger, 1988). A student of Spemann, Alfred Marx, transplanted only the involuted endomesoderm to a position under the flank ectoderm and a perfect secondary axis was induced, indicating that the neural plate could be induced entirely by the subjacent endomesoderm. J. Holtfreter supported this conclusion with exogastrula experiments in which early gastrula developed with the endomesoderm evaginated from the ectoderm and the hollow ectodermal sphere failed to differentiate into a neural plate or other neural structure. More recent experiments involving "Keller sandwiches," however, have indicated that planar signals may also be capable of induction and, therefore, that vertical inductive signals do not act alone to induce and pattern the neuroectoderm (Kintner and Dodd, 1991; Doniach et al., 1992; Keller et al., 1992). The notion that there are redundant signals involved in neurulation was introduced by Spemann.

Double Assurance

In some species (e.g., *Rana esculenta*) the mesoderm has the ability to induce neuroectoderm, but the ectoderm also has the capacity to self differentiate. Spemann called this redundancy of task "double assurance" or the "synergistic principle" (Spemann, 1931; Spemann, 1938). He believed that the capacity for self-differentiation by the ectoderm was due to a planar signal passed from the cells of the blastoporal lip directly into the ectoderm before invagination of the mesoderm. Whether this duplication of induction was completely redundant, or whether the two signals were complimentary or sequential, was not completely obvious. In fact, several cases of consecutive events are now known to be required for induction of structures including the otocyst and lens (for review see Hamburger, 1988; Jessell and Melton, 1992).

There remained a question, however, about whether there is a two-step process for neural induction, the first between the organizer and the ectoderm before involution of the chordamesoderm, and the second a vertical induction between the chordamesoderm and ectoderm after involution. Yamada, in sandwich experiments using guinea pig bone-marrow (a mesoderm inducing agent), induced well organized notochord and adjacent rows of somites within the gastrula animal cap. These mesodermal structures, however, failed to secondarily induce neuroectoderm in the sandwiches (Yamada, 1959), perhaps because the "organizer" component of the induction was lacking. Experiments on amphibian gastrula have demonstrated that dorsal ectoderm is predisposed or conditioned to become neuroectoderm and to show a synergistic relationship between planar signals from the dorsal mesoderm prior to involution and vertical signals after (Sharpe, 1987; Dixon and Kintner, 1989).

Hensen's Node, the Organizer in Birds

Hensen's node, named for the scientist who first described it (Hensen, 1876), is the area lying immediately around the primitive pit at the rostral end of the primitive streak in mammals and birds. The primitive streak was first identified as a location of mesoderm ingression and, therefore, considered as a possible homologue of the organizer in amphibia. The rostral streak, including the node, could differentiate into head and trunk structures, however, nodeless streak could form only gut and cartilage. Proof that Hensen's node was an organizer was obtained by explanting the node to an ectopic site where it could induce flank ectoderm to form a neural plate. These experiments were carried out using chick and duck embryos by Waddington and co-workers (Waddington, 1930; Waddington, 1932; Waddington and Schmidt, 1933). Although a histological distinction could not be made between the tissues, the induction could nevertheless be concluded on morphological grounds (cell size). That the mesoderm from the graft was responsible for the induction was definitively shown considerably later by Hara (Hara,

1961). Thus, in chick as in amphibians, Hensen's node/dorsal lip is the site of ingression/involution of endoderm, prechordal plate mesoderm and notochord. The notochord cells remain in the regressing node, or dorsal lip in amphibia, throughout the duration of gastrulation, with earlier node/lip giving rise to endoderm and rostral notochord and later node/lip to caudal notochord. Both node and lip can induce a secondary axis when transplanted into an ectopic site (for reviews see Hara, 1978; Hamburger, 1988). In fact, Hensen's node transplanted into *Xenopus* ectodermal caps induces neural tissue in the amphibian ectoderm, indicating that the signals for induction are also maintained across evolution (Kintner and Dodd, 1991).

For gastrulation and neurulation, Hensen's node and the organizer seem to be responsible for the differentiation of the neuroectoderm through two pathways: directly through planar signals from the node/organizer to the ectoderm and indirectly as the source and location for involuting inductive mesoderm. Primary induction should result in the formation of cytodifferentiated neuroectoderm. To date, however, this step in neural development is inextricably linked to the other two steps: segmentation and regionalization. The role of the organizer in these has not been clarified.

SEGMENTATION

Segmentation is clearly evident in both embryonic and adult insects and vertebrates. In *Drosophila*, the metameric nature of the body plan becomes obvious as parasegment boundaries constrict along the length of the embryo body. These later divide again, giving rise to the three thoracic and eight abdominal segments of the adult (for review see Ingham and Martinez Arias, 1992). In vertebrates, an obvious embryonic segmentation occurs in the subdivision of the segmental plate into the somites, which give rise to the vertebrae and segmented axial musculature of the adult. In addition, specific nerve roots grow out of the neural tube at each vertebral level, indicating that the spinal cord is also segmented. Neuromeres, regular compartments within the hindbrain, indicate that this level of the

neural tube is also segmented. Thus, in insects, segmentation occurs before the first round of differentiation and in vertebrates it appears to occur after, but for both it is an integral part of development of the body plan.

Segmentation of the vertebrate head has been controversial since the early days of comparative anatomy. In embryonic vertebrates, the branchial arches, cranial nerves, chondrocranial cartilages, somitomeres, neuromeres and rhombomeres all seem to be segmental components of the head. The anterior parts of the head that do not appear segmented might be divergent specializations of the anterior most segments. This model is conceptually similar to that proposed for the origin of the arthropod head (Raff and Kaufman, 1983). An alternative argument holds that the anterior portion of the head (face and forebrain) is not an extension of some segment, but rather a *de novo* structure of vertebrate evolution (Northcutt and Gans, 1983).

The molecular mechanisms behind segmentation and patterning of the *Drosophila* embryo appear to be repeated in the formation of the vertebrate CNS. Segmentation in *Drosophila* is established by the coordinated expression of a group of genes, many of which are transcription factors that contain a conserved region called the homeobox. Homologues to these genes have been identified in vertebrates, although their function remains to be determined. Pattern, or positional identity in *Drosophila* is established at segmental intervals, apparently by region-specific, master regulatory genes that in many cases contain a homeobox. Expression of homologous homeobox-containing genes has been identified in a sequential pattern within the neuroectoderm of vertebrates (see below). At least one homeobox-containing gene in *Drosophila* expresses a protein that provides both segmentation and patterning functions; *engrailed* (Poole et al., 1985). The *Drosophila engrailed* gene product is required for the maintenance of a normal segment boundary caudal to the expressing region and for the posterior compartment of each segment to develop the appropriate phenotype (Garcia-Bellido and Santamaria, 1972; Morata and Lawrence, 1975; Kornberg, 1981a,b; Lawrence and Struhl, 1982). The sequencing and

characterization of the expression pattern of one *Drosophila engrailed* homologue in chick, *Engrailed-2* (*En-2*) is the early focus of this dissertation.

PATTERN

Like the *Drosophila* embryo, the vertebrate neuroectoderm becomes both segmented and patterned. For early experimental embryologists like Hans Spemann, who had inferred that mesoderm induced neuroectoderm differentiation, a second question presented itself: did different parts of the mesoderm induce different parts of the neuroectoderm. The confounding question was whether rostral ectoderm has a propensity to form head ectoderm and trunk ectoderm to form spinal cord. By using early or late blastula dorsal lip transplanted into head-level or trunk-level flank, Spemann determined that the mesoderm did indeed have positional information which could induce a positional address in the neuroectoderm, but that the level of the ectoderm also influenced the response. Spemann coined the terms "head organizer" and "trunk organizer" to describe the region specific potency of the mesoderm. A similar experiment by Otto Mangold in which rostrocaudal quarters of the involuted mesoderm were transplanted by the "einsteck" method (implantation of graft fragments into the blastocoels of the host) induced appropriate rostrocaudal ectodermal structures, confirming the regional induction concept. However, because there seemed to be some overlap in the patterning potential of the chordamesoderm, he could not determine whether the regional induction was just of some general regional field or was of specific regional character.

At Cambridge, Needham and his co-workers suggested that the induction and patterning of the neural axis involved two processes. First, the determination that an embryonic axis shall be developed and second, the determination of the character of the axis (Needham et al., 1934). They called these two events "evokation" and "individuation." This dual nature of induction and patterning was reiterated by P.D. Nieuwkoop and co-workers, who called the two processes "activation" and

"transformation" (Nieuwkoop, 1952). Several other models have also been developed to explain induction and patterning of the neuroectoderm (reviewed by Hamburger, 1988).

Scientists continue to attempt to elucidate the mechanisms of action or capacities of tissues to regionalize and be regionalized. Recent experiments have taken advantage of a LiCl treatment of *Xenopus* embryos. Treatment at the 64-cell stage results in embryos that fail to gastrulate and, therefore, have an animal cap which is inducible, but naive. Co-culture of this ectoderm with various mesoderm isolates has been used to reveal inducing ability as well as regionalizing potential. Co-culture with anterior notochord results in the induction of anterior neural plate with high frequency, whereas co-culture with paraxial mesoderm or posterior notochord did so less frequently (Hemmati-Brivanlou et al., 1990). In previous *in vitro*, sandwich type experiments, heterogeneous inducing agents (HeLa cells, perch kidney, viper liver, etc.) have experimentally induced forebrain, midbrain or spinocaudal regional neuroectoderm. Some growth factors have also been implicated in neural pattern formation (Jessell and Melton, 1992). However, to my knowledge, there is still no experiment in which differentiated neural tissue has been induced and then separately regionalized. Such a separation will be required to determine which, if any, of the current developmental models are correct. In the mean-time, experiments such as these, in which the inducer or induced tissue can be isolated from some influential interactions, will help with identification of what is required or sufficient for the generation of pattern within the neural tube.

Morphogens

One central concept in the formation of pattern is that of morphogen gradients and positional information. In order for cells or tissues in a given region to alter their cell type, state, growth or motility based on their location, some information about that location must be available to the cells. One possible source of such positional information could be a chemical gradient. The chemical that could impart positional information has been called a

morphogen (for review see Wolpert, 1989). Whereas an inducer can alter cell fate in one way, a morphogen must be able to alter cell fate in two or more ways, depending on concentration (Slack, 1991).

A system that has lent itself to the investigation of segmentation and positional information or patterning is the fly embryo, *Drosophila melanogaster* (reviewed by St Johnson and Nüsslein-Volhard, 1992) Through observations and descriptions of *Drosophila* normal and mutant development, advances have unraveled some of the molecular signals involved in these two steps. Genes that generate the segmented body plan, and genes that create and respond to morphogen gradients, have been identified. A common, although not ubiquitous, component of these genes is a 180 nucleotide conserved region called the homeobox (see below).

The best example of a putative morphogen that specifies positional signalling during development is probably the product of the Drosophila gene, *bicoid* (Driever and Nüsslein-Volhard, 1988). The *bicoid* gene is expressed in the rostral pole of the *Drosophila* embryo and generates a gradient of a transcription factor that appears to establish positional information or coordinates along the rostrocaudal body axis. A 10% concentration change in the *bicoid* protein alters the position of the *Drosophila* head/thorax boundary by 15% (Driever and Nüsslein-Volhard, 1988). Presumably, a certain threshold of the *bicoid* protein designates a boundary between head and thorax, head-specific genes being expressed at values higher than this threshold and thorax-specific genes at values lower. In response to this gradient, segmentation and possibly other patterning genes are expressed (e.g., hunchback; for review see St Johnson and Nüsslein-Volhard, 1992). Another possible morphogen has been identified in vertebrate development: all-*trans* retinoic acid (RA).

Retinoic Acid

The hypothesis that any chemical or other signal acts as a morphogen in any region of vertebrate development requires a combination of several lines of evidence. First, it must be shown that too much or too little of the proposed morphogen causes developmental defects, and second, that it can be found normally in those same areas of the developing embryo, preferably in a gradient. Both of these criteria have been met for the proposed morphogen RA (Kalter and Warkany, 1959; Morriss, 1972; Shenefelt, 1972; Rosa, 1983; Lammer et al., 1985). Additional support for the hypothesis depends on the demonstration that RA occurs in the presence of binding proteins and receptors, ensuring that there is some mechanism by which it can influence development (Dollé, 1989; Maden, 1989; Dencker, 1990; Maden, 1991; Ruberte, 1991). For these reasons, RA has been implicated as a morphogen in the development and regeneration of the limb, as well as in the development of the central nervous system (Eichele et al., 1985; Maden et al., 1989; Smith et al., 1989). For example, a three-fold difference in concentration is sufficient to specify digit 2 from digit 4 during chick limb differentiation (Tickle et al., 1985) and an eight-fold difference is sufficient to specify wrist vs. whole arm during amphibian limb regeneration (Maden et al., 1985).

RA is also known to influence nervous system development at certain stages in fish, frog, chick, mouse and human. RA treated embryos have an increased probability of developing neural tube defects, including the apparent loss of rostral structures or the cerebellum. During early development in *Xenopus*, RA excess causes an apparent shift whereby anterior structures in the embryo are lost, and posterior structures are concomitantly enlarged (Durston et al., 1989; Sive et al., 1990), perhaps by arresting the extension of dorsal mesoderm during gastrulation (Ruiz i Altaba and Melton, 1989). Whether this transformation is mediated entirely through the mesoderm via a loss of "head organizer" or also via the ectoderm in its capacity to respond has been analyzed by Sive and Cheng (1991) who demonstrate that both ectoderm and mesoderm are effected by RA

treatment. This is demonstrated in the change in the pattern of expression of homeobox-containing genes expressed in these tissues.

In mouse, RA apparently suppresses the development of the rostral hindbrain when administered during neurulation but before first somite formation (Morriss-Kay et al., 1991). This is consistent with observations in human infants exposed to RA excess *in utero* who have cerebellar abnormalities including cerebellar hypoplasia and aplasia (Lammer et al., 1985). Addition of exogenous RA to zebrafish embryos interfered with the development of the CNS by specifically inhibiting development of the caudal midbrain and rostral hindbrain and eliminating the expression of *En-2* in this region (Holder and Hill, 1991). Differences between the RA effects on amphibians, fish and mammals may be the result of differences in RA receptor and binding protein distribution. Cellular retinoic acid binding protein (CRABP) expression in mouse mesencephalon and rostral metencephalon closely mirrors *En-2* expression with expression higher caudally than rostrally and lacking entirely in the floor plate (Ruberte et al., 1991). To determine whether RA treatment would effect *En-2* expression in chick, early chick embryos were treated with three concentrations of RA and screened for immunolabeling with MAb-4D9, an antibody against the *Drosophila invected protein* that recognizes the En-2 protein in chick (Chapter 4).

In addition to sensitivity to disruption of normal pattern in the presence of RA, there is other evidence of similarity between mechanisms of neuroectoderm and limb patterning, including similar signaling molecules and regionally specific homeobox-gene expression. RA is known to be able to influence the transcription of homeobox genes (Gudas, 1991; La Rosa and Gudas, 1988; Mavilio et al., 1988; Simeone et al., 1990; Sive and Cheng, 1991), likely through DNA binding of the RA activated, nuclear RA receptors (Yang et al., 1991). The cellular RA binding protein (CRABP) appears in a limited region within the central nervous system and in the anterior margin in the limb. These patterns of expression implicate RA in Hox 2.9, Ghox 2.1 and potentially other homeobox gene regulation within these structures (Maden et al., 1991; Wedden et al., 1989). Thus, homeobox genes,

retinoid binding proteins and retinoids may be intimately involved in the patterning and development of both limbs and CNS.

Homeobox Genes

The expression of homeobox-containing genes results in the accumulation of homeodomain-containing proteins. These have been implicated in the regulatory cascade of positional information for both *Drosophila* and vertebrates because they are putative transcription factors; they are found in restricted regions of the embryo or neural tube, respectively; and their over or under expression often results in alterations in segmentation or pattern. For example, the *engrailed* gene in *Drosophila* (Poole et al., 1985) is expressed in the posterior region of each segment and loss of *engrailed* expression results in a change in phenotype of the posterior region into an anterior identity. Because a juxtaposition of a rostral posterior identity with a caudal anterior identity is required for appropriate segment boundary formation, loss of *engrailed* expression also results in segmentation defects (Garcia-Bellido and Santamaria, 1972; Morata and Lawrence, 1975; Kornberg, 1981a,b; Lawrence and Struhl, 1982). Thus, *engrailed* appears to be a cascade-regulatory gene for both segmentation and positional information (posterior compartment identity for each segment) in *Drosophila.* A model of part of the cascade involving *engrailed* has been proposed (DiNardo and O'Farrell, 1987).

In 1984, at the time this dissertation research was begun, the only information about homeobox-containing genes in vertebrates came from a few Southern blots of vertebrate embryo DNA that cross-hybridized with *Drosophila* homeobox probes. Through cloning and hybridization experiments such as those in Chapters 1 and 2 of this dissertation, the homology and expression patterns of many homeobox-containing genes have been elucidated in vertebrates. The resulting cumulative data show that many homeobox-containing gene homologues are expressed in the vertebrate central nervous system (for review see McGinnis and Krumlauf, 1992). In the vertebrate hindbrain and

spinal cord, homeodomain proteins are restricted at their rostral margin to a specific segment boundary along the rostrocaudal neural axis but are often unrestricted caudally. Some genes are also expressed in the underlying mesoderm in an initially identical pattern. Although many of the genes are expressed caudally for some distance from the rostral margins and, therefore, overlap with other genes, because of their differing rostral boundaries, the sum of genes expressed at any level may still be unique to that level and satisfy the requirement of an address for that position. As transcription factors, each unique combination of genes could then act to coordinate the cascade of structural and regulatory genes appropriate for the development of that neural level.

This restricted localization also makes homeodomain-containing proteins good markers for regional differentiation. A large number of homeobox/domain probes are now available for use in assaying the patterning of the ectoderm and mesoderm during neurulation in the chick. The cloning and mapping in chick of a *Drosophila engrailed* homologue, *En-2*, with the intention of generating a probe for development regionalization (or segmentation) was the first task undertaken for this dissertation research (Chapter 1). Using RNA blots of various regions and ages of the developing chick, I ascertained that *En-2* was expressed in the brain or head at or before day 2 (approximately stage 10) of development and continued its expression in the midbrain through day 18 (approximately stage 44) and beyond (Chapter 2). Collaborators, using subclones derived from my cDNA and genomic clones, elaborated on the localization of chick *En-2* expression, demonstrating that its neural expression was localized to the midbrain and rostral hindbrain (Gardner et al., 1988; Martinez and Alvarado-Mallart, 1990). While this work was underway, a monoclonal antibody was developed against the protein from the *Drosophila invected* gene, which is a member of the engrailed-gene family. This monoclonal, 4D9, cross-hybridized with the chick *En-2* protein (Patel et al., 1989) and was thereafter used as a probe for the *En-2* expressing region (Gardner et al., 1988; Chapters 3 and 4).

Neural Regionalization

Just as Spemann proposed that ectoderm might both self differentiate and be induced by mesoderm to form neuroectoderm (differentiation "double assurance"), it is possible that both the mesoderm and neuroectoderm respond to positional information signals and positional induction and self-differentiation doubly assure that neural regionalization will occur. Several studies in *Xenopus* have indicated that notochord is responsible for both neural induction and neural patterning. Experiments using isolated, naive, animal cap ectoderm cultured with regional, isolated notochord have demonstrated regional character within the induced neuroectoderm (Hemmati-Brivanlou et al., 1990; Sharpe and Gurdon, 1990). I sought to determine if notochord was required for the establishment of regional character during chick neural differentiation as assessed by *En-2* expression (Chapter 3).

Other questions pertaining to the positional specification within the neural tube were also considered (Chapter 4). I sought to determine if there was a requirement for a continuous signal or association between rostral and caudal parts of the embryo responsible for establishing axial, rostrocaudal positional information. To accomplish this, I transected embryos transversely (perpendicular to the rostrocaudal axis) at various stages and rostrocaudal levels and looked for loss or maintenance of position specific development within the separated halves. Also, I attempted to surgically separate the future neural ectoderm from the organizer prior to chordamesoderm invagination to assess whether such a separation would interfere with the patterning of the future neuroectoderm.

Next, I addressed the question of competence for patterning at the level of gene expression by asking whether neural ectoderm is predisposed to neural regionalization. That is, whether the mesodermal induction is instructive or permissive with respect to the patterning regulatory cascade within the ectoderm. Does non-neural ectoderm have the capacity to respond with position specific gene expression to positional signals from the organizer or chordamesoderm? Regional differentiation in non-neural ectoderm, which

was described by Spemann as resulting from "head organizer" and "trunk organizer" induction in amphibians, has been observed in chick (Gallera, 1970; Ning et al., 1965; Tsung et al., 1965; Vakaet, 1965; Waddington and Schmidt, 1933). These data are suspect, however, due to the lack of a definitive graft or host marker. Caudal neural structures identified in the absence of rostral neural structures may have been self-differentiated graft neuroectoderm. One recent experiment in which a cellular marker was used to distinguish graft from host neuroepithelium has shown by morphological criteria that induced neuroepithelium has rostral or rostral and caudal regional character (Dias and Schoenwolf, 1990). To further define the regional character acquired in ectopic embryos, molecular neural markers needed to be used. I transplanted Hensen's node to a site underneath non-neural ectoderm in the chick germinal crescent, induced an ectopic neural tube, and assayed for region-specific expression of *En-2* (Chapter 4). Similar experiments have recently been published by Storey and her co-workers (Storey et al., 1992).

Conclusion

A more detailed version of the model being considered above is as follows: the endomesoderm, via planar or vertical signals, induces ectoderm to differentiate into neuroectoderm by instructing the ectoderm to express neural specific master regulatory genes (still unidentified). Concurrently, some type of wave prepattern regularly subdivides the neural axis and neighboring paraxial mesoderm into segments. In addition, patterning of either the mesoderm or the neuroectoderm by a morphogen gradient (such as RA or other signal) results in the acquisition of positional information by the neural tube. In response to this positional information, region specific regulatory genes, including homeobox genes, are activated within the neuroectoderm to turn on a cascade of genes, both regulatory and structural, through which the new identity of the region can be evoked. The characterization of one homeobox gene, *En-2*, and the investigation of its regulation of expression as a marker of regionalization are the basis for this dissertation.

Chapter 1

CLONING AND SEQUENCE OF THE *ENGRAILED-2* GENE FROM THE EARLY CHICK

INTRODUCTION

Advances in *Drosophila* developmental genetics in the early 1980s have led to the cloning of genes controlling morphogenesis in flies (for review see St Johnson and Nüsslein-Volhard, 1992). Many of these genes share a conserved region encoding 60 amino acids known as the "homeobox." Within homeobox-containing genes, the *engrailed* and *invected* genes share a distinctive homology both within and outside the homeobox (Coleman et al., 1987; Poole et al., 1985). Since the *engrailed* gene in flies is involved in the control of segmentation and positional identity during early development, I sought to determine if a vertebrate equivalent of *engrailed* existed and functioned similarly in chick development. Preliminary data from our lab indicated that the *Drosophila engrailed* probe cross-hybridized with a band on a chick genomic Southern blot. I subsequently cloned and sequenced both genomic and cDNA clones containing the conserved coding regions and 3' untranslated end of an *engrailed*-like chicken gene. The coding region of the gene contains a single 1 kilobase (kb) intron, an *engrailed*-like homeobox and extra-homeobox conservation unique to the *engrailed* class of genes. Sequence homology in this region was most similar to the mouse *En-2* gene (Joyner and Martin, 1987). The 3'-untranslated region was also sequenced and two poly $(A)^+$ addition sites were identified. The 3'-untranslated region contains some homology with the *Drosophila invected* and *Xenopus engrailed* 3'- untranslated region.

MATERIALS AND METHODS

Genomic DNA

The Amplified Axel, Charon 4A genomic library was grown in K802 or LE392 bacteria and screened using standard protocols (Maniatis et al., 1982). In short, phage were used to infect bacteria, that were then plated out so that 1-2mm phage plaques (regions of bacterial lysis) reached nearly confluent density. DNA from the lysed bacteria was lifted onto nitrocellulose filters and screened with a nick-translated 2.1kb or 0.7kb Drosophila *engrailed* cDNA probe (cDNA-2d provided by Tom Kornberg). Phage plaques that hybridized were used to reinfect bacteria and these were re-plated until all plaques on a plate hybridized (plaque purity). Phage DNA was isolated from a plaque descendant of each original hybridizing plaque. Phage DNA was restriction endonuclease mapped by agarose gel electrophoresis. Selected DNA fragments were subcloned into the SP64 vector. Southern blots of restriction fragments from this chick genomic clone were probed with a *Drosophila* 237 base pair (bp) homeobox probe under high stringency conditions. Subclones for sequencing were made from fragments isolated from restriction endonuclease digestions or from ExoIII/mungbean exonuclease digestion.

The 5' end of the homeobox-containing exon was mapped by S1 analysis using a kinase labeled EcoRI-BglII probe annealed to day 4 embryonic chick brain poly $(A)^+$ RNA.

cDNA Library

RNA was isolated from day 6 chick brain using the LiCl-Urea protocol (Auffray and Rougeon, 1980). Poly $(A)^+$ RNA was further isolated using oligo dT (Collaborative Research). Poly $(A)^+$ RNA was reverse transcribed, ligated to an EcoRI linker and cloned in to the lambda phage arms provided in the cDNA library kit (BRL). Bacteria were infected with phage and were plated so that plaques reached nearly confluent density. Phage DNA was lifted onto GeneScreen filters and screened with the ChickEn gCE-RX

genomic probe. cDNA inserts from phage plaques that hybridized to ChickEn were subcloned into the EcoRI site in the Bluescript KS plasmid and the EcoRI linkers added in the original library cloning. Subclones for sequencing were made from fragments isolated from restriction endonuclease digestions or from ExoIII/mungbean exonuclease digestion.

PCR RACE

The downstream poly(A)$^+$ site was identified in a cDNA clone generated by the polymerase chain reaction rapid amplification of cDNA ends (PCR RACE) techniques and primers developed by Mike Frohman and co-workers (Frohman et al., 1988; Frohman and Martin, 1989). The internal primer, CEP2 (5'GAC TTT TGC GGA CAG ATG TG3'), bound 5' to a NarI restriction site within the c6 cDNA clone and the RACE primers contained restriction sites XhoI, SalI and ClaI for cloning. A NarI-SalI restriction fragment was cloned from PCR generated cDNA into a Bluescript KS vector.

Sequencing

Both cDNA and genomic clones were sequenced by the double stranded method of dideoxy chain termination or using the Sequinase kit method. Both strands were sequenced for the coding region of the cDNA. For the intron and 3' untranslated region, some regions were only sequenced on one strand and are unverified and, therefore, may contain errors.

Southern Blot

Genomic DNA cut with PvuII was probed with a 106 base pair BglII-PstI fragment subcloned from the homeobox region of the chick *En-2* genomic clone. Southern hybridization was done on GeneScreen membrane using the standard dextran sulfate technique (Method III; NEN Research Products). This involved prehybridization for 6 hours at 42°C in 50% deionized formamide, 0.2% polyvinyl-pyrrolidone, 0.2% bovine

serum albumin, 0.2% Ficoll, 0.05M Tris-HCl (pH 7.5), 1.0M NaCl, 0.1% sodium pyrophosphate, 1.0% SDS, 10% Dextran sulfate and 100μg/ml denatured salmon sperm DNA. A nick translated P^{32} labeled probe was added for hybridization over night at 42°C and blots were washed the next day at moderate stringency and exposed to film.

RESULTS

Genomic Cloning and Mapping

The Amplified Axel, Charon 4A genomic library was screened twice using a nick-translated 2.1kb or 0.7kb *Drosophila engrailed* cDNA probe. Four hybridizing phage clones were selected and plaque purified. All four clones were found to be identical by restriction endonuclease mapping. The total insert length of chick genomic clone 1-2 was approximately 15 kb and it contained one internal EcoRI restriction site. Based on Southern hybridization using the 0.7kb *Drosophila engrailed* probe; a 12.8 kb, EcoRI restriction fragment from genomic clone 1-2 was selected and subcloned into the SP64 vector. High stringency hybridization with the *Drosophila* 237 bp homeobox probe to Southern blots of restriction fragments from this chick genomic clone indicated that this clone contained a homeobox. Fragments hybridizing to the *engrailed* homeobox were subcloned and sequenced, verifying that this clone contained an *engrailed*-like homeobox.

The gene containing this homeobox was named *ChickEn* (Darnell et al., 1986). A restriction map of the genomic clone that cross hybridizes with the *Drosophila engrailed* probe is shown in Figure 1.1. The cloned coding region and 3' untranslated region of *ChickEn* were determined to be contained in a 3.5 kb EcoRI-XhoI fragment which was subcloned and called gCE-RX. Hybridization of Southern blots of the restriction digested gCE-RX to a probe containing the Drosophila *engrailed* homeobox revealed that the region of homeobox homology lay approximately 1500 nucleotides from the 5' end of the genomic clone.

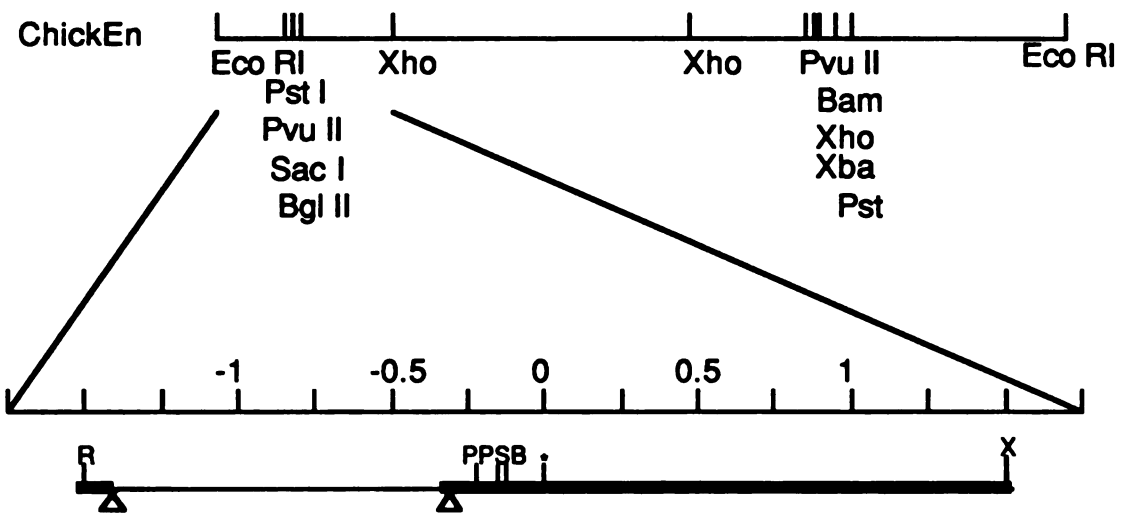


Figure 1.1 A restriction map of the genomic clone and the subclone gCE-RX that contains the homology with *Drosophila engrailed*. The * indicates the 3'-end of the coding region. The ruler is marked in kilobases. Restriction enzyme sites are identified by standard abbreviations. The thin line represents intron sequence and the thick line exon sequence within this genomic clone.

Sequence analysis of a PstI-BglII subclone of this region revealed that the orientation of the clone was 5' left to 3' right as mapped in Figure 1.1.

cDNA Cloning and Mapping

Northern blot analysis of various stages and regions of developing chick embryos revealed the strongest expression of the *ChickEn* gene in the early brain (see Chapter 2). For this reason, the gCE-RX clone was used to screen a cDNA library made from embryonic day 6 brain. Eight cDNA clones that hybridized with gCE-RX were identified and further characterized (Figure 1.2). All eight of the cDNA clones were sequenced. Two of these clones, c4 and c9, contained the homeobox and extended a significant distance in the 5' and 3' direction, respectively. The 5' ends of the genomic gCE-RX clone, and cDNA clones c4 and c9, were sequenced to determine the extent of their homology to known *engrailed*-like genes, and to compare them with one another. The 5' end of the genomic clone extends approximately 1.14 kb 5' of the beginning of the homeobox. Mapping and sequence for clone c9, when compared to the genomic clone, indicated that an intron existed within gCE-RX at a point 40 bp upstream from the beginning of the homeobox and extending 5' for 1kb (thin line; Figure 1.1 and 1.2). This was confirmed by sequence comparison. Within c9, an open reading frame extended for 723 bp from the 5' end of the clone to a TAG stop codon. At the 5' end of c4, a string of 94 Ts is attached to the otherwise normal cDNA. A similar 5' poly (T) sequence was identified by Kimmelman and Kirschner (Kimmelman and Kirschner, 1987) and identified as the poly $(A)^+$ tail of an opposite strand message. I was unable to detect any message coded off of the opposite strand of clone c4 using a single stranded riboprobe (data not shown) and, therefore, concluded that this poly (T) tail was probably an artifact of the cDNA library. Sequence within the cloned coding region was obtained by sequencing on both strands of either c4 or c9.

As shown in Figure 1.3, there is extensive sequence homology with other engrailed

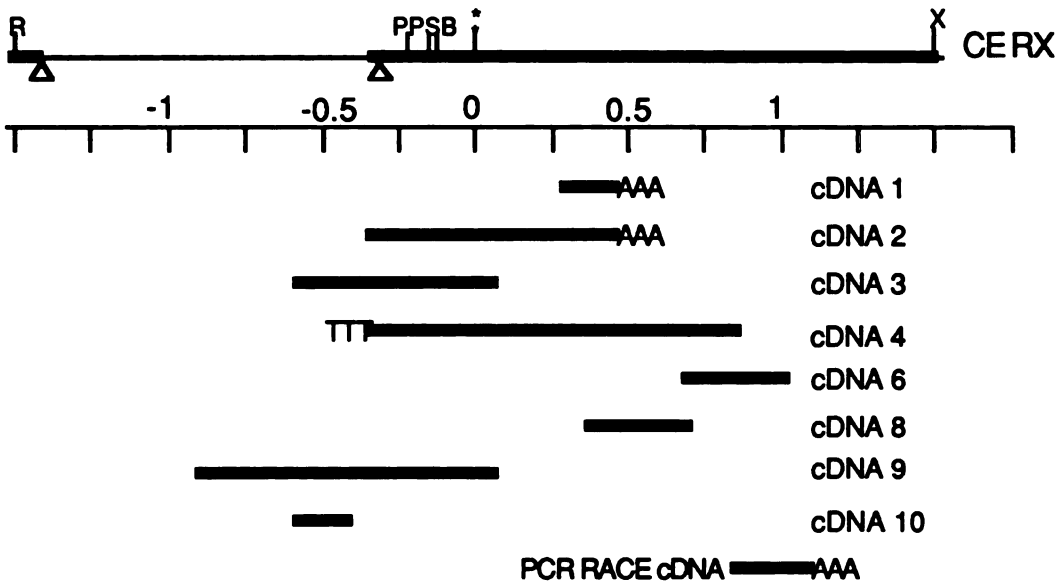


Figure 1.2 A map of nine cDNA clones. Top map shows the genomic clone CE RX with the intron indicated as a thin line. Restriction sites are identified by single letters: R=EcoRI (linker added for subcloning), P=PvuII, P=PstI, S=SacI, B=BglII (these four are found in the homeobox) and X=Xho (natural site used for subcloning). The * represents the 3' end of the coding sequence for translation and aligns with 0 on the ruler. The ruler is marked in kilobases. Below, clones 1-4, 6, 8-10 are cDNAs isolated from a library. Clones 1 and 2 contained a 3' poly $(A)^+$ tail at an identical location 504 nucleotides downstream of the translation stop codon. Clones 4, 6 and 8 contained sequence downstream to that poly adenylation site. The PCR RACE method was used to obtain the cDNA shown at the bottom of the figure. Sequence analysis of this clone located the downstream poly $(A)^+$ addition site 1061 bases 3' of the stop codon. Clone 4 had a string of 94 Ts located at the 5' end of the cDNA (artifact).

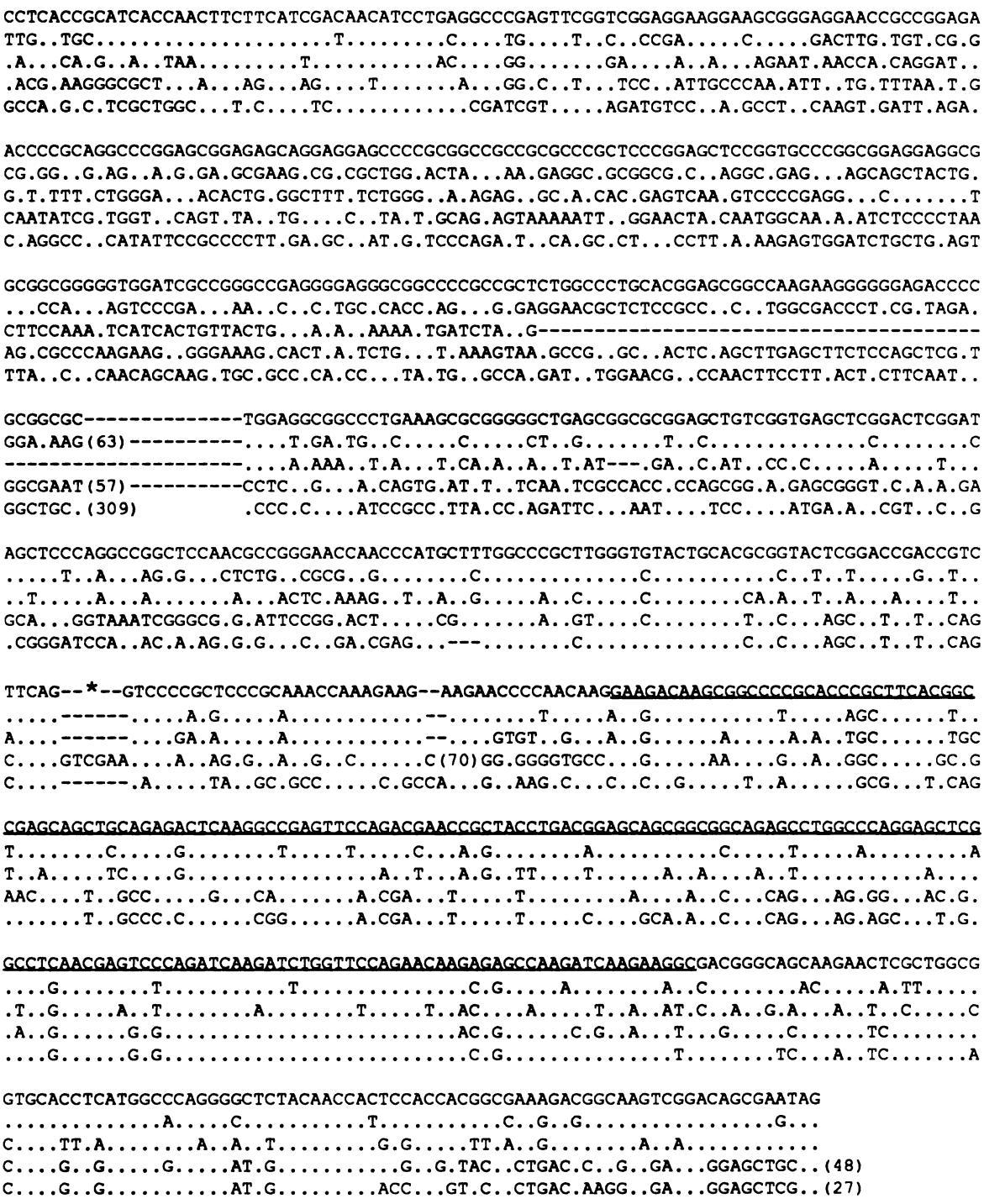


Figure 1.3 Aligned sequence showing homology within the coding region for related *engrailed*-like genes. The lines are ordered from the top down: chick *En-2*, mouse *En-2*, *Xenopus En-2*, *Drosophila invected* and *engrailed*. Homologous bases are represented by periods (.). Spaces to conserve the homology are dashes (-). The homeobox region is underlined. The chick exon boundary is identified by an asterisk. Sequence begins at the 5' end of cDNA clone c9. The remaining 5' end of the coding region has been sequenced from a genomic clone by others.

class genes both within and outside of the homeobox. The closest homology was with the mouse *En-2* gene. For this reason, the name of the gene was changed from *ChickEn* to chick *En-2*. The homeobox region is highly conserved between chick, mouse, *Xenopus*, and *Drosophila* (mouse *En-2*: 153/180=85%; *Xenopus En-2*: 138/180=77%; *Drosophila invected* 129/180=72%; *Drosophila engrailed* 132/180=74%). Outside the homeobox, three other prominent areas of "*engrailed*-like" conservation can be identified. Between the homeobox and the translation stop codon homology with chick *En-2* ranges from a low of 61% identity with *Drosophila engrailed* to a high of 88% identity with mouse *En-2*. In the 197bp region immediately upstream of the homeobox, identity with chick *En-2* ranges from 79% for mouse *En-2* to 45% for *Drosophila invected*. Further upstream at the 5' end of the c9 cDNA clone, sequence identity over a 71 bp stretch ranged from 76% with mouse *En-2* to 46% with *Drosophila engrailed*.

Similar regions of conservation are seen in the putative protein sequence (Figure 1.4). The amino acid sequence of the homeodomain for chick *En-2* and mouse *En-2* are nearly identical, save for one amino acid (98%). The chick *En-2* homeobox differs from *Xenopus* and *Drosophila engrailed* by 3 and 12 amino acids, respectively (95% and 80% homology). The region of homology flanking the homeobox is also present at the protein level, as is the 5' conserved region.

Unusual Nucleotide Concentrations

Given the expectation that the nucleotides within a gene would appear generally random in order, the chick *En-2* gene contains some unusual sequence. In both coding and non-coding regions of the gene, one or two nucleotides are repeated to the exclusion of the others over significant distances. Although these regions are most extensive in the 3' untranslated region of chick *En-2*, they are also present in the coding region of chick *En-2* and in other genes of the *engrailed* class (Figure 1.5). In one region 79 nucleotides in length the "GC content" is as high as 88%. In several labs, a GC concentration of this

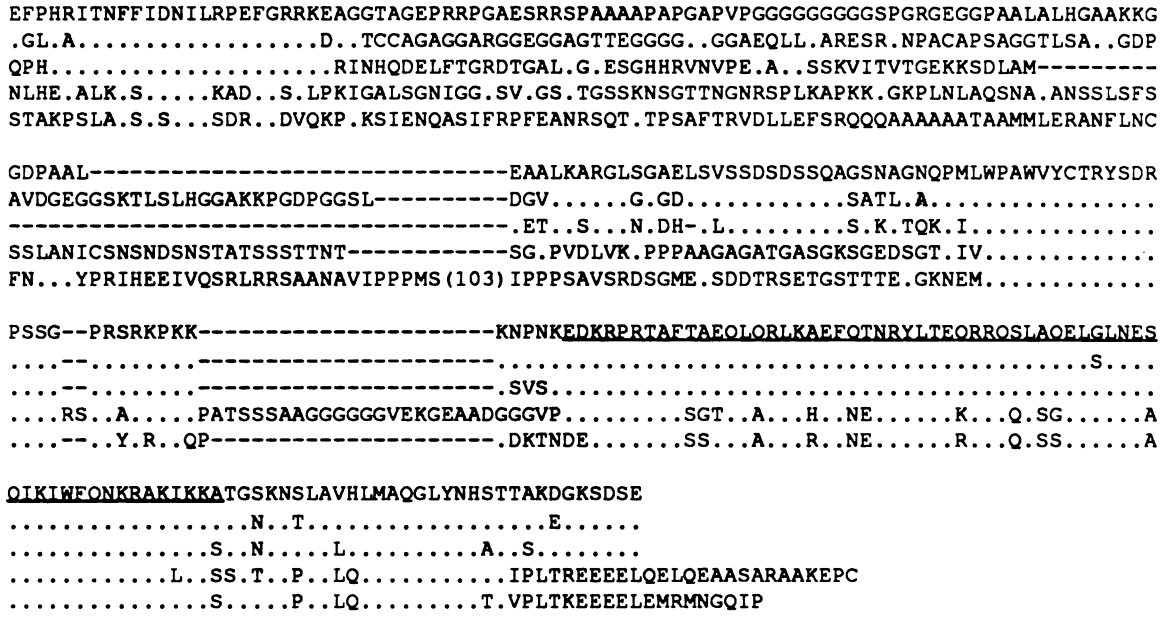


Figure 1.4 Aligned conceptual translations showing homology within the homeodomain (underlined) and in extended regions both N-terminal and C-terminal to it. Dots (.) indicate identical amino acids. Dashes (-) indicate spaces added to improve alignment. The sequences are from chick En-2, mouse En-2, frog En-2, fruit fly invected and engrailed in descending order. The N-terminal of this conceptual translation begins at the 5' end of cDNA clone c9 for this figure. The actual start of translation and transcription are further 5' than any clones isolated for this analysis.

Chick En-2

coding ATATTTTTTTATACATATTTTTAA
TTATTTTTAATTGTTTGTTTTATTT
CGGGGGCGGGGATGAAGCGCGGCGGCGGCGGCCGCGGGACGCGGCGAGCGGCGGCGGCGGCGGCGGCGACGGGAGGAGC
TTTTTGTTTTGTTCGATTTGATTTTTTAAATTTTTTTTTTTTTTTTTTTT
TGTGCGTGTGTATATAGAAAAATATATATATAT
AAAAAAAAAAAAATTAAAATAA
CCGCGGCAGCCGCCCCCAGGCAGCGCCCGGCGGGGCGGGCAGGAGCCGC
CCCCCCGGCGACCCCCCCGCACCCCCACCCGCAC
GGAGGAAGGAAGCGGGAGGAA
GCCCCGCGGCCGCCGCGCCCGC
GCCCGGCGGAGGAGGCGGCGGCGGGGG
AAACCAAAGAAGAAGAACCCCAACAAGGAAGACAA
3'UT GGGAAGGGGGGGAAAGGGGGGGGAAAGGGGGGGGGGGGGGGGAGGGGGCGAAG
AAAAAAGAAAAAAAGGAAAAAGGAAGGAAAAAAAAAAAA
TTTCTTTGTTTTGCTTTTTTCTTTTTT
TTTTGCTTTCCTTTTTCGTTCGGCGGCTTTGGCGTTTGCTGGTTTTCTTTTAATTGCTTTCTTTCTTTTTTTTTTCCTT
AATTGGAGATTTTT
TCTTTATGTTTTGGTTTTTTTTTTTTTTTTTATGCTTAAGTTCCGTTGTTTTT
AAAATAATAATAATAATTAAAAAA
TTTTACCTTTTTTTTCCCCCCCCACCCTTGCCCCTTTTCATTTTCTTCTTCTTATTTTTTAAGTTCTCTTTATTTTATTA
TATTTTTATTTTTAGTTGTATTTTTTTTTCTTTT
TATATATAGTGTGTGTGTATGTGT
GGGGGCCAGCAGGGGGGCGCCGCTGGGTGAGGGGGCG
CCCCCCCCTTTTCCCCCTTCC
AAAAAATAAAAGAAGGGTGGGGGGAAAAAAAAATAAAATAAGCCAAAAAAAAAACAAA
CCCTCCCCCCCCTCCTCCCTCCATCTCTTCTTCC

Xenopus En-2

coding TTTCTATTATTTTATATCTATTATATGT
GGAAGGAGGGGGG
GGGGAAGAAGAAGAAGAAGA
AGAAGTGAAACATTAAAAATCTGCAGAGGAACCAACAGAGGAACCAAGAGAACAAGAAGAA
3'UT ACAGACAAACAAACAAAAGGAAAAA
TATTTTTTCTTTGATTTCATTATTATTTTATTTTGTTTAATCCTTTCCAGTCCTTTTTTTTCCTGCCTTTAGATTTTAT
AGAGAAAAAAAAAAATCAAAAAAAGTCAACCTTTTTTTTTTTTTT
TTTAATTAAAAAAAAAAAAAAGCTAATATTTGTTGTGTTTTTTTTTGTTTTTGTTAATATTTGAAACTTTTTGTT
TGTATTTTAATTGATTTAATTTTTTTGTTTTTATATGCATTTTTTTT
TTTATAGTTTTGCACTTAGTGTTTTTTTTTT
TTTTTTTTTTTTT
CCCCCCTTCCCCACTCTGCTGCACCCCCCTTCCC
AATTTTAAAAAAA
TTTCTTTTGTTACAGTTCATTTTTTTTTTAAAGATATTAAAGTTTTTTTGTTTTTTTAAAT
AAAACTTAAAAATCATGCAAAAGAA
TTTATTCTCTTACTGTATATGTACCATTTGCCTTCTTTTAATTGATTTTTTTTTTTTT
AAATTAAAAAACATAAAAAGGAAAAAAAAAAATCTGGCAAGACAAAGCCAGAAA

Drosophila invected and engrailed

coding CACCCAACCCAAAAACCAA
3'UT TTTTTGTATATATCTCTAAAAATATATATATATATA
TAAATAATTTAATAAACATTTAAATTATAAAAAA
AAAGAAGTTCATGAAAGAAATTAAAAATAAAATAAAACAATTATGGGAAAAAAAAATTAAAAAAAAAAAAAAAAAAAAAA

Figure 1.5 Unusual nucleotide sequences within the coding and 3' untranslated regions of *engrailed*-like genes. Chick *En-2* contains many lengths of sequence that are composed almost entirely of one or two nucleotides. Similar unusual concentrations of nucleotides are also found in *Xenopus En-2* and *Drosophila invected* and *engrailed*, especially in the 3'UT region. Each line in this figure shows an independant region of unusual sequence.

type has confounded attempts to generate cDNAs of the 5' end of the *En-2* gene in both mouse and chick. The 5' ends of both *En-1* and *En-2* have recently been isolated from genomic libraries for chick, mouse and human. The complete putative coding sequence for these six genes is proposed in a manuscript currently being prepared, in collaboration with me, by Cairine Logan and co-workers in Alexandra Joyner's laboratory.

cDNA 3' Untranslated Region

Seven of the eight cDNA clones extended into the 3' untranslated (UT) region of the gene. Of these, two terminated in a poly $(A)^+$ tail 503 nucleotides 3' of the translation stop site (cDNAs 1 & 2; Figures 1.2 and 1.6) and three contained sequence 3' to that poly adenylation site (cDNAs 4,6 and 8; Figure 1.2), indicating that there were two or more 3' ends for chick EN-2 messages. The mRNA from an embryonic day 6 brain was used as a substrate for PCR RACE (rapid amplification of cDNA ends) amplification of a more 3' poly adenylated cDNA (Frohman et al., 1988; Frohman and Martin, 1989). A PCR cDNA containing a poly $(A)^+$ consensus sequence and addition site that mapped 3' to the previously identified site was cloned (Figures 1.2 & 1.6). The two transcripts differ in length by 594 nucleotides.

Conserved Sequences

Obviously, sequence conservation in the coding region of gene families is expected. Several sequence motifs are also conserved in the promoter regions of eukaryotic genes. Sequence conservation in the 3'untranslated region is less common. From my sequence analysis, I have identified one region of homology in the 3' UT regions of the chick *En-2* and *Drosophila invected* genes and three regions of sequence homology in the 3'UT region of the *Xenopus* and chick *En-2* genes (Figure 1.7). The region of homology between chick *En-2* and *invected* gene is 31 bases long with 67% (21/31) homology. No such homology could be found in the *Drosophila engrailed* gene 3'UT region.

AGCGAATAGCCGGGAAGGGGGGGAAAGGGGGGGGAAAGGGGGGGGGGGGGGGAGGGGGCGAAGTTTATA
CAATGCAATAATTTAATTAAAAAAGAAAAAAAGGAAAAAGGAAGGAAAAAAAAAAAAAGGGCCAGTGTATAAA
GATTATACCAGCATTCATAGCGAAAATATGGTGTATTAGATATAATTCTGCAATATTCTATGTATATATAAT
TTACAGGTAAGGTGGTGTAAAAATACGAGATATCGGATTATAAAGTATTTCTTTGTTTTGCTTTTTTCTTTT
TTCGGGTTCGTGTTGGTTCCATCGTTTTGCTTTCCTTTTTCGTTCGGCGGCTTTGGCGTTTGCTGGTTTTCT
TTTAATTGCTTTCTTTCTTTTTTTTTTCCTTTTGGTTGAATTGGAGATTTTTAGACGCTGTCTTTATGTTTT
GGTTTTTTTTTTTTTTTTTATGCTTAAGTTCCGTTGTTTTTATAGACTTAAGCGCTGTTGGATGGACATTGG
ACGCTGTTTTTTTGACATTCAAAATAATAATAATAATTAAAAAACA***ACTTTTGCTGAAGTCCAAAGATT
TTTATTGCTGCATTTACACGACTGTGAACCGAATAAATAGTTCTCCTATGTGTTCCACGAGTTTTACCTTTT
TTTCCCCCCCCCACCCTTGCCCCTTTTCATTTTCTTCTTCTTATTTTTTAAGTTCTCTTTATTTTATTATTAT
TATTATTTTTATTTTTAGTTGTATTTTTTTTTCTTTTAGGGCTCTGGGTGTGTCTGGGGCTGACTTTTGCGG
ACAGATGTGGGGATTATAATTTAAAAAGCAAATGATATATAGTGTGTGTGTATGTGTAAGGATAAGGGAAAG
ACATCTCAGCCCAGCCGCCCCCTGCGCCGGTGCTGCCCCCGCGCCGTTTGCGGGACACGGGGGAGGGGTTTA
TCCCCTCCACCCCTCAAAAAAATATACATCCAACTTAAGGGCCACTTTGGGGTTCCCCTCGCCAATAAGGTA
TGACCCCCCCCCTTTTCCCCCTTCCACTCCTACCCGGGGCTGGGACAGCGCCCGCTTCTGCCTTCCCCGCGAG
TTTGGGTGCGTTTTCCACAAAAAATAAAAGAAGGGTGGGGGGAAAAAAAAATAAAATAAGCCAAAA***AAAA
ACAAAGTGGCATTTTTGGTTTCCCTCCCCCCCCACCTCCCTCCATCTCTTCTTCCCGGCCGCTG

Figure 1.6 Sequence of the 3' untranslated region of the chick *En-2* gene. The first nine nucleotides (**bold**) code for the last two amino acids and the translation stop codon. Putative poly $(A)^+$ addition consensus sequences are underlined and approximate poly $(A)^+$ addition sites are marked with an ***. The poly $(A)^+$ addition site at 503-505 nucleotides 3' of the stop codon was identified from cDNA clones c1 and c2. The poly $(A)^+$ addition site at 1057-1065 nucleotides 3' of the stop codon was identified from a PCR RACE cloned fragment. Sequence was obtained from both cDNA and genomic clones, however, not all regions were sequenced on both strands. This sequence may, therefore, contain nucleotide errors.

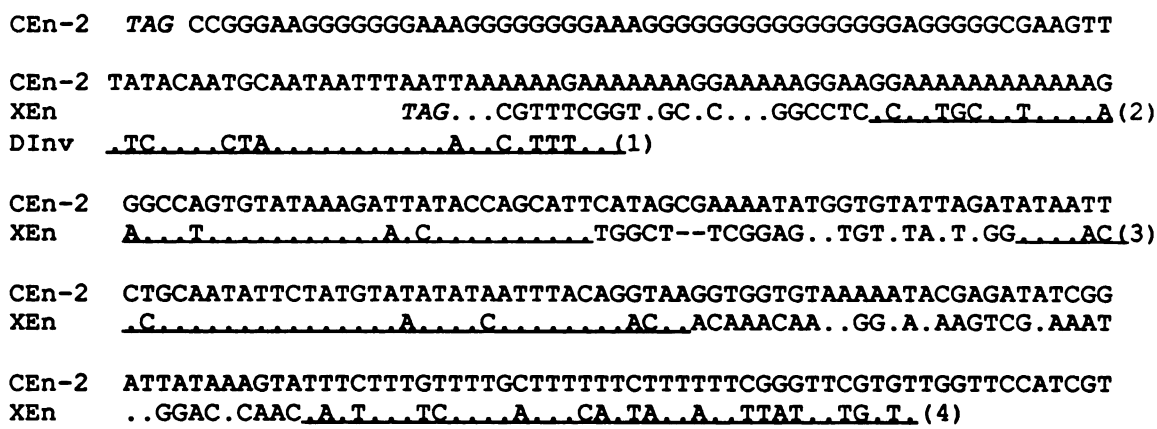


Figure 1.7 Conserved sequence in the 3' untranslated region of *En-2* from chick and *Xenopus* and from *Drosophila invected.* Both vertebrate sequences shown begin at the translation stop codon (*TAG*) and the *Drosophila inv* sequence shown begins 210 bases downstream from the stop codon. Periods (.) represent identical nucleotides and the regions of homology are underlined. The region of homology between chick *En-2* and *invected* (1), is 31 bases long with 67% (21/31) homology. No such homology could be found in the *Drosophila engrailed* gene 3'UT. The first short region of homology between chick and frog (2), contains a high concentration of adenine (A) nucleotides followed by variable and highly conserved sequence (34/44 or 77%). Considering only the variable region, the homology is 25/28 or 89%. The next downstream region of homology between chick and frog (3), has 34/41 conserved bases or 83%. The most downstream region (4), is due entirely to conserved thymidine (T) nucleotides (See Figure 1.5).

The first region of homology in the chick and *Xenopus* 3'UT region (Figure 1.7, region 2) contains a high concentration of adenine (A) nucleotides followed by variable and highly conserved sequence (34/44 or 77%). The conserved adenine stretch is similar to several other regions with a highly reiterated single nucleotides. Considering only the variable region, the homology is 25/28 (89%). The next region of homology between chick and *Xenopus* (Figure 1.7, region 3) has 34/41 (83%) conserved bases. The third region of homology just downstream (Figure 1.7, region 4) is due entirely to conserved thymidine (T) nucleotides (see Figure 1.5).

Intron Boundaries and Consensus Sequences

By comparing the map of the genomic clone ChickEn with that of cDNA clone c4, a discrepancy of approximately 1kb was identified 5' to the homeobox region, but 3' to the most upstream conserved region. S1 analysis between the genomic clone gCE-RX and day 6 and 7 brain poly $(A)^+$ RNA revealed an intron-exon boundary 185 nucleotides 5' of the BglII site within the homeobox (Figure 1.8). This location was confirmed by comparing cDNA and genomic sequence in this region. Sequence analysis also revealed the 5' exon-intron boundary located 418 nucleotides from the 5' end of the c4 cDNA clone and 98 nucleotides from the 5' end of the chick En-2 genomic clone. The intron-exon boundary sequences and intron splice consensus sequences are highlighted in Figure 1.9. The expected splice site consensus sequence for the 5' splice junction of the intron is (A/C)AG|GT(A/G)AGT (for review see Harris and Senapathy, 1990) . The splice site for chick En-2 matches 8 of 9 nucleotides, with an A instead of a T in the 3' position. The 5 nucleotide lariat branch point is expected to lie 10-50 nucleotides upstream of the acceptor splice site and have a consensus sequence (50% probability) of (C/T)T(A/G)A(C/T) with the fourth position A acting as the lariat branch point. A 4 of 5 match, CTGAG was identified 59 nucleotides upstream of the 3' intron/ exon boundary. The 3' splice acceptor is usually characterized by a pyrimidine rich region followed by a (C/T)AG. This region in

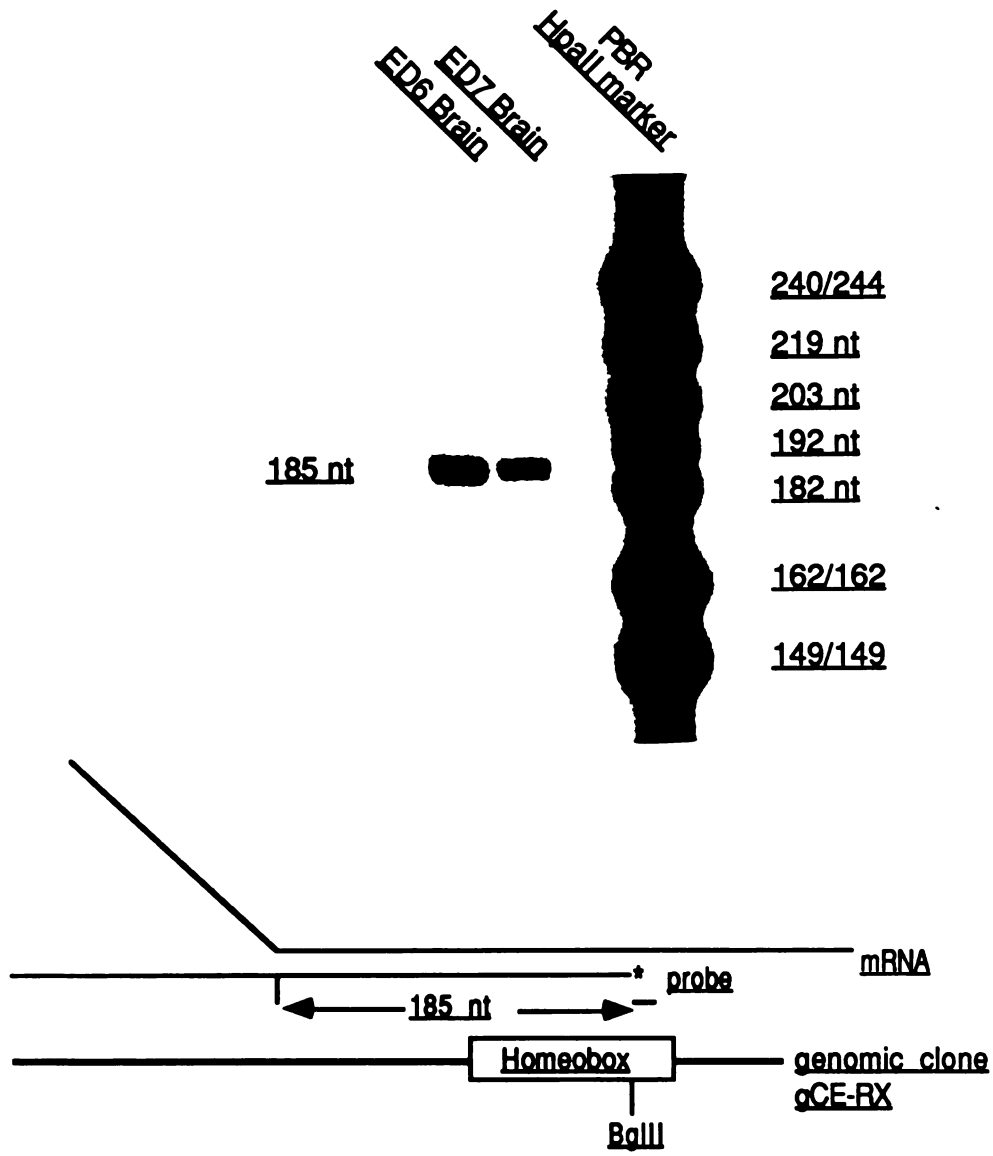


Figure 1.8 S1 map of intron/exon boundary 5' of homeobox. The probe was made by dutting and kinase labeling a genomic fragment at the BglII site within the homeobox. This labeled fragment was denatured and renatured with mRNA prepared from embryonic day (ED) 6 and 7 chick brain. S1 nuclease cuts where genomic DNA and mRNA diverge, at an intron/exon boundary or the 5' or 3' end of the message. In this case, a 185 nucleotide fragment corresponds to the intron/exon boundary 5' to the homeobox.

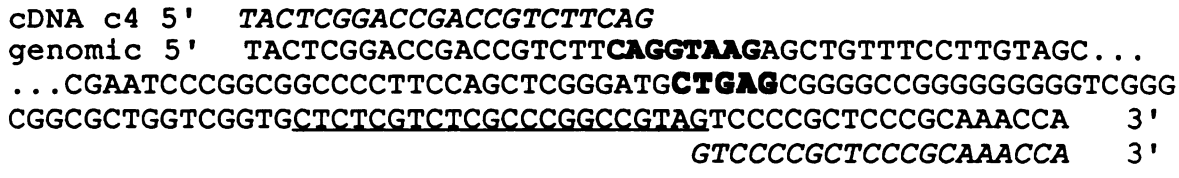


Figure 1.9 Intron-exon splice boundaries and lariat consensus sequence. The expected splice site consensus sequences for the 5' splice junction of the intron is (A/C)AG|GT(A/G)AGT. The splice site for chick engrailed-2 matches 8/9 and is highlighted in **bold** lettering. The 5 nucleotide lariat branch point is expected to lie 10-50 nucleotides upstream of the acceptor splice site and have a consensus sequence (50%) of (C/T)T(A/G)A(C/T) with the fourth position A acting as the lariat branch point. A CTGAG sequence is found in this gene 59 nucleotides upstream of the 3' intron/exon boundary, conserved at 4 of the 5 consensus nucleotides. The 3' splice acceptor in the intron is usually characterized by a pyrimidine rich region followed by a (C/T)AG. This region in the chick engrailed-2 gene has such a region (14/19 pyrimidines followed by TAG) and it is shown underlined above. The cDNA is written in *italics* and approximately 900 nucleotides have been omitted from the intron sequence between the ... ...

the chick En-2 gene has such a region (14 of 19 pyrimidines followed by a TAG). Thus, in addition to 1kb of sequence present in the gCE-RX clone and absent in cDNAs 3 and 9, the intron was confirmed by splice consensus sequences.

Genomic Southern

Genomic DNA cut with Pvu II was Southern blotted and probed with the chick En-2 homeobox probe. Two bands, at 2.6 and 1.35 kb were identified (Figure 1.10), implying that there are two engrailed-like genes within the chick genome. The 1.35 kb band corresponds to a Pvu II fragment in the chick En-2 gene by genomic mapping.

DISCUSSION

Since the *engrailed* gene in *Drosophila* is involved in the control of segmentation and positional identity during early development, I sought to discover if a vertebrate equivalent of *engrailed* existed and functioned similarly in chick development. I subsequently cloned and sequenced both genomic and cDNA clones containing the conserved coding regions and 3' untranslated end of an *engrailed*-like chicken gene. The coding region of the gene contains a single 1 kilobase (kb) intron, an *engrailed*-like homeobox and extra-homeobox conservation unique to the engrailed class of genes. Sequence for chick was very highly conserved with the mouse *En-2* gene (Joyner and Martin, 1987) both within and outside the homeobox. The homeodomain region of the protein acts as a DNA binding and transcription factor (Desplan et al., 1988; Levine and Hoey, 1988), and the extremely high conservation between chick and mouse (98%) in this region would imply that the binding site for this transcription factor is also conserved. In addition to the transcriptional regulation functions mediated by the homeodomain, *Drosophila engrailed* can also act as an active transcriptional repressor (Jaynes and O'Farrell, 1991), a function of a domain near the N terminal of the protein. The sequence coding for this region of chick En-2 is not present in my clones, which lacked the 5' end of

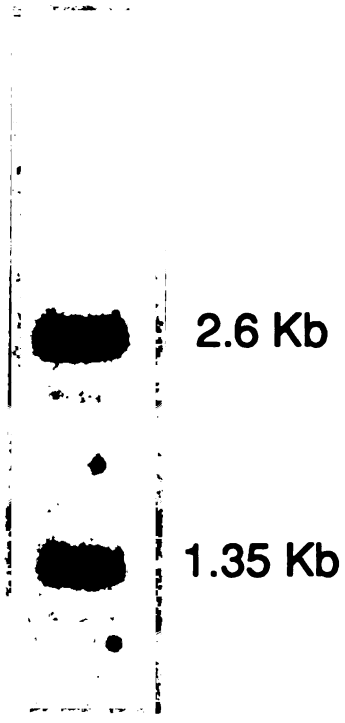


Figure 1.10 Southern blot of PvuII cut genomic DNA probed with chick *en-2* homeobox probe (106 nucleotide BglII-PvuII fragment). A 1.35 Kb band corresponds to the expected size for the chick *en-2* gene based on map distances in the genomic clone.

the gene. It is therefore unknown whether structural and functional homology in this region exists or would result in a conservation of active repression in the chick En-2 protein. Three other regions outside the homeodomain are, however, conserved within the engrailed-class of proteins. In light of the evidence that transcriptional regulatory functions can be mediated by regions outside the homeodomain, it is possible that these conserved regions represent additional regulatory capacity or protein-protein binding sites that have been conserved over millions of years of evolution.

The 3'-UT region was also sequenced and two poly $(A)^+$ addition sites were identified, indicating that at least two transcripts are produced from this gene. Because both transcripts were isolated from the day 6 brain, it is unclear whether there is any significance to this variability. The 3'-untranslated region contains some homology with the *Drosophila invected* and *Xenopus engrailed* 3'-untranslated regions. These regions of homology may be coincidental, or they may act as 3' transcriptional regulatory sequences, for example as an enhancer. A comparison of the *Xenopus*/chick 3'UT conserved regions with other vertebrate engrailed 3'UT sequences should be considered when these sequences become available.

In both coding and non-coding regions of the *engrailed*-like genes, one or two nucleotides are repeated to the exclusion of others over significant distances (Figure 1.5). Although these regions are most extensive in the 3' UT region of chick *En-2*, they are also present in the coding region of chick *En-2* and in other genes of the *engrailed* class. Although these regions translate into stretches of single amino acids occasionally (GC rich regions lead to alanine and CA rich regions to glutamine repetition in *engrailed* and GA rich regions result in glycine repetition in chick *En-2*), for the most part, the amino acids do not reflect the redundancy of the DNA sequence. The cause or effect of such redundancy within the DNA sequence is unclear.

The general structure of the vertebrate *engrailed*-like genes is similar. The stop codon location is similar in relation to the homeobox, as are the intron/exon boundary

locations. Sequence conservation is high over extended regions of the gene, including some conservation in the 3'-UT region. The homeobox regions are nearly identical, as are the timing and pattern of expression (see Chapter 2). The strength of conservation in so many features of this vertebrate gene group enhances the probability that conclusions that can be drawn for one family member will likely also be true for other *engrailed-2* like genes and proteins.

CHAPTER 2

EXPRESSION OF CHICK *EN-2* IN EARLY EMBRYOS

INTRODUCTION

To begin to define the possible functions of the chick *En-2* gene, I analyzed its expression during early development, first by northern blot, and subsequently, using a monoclonal antibody specific for *En-2* in the chick, 4D9. My results show that although *En-2* expression can be detected at low levels in several tissues by northern blot, a high level of expression is found only in the midbrain and rostral hindbrain regions of the central nervous system, beginning fairly early development (stage 8). Using in situ hybridization and immunolocalization, others have further defined that expression to a region restricted to the caudal mesencephalon/rostral metencephalon. In addition, chick *En-2* expression is observed in overlying ectoderm and in a subset of neural crest cells in vivo (Chapter 3) and in culture (Gardner et al., 1988), suggesting that expression of this gene may also be important in the development of neural crest derivatives *in vivo*.

MATERIALS AND METHODS

Northern Blot Hybridization

Chick embryos were dissected and total RNA was extracted from tissue samples using LiCl (Auffray and Rougeon, 1980). RNA was isolated from whole embryos (Embryonic Day (ED) 2, 4 and 6), brain (ED 4, 6, 7, 10, 11, 14 and 18), neck, flank, heart, liver and viscera (ED 6). Poly $(A)^+$ RNA was isolated from total RNA by elution over an oligo-dT cellulose column (Collaborative Research, Inc.). Twelve micrograms of poly $(A)^+$ RNA were loaded in a denaturing formaldehyde-agarose gel, fractionated by electrophoresis and transferred to GeneScreen (NEN Research Products). RNA was bound to the membrane using UV irradiation (Church and Gilbert, 1984). Blots were prehybridized for 10 min and hybridized for 16 hrs at 65°C with 10^7 cpm/ml in 10 ml of "moderate hybridization buffer" consisting of 1% bovine serum albumin, 0.2M $NaPO_4$

(pH 7.2), 1mM EDTA, 7% SDS and 35% deionized formamide. All probes used for Northern blot hybridization were prepared by nick translating isolated fragments of the genomic clone gCE-RX. (The cDNAs had not been cloned and characterized at the time this work was done.) These probes consisted of: a 680 bp SacI-EcoRI fragment (probe A, Figure 2.1), a 520 bp SacI fragment (probe B, Figure 2.1) and a 106 bp BglII-PstI fragment that contained only homeobox conserved sequence (probe C, Figure 2.1). After hybridization and washing, the blot was exposed to Kodak X-Omat film at -80°C with an intensifying screen. Markers were end labeled lambda DNA digested with HindIII restriction endonuclease.

Immunolocalization

Embryos were immunolabeled whole as previously described (Patel, 1989), except that the peroxidase reaction product was enhanced by adding $CoCl_2$ (0.025% aq.) and $Ni(NH_4)_2(SO_4)_2$ (0.02% aq.) to the immunolabeling reaction. Hydrogen Peroxide was diluted to 0.03% and embryos were observed under the microscope for 1-2 minutes until the reactions were complete.

RESULTS

Northern Blot Hybridization

Northern blots of total or poly $(A)^+$ RNA from tissues of early chick embryos were hybridized to nick translated probes A, B and C derived from the Chick En-2 genomic clone gCE-RX (Figure 2.1). Tissues tested included whole embryo, head, trunk, neck, limb, liver, heart, brain, flank and viscera from embryos from embryonic day 2 to day 18. Nothing was known at the time about the location of probable expression for any vertebrate homeobox-containing gene. Three major hybridization bands of approximately 2.2, 3.5 and 5 kb in length were detected (probes A and B, Figure 2.2; probe C, Figure 2.3). Hybridization signal was highest in early embryonic head and brain. Hybridization to brain

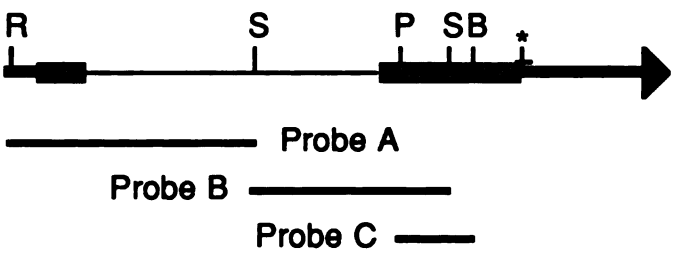
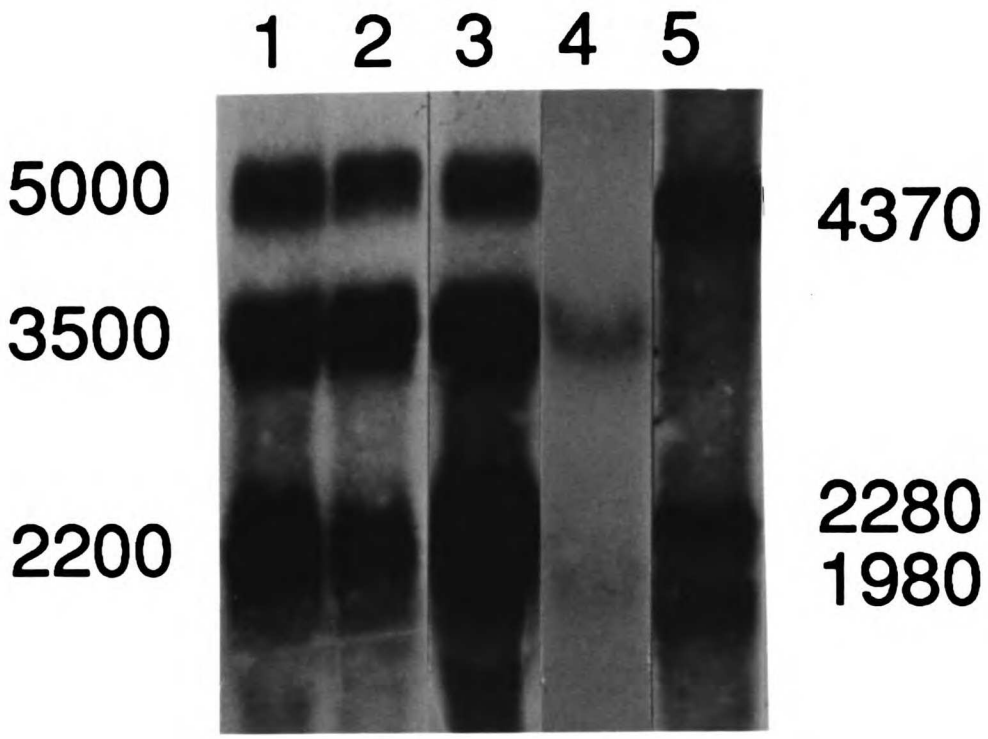


Figure 2.1 Maps of genomic probes used in Northern and Southern blots. Probe A contains conserved sequence 5' to the intron/exon boundary. Probes B and C contain conserved sequence 3' to the intron/exon boundary including some of the homeobox.

Figure 2.2 Northern blots of chick poly $(A)^+$ RNA. Lane 1: ED4 head, probe A; lane 2: ED6 brain, probe A; lane 3; ED6 brain, probe B; lane 4: ED6 flank, probe C; lane 5: HindIII digested, end-labeled lambda marker. Lanes are not quantitatively comparable due to RNA concentration and probe variability.


1 2 3 4 5
5000
3500
2200
4370
2280
1980

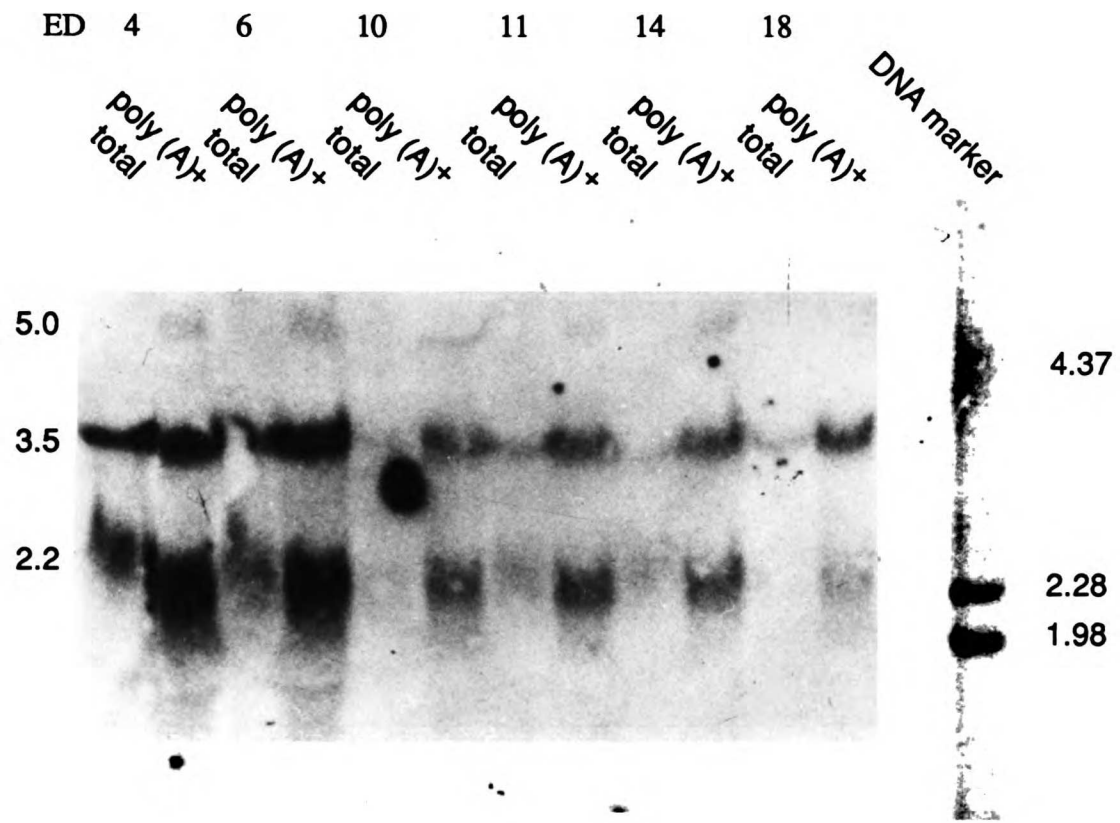


Figure 2.3 RNA hybridization to probe B (figure 2.1) for different ages of embryonic chick brain. Fifteen micrograms of total or poly (A)+ enhanced RNA were loaded per lane. Sizes of RNA bands were determined by comparing to DNA standards shown on the right. All sizes are in kilobases.

poly $(A)^+$ RNA was seen at all developmental stages tested (Figure 2.3). The more prominent 2.2 and 3.5 kb bands are detectable in total RNA from day 2 chick whole embryo and are also detected at lower levels in poly$(A)^+$ RNA from other dissected embryonic tissues (day 4 body; day 6 body, viscera, flank, and neck) using probe C, the homeobox probe. In some cases, the 5 kb band is also detected (data not shown). All probes used contain at least one of several regions of Chick *En-2* DNA now known to be conserved among *engrailed*-like genes in flies and vertebrates (see Chapter 1).

Monoclonal Antibody Staining

In whole mount embryos, immunolabeling with MAb-4D9 localizes En-2 expression to the midbrain and rostral hindbrain during early embryonic development (Gardner et al., 1988; Patel et al., 1989). I was able to confirm this labeling, however, I did not pursue the specifics of chick *En-2* expression in normal embryos using this antibody because this line of inquiry was being investigated by my collaborator, Charles Gardner. Immunolocalization of chick *En-2* protein was consistent with the mRNA detected by Northern blot hybridization in the midbrain and rostral hindbrain of embryonic day 2 and older chicks. The low levels of RNA signal seen for neck, flank, back and viscera were not corroborated at the protein level by 4D9 localization.

DISCUSSION

Three sizes of transcript were detected in Northern blots of brain and other tissues from early embryos when these blots were probed with any one of several inserts from the genomic clone gCE-RX. Since not all of these probes contained regions now known to be conserved between homeobox-containing genes, it seems likely that they represent En-2 or engrailed-like transcripts. Because one other engrailed-like gene appears to exist in the chick genome (Figure 1.10) there remains a possibility that one or more of these bands represents cross-hybridization with this other gene (*En-1*?).

Two transcripts from the En-2 gene were identified by sequencing cDNA clones (Figures 1.2 and 1.6). These two transcripts were due to poly-adenylation at different sites. The approximately 555 nucleotide difference between these two transcripts cannot account for the differences in size for any of the three bands detected on Northern blots (2.2, 3.5 and 5.0 kb). This discrepancy may be the result of the use of DNA markers (Lambda HindIII) to determine the sizes of mRNA transcripts. Alternatively, there may be other variations between the transcripts that have not yet been detected because a full length cDNA has not been cloned.

Northern blot hybridization of the *En-2* homeobox probe to RNA from non-brain tissue was not corroborated by immunolocalization using the 4D9 antibody. One possibility is that the mRNA is not translated in these tissues. I cannot, however, rule out a second possibility that this signal is the result of contamination of non-brain tissues with brain during isolation, although I attempted to cleanly dissect each tissue or region and the signals were seen in several experiments. Another third possibility is that the signal in back, flank, neck, etc. may reflect cross hybridization with other homeobox containing genes, which are now known to be expressed in many regions of developing vertebrate embryos. However, the sizes of the bands in these other tissues were always the same as those in the brain mRNA, introducing a fourth possibility. There may be low levels of expression in tissues outside the brain that have not yet been detected above background by antibody or by *in situ* hybridization analysis. Further experiments and perhaps more sensitive techniques will be required to select between these and perhaps other yet unconsidered possibilities.

CHAPTER 3

CHANGES IN DORSOVENTRAL BUT NOT ROSTROCAUDAL REGIONALIZATION OF THE CHICK NEURAL TUBE IN THE ABSENCE OF CRANIAL NOTOCHORD, AS REVEALED BY EXPRESSION OF ENGRAILED-2

INTRODUCTION

In 1938, Spemann proposed that vertebrate neuroectoderm might acquire its regional character in response to inductive signals from underlying, patterned dorsal mesoderm (Spemann, 1938). Recent *in vitro* experiments in *Xenopus* have suggested that a subset of this mesoderm, the anterior notochord, can provide sufficient positional information to the neuroectoderm to result in the expression of a spatially-restricted molecular marker, *engrailed-2* (*En-2*), in the mesencephalon and anterior metencephalon (Hemmati-Brivanlou et al., 1990). In a variety of vertebrate species, *En-2* expression has been detected in this restricted region of the developing central nervous system beginning around the time of formation of the first somite (Davidson et al., 1988; Davis et al., 1988; Fjöse et al., 1988; Gardner et al., 1988; Hemmati-Brivanlou and Harland, 1989; Patel et al., 1989). In avian embryos, normal expression of the *En-2* protein has been demonstrated specifically in nuclei of the mesencephalon and cranial metencephalon (Gardner et al., 1988; Patel et al., 1989; Martinez and Alvarado-Mallart, 1990) and in a subset of adjacent neural crest cells (Gardner et al., 1988). We have also found *En-2* expression in these regions as well as in the overlying surface ectoderm (Figure 3.1 A-C). However, *En-2* expression is not found in the floor plate region overlying the notochord (Figure 3.1B).

To determine whether the restricted, regional expression of *En-2* requires signals from the notochord, Hensen's node was removed from 199 cultured embryos (stages 3c-5; Hamburger and Hamilton, 1951, as modified by Schoenwolf et al., 1992; Figure 2A, B); an additional 26 cultured embryos received no surgery and served as controls. Hensen's node contains the precursor cells of the notochord, and its extirpation can result in the loss

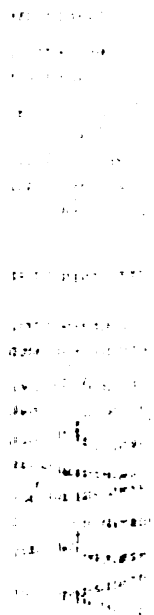
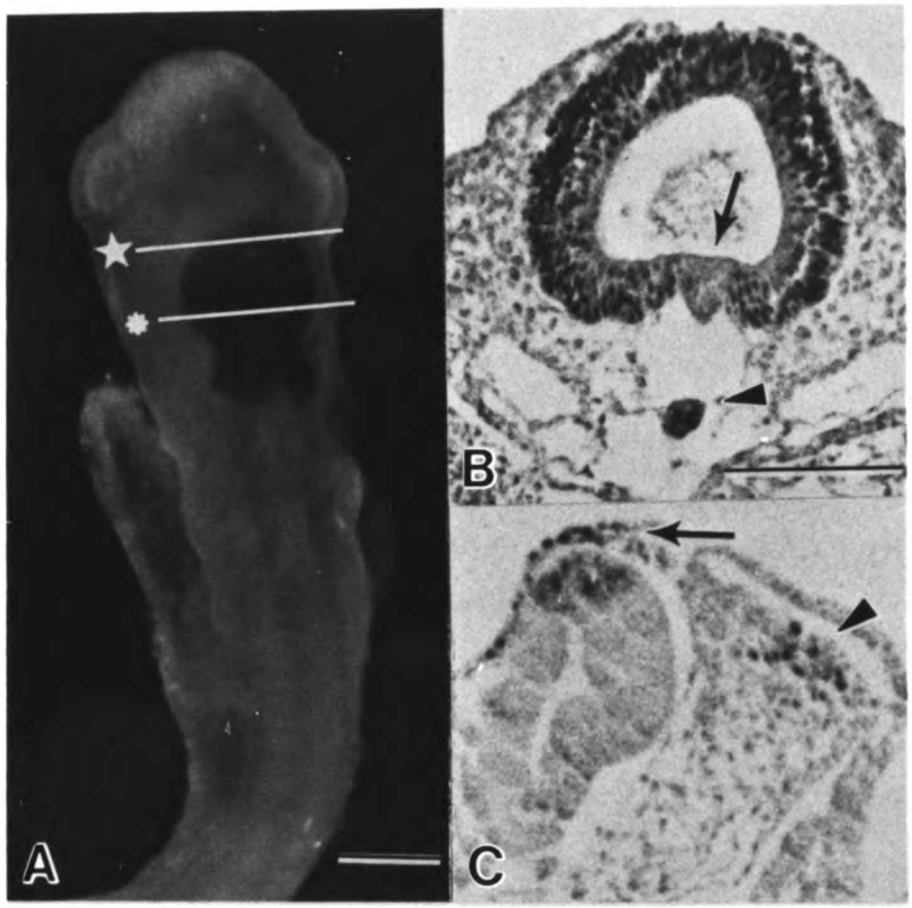


Figure 3.1 Stage 12 control embryo labeled with 4D9 in the mesencephalon and cranial metencephalon and Not-1 in the notochord. (A) Whole mount showing 4D9 label in the mes- and rostral metencephalon. Not-1 label not visible in this view. A transverse section at the level of the asterisk (B) shows that 4D9 labels a nuclear protein expressed in cells located in the roof plate and lateral walls of the neural tube (but not in the floor plate), and that Not-1 labels a cytosolic protein in the notochord. A transverse section at the level of the star (C) shows 4D9 label in the overlying ectoderm (arrow) and in the neural crest migratory pathway (arrowhead). Scale bar = 0.1mm.


A
B
C

of the cranial notochord from developing embryos (Grabowski, 1956). After 24 hours in culture, all 225 embryos were grouped in the following categories based on their gross appearance (Table 3.1): "good," "fair," "inverted," or "poor" (see Figure 2C-E and experimental procedures section for evaluation criteria). "Poor" embryos were excluded from subsequent analysis. To detect regional differentiation of the neuroectoderm, embryos were immunolabeled with 4D9, a monoclonal antibody that recognizes *En-2* (Patel et al., 1989), and subsequently sectioned for histological analysis.

MATERIALS AND METHODS

Embryos

Embryonic day 0, fertile White Leghorn chicken eggs *(Gallus gallus domesticus*; Feather Hill Farms, Petaluma, CA; Butterfield Farms, Riverton, UT) and Japanese quail eggs (*Coturnix coturnix japonica*; Strickland Farms, Pooler, GA) were incubated at 38°C in a forced-draft incubator. At 22-24 hours of incubation, embryos were removed from the shell, washed with 123 mM NaCl solution, and staged according to the following criteria: elongating primitive streak (stage 3c), fully elongated streak (stage 3d), initial head process (stage 4), elongating head process (stage 4+), and definitive head process (stage 5) (Hamburger and Hamilton, 1951, as modified by Schoenwolf et al., 1992).

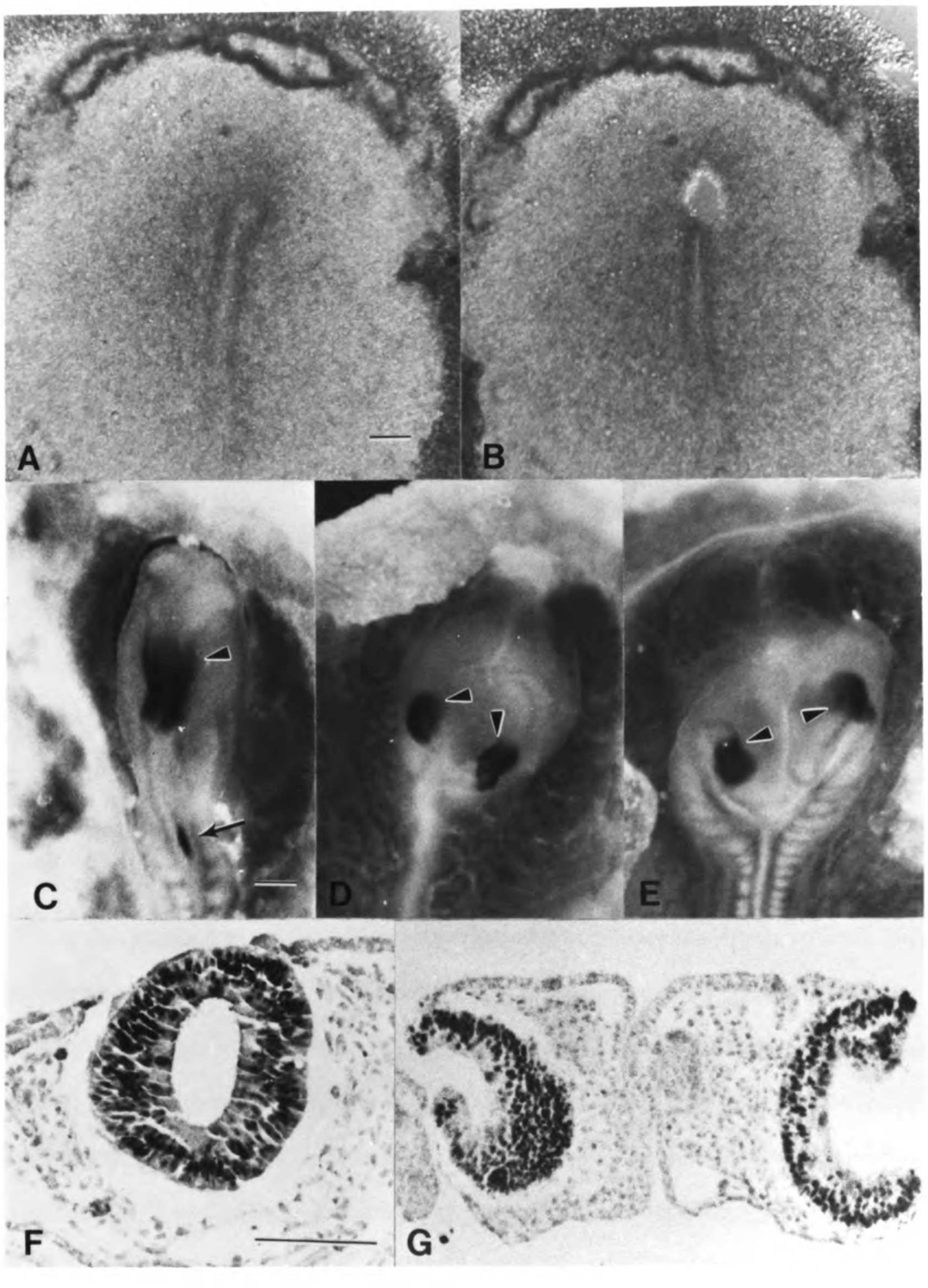
Surgery and culture

Hensen's node and visible head process (when present) were removed from experimental embryos by cutting through the blastoderm with a glass needle (Figure 3.2A, B). Experimental (E) and control (C) embryos were cultured dorsal-side up in culture dishes (Smith and Schoenwolf, 1989) for 24-30 hours at 37°C and 100% humidity. Cultured embryos were categorized as follows: as "good" (normal and/or stunted rostrocaudally or with small neural tube defects; E 35%, C 46%); "fair" (with large neural tube defects; E 28%, C 27%); "inverted" (head inverted into the hole created by surgery; E

Table 3.1. The percentages of all experimental and control embryos in each of the four quality categories. See Figure 3.2C-E and experimental procedures section for description of categories.

Categories	Experimental Embryos	Control Embryos
Good	35%	46%
Fair	28%	27%
Inverted	24%	0%
Poor	13%	27%

Figure 3.2 Extirpation of Hensen's node influences dorsoventral but not rostrocaudal patterning in the neuroectoderm. In experimental embryos, Hensen's node and visible head process (when present) were removed by cutting completely through the blastoderm with a glass needle. (A) Prior to extirpation. (B) Immediately after extirpation. Experimental and control embryos were cultured and immunolabeled with 4D9 (arrowheads in C-E) and in some cases with Not-1 (arrow in C). Examples of the following categories of cultures embryos are shown: (C) "good," (D) "fair," and (E) "inverted." Transverse sections through (F) "good" and (G)"inverted" embryos show 4D9 immunolabeling completely "encircling" the neural tube in the absence of the notochord. Scale bar = 0.1 mm A and B; 0.2 mm C-G.


A
B
C
D
E
F
G

24%, C 0%); or "poor" (not shown; failed to develop; E 13%, C 27%). Similar morphology after this type of surgery and culture has been described previously (Grabowski, 1956; Smith and Schoenwolf, 1989, 1991).

Immunolabeling

Embryos were immunolabeled as previously described (Patel et al., 1989), except that the peroxidase reaction product was enhanced by adding $CoCl_2$ (0.025% aq.) and $Ni(NH_4)_2(SO_4)_2$ (0.02% aq.) to the immunolabeling reaction. Hydrogen peroxide was diluted to 0.03%. The antibodies used have been previously described: 4D9 (Patel et al., 1989) and Not-1 (Yamada et al., 1991).

RESULTS

Rostrocaudal expression of *En-2*

In all "good" and "fair" embryos, 4D9 intensity and location appeared normal in gross view (Figure 3.2C, D). Fifty-three of the experimental embryos in these groups were serially sectioned transversely to determine whether cranial notochord was absent. In sections, 4D9 nuclear immunolabeling was clearly discernable (Figure 3.2F). Notochord cells, when present, were identified by location and morphology. Additionally, to confirm our ability to identify these cells, 18 experimental embryos were also immunolabeled with the monoclonal antibody Not-1, which recognizes a cytosolic protein in the notochord (Yamada, 1990; Figures 3.1B and 3.2C). Embryos varied in the extent to which notochord failed to form (Table 3.2). Ten embryos with "good" morphology completely lacked notochord under the entire rostrocaudal extent of the neural tube in which 4D9 immunolabeling was detected. The length of the 4D9 immunolabeling region in these embryos ranged from 165-295 μm. Of the 43 remaining sectioned embryos, 23 had partial notochord loss and 20 had no detectable loss. The 4D9 immunolabeling region of embryos with partial loss of the cranial notochord varied in length from 135-340 μm, and the part of

Table 3.2. Range of lengths (in micrometers) of the 4D9 immunolabeling region as a function of the percentage of underlying notochord remaining for the 53 sectioned "good" and "fair" experimental embryos. Stage at the time of Hensen's node extirpation was included as a co-variable in a multiple regression analysis. Neither stage nor percentage of notochord remaining had a significant effect on the length of the 4D9 immunolabeling region ($p = 0.85$).

% notochord	No. embryos	4D9 min. length	4D9 max. length	4D9 avg. length
0%	10	165	295	272
1-25%	13	135	340	223
26-50%	5	170	220	192
51-99%	5	185	305	236
100%	20	135	315	224

this length that included notochord varied from 15 μm (6.5%) to 230 μm (91.2%). For embryos in which the notochord completely regenerated, the length of the 4D9 immunolabeling region ranged from 135-315 μm. To determine if the *En-2* expressing region was diminished or expanded in length as the result of notochord loss, a multiple regression analysis was done on the numerical data from the 53 sectioned embryos. Both embryonic stage at the time of surgery and the percentage of 4D9 immunolabeled sections with notochord (0-100%) were tested as independent variables to determine whether either variable significantly influenced the length of 4D9 labeled neural tube. Neither stage nor percentage notochord remaining after Hensen's node extirpation correlated with significant changes in the length of the region that expressed *En-2* ($p = 0.85$). We conclude that notochord directly underlying the mesencephalon and cranial metencephalon is not required to establish rostrocaudal regional character in the neuroectoderm, as assessed by *En-2* expression.

An alternate method of pattern regulation could be the suppression of regionally restricted genes outside their normal territory of expression. The rostrocaudal extent of the *En-2* expressing region does not extend after Hensen's node extirpation into the prosencephalon, caudal rhombencephalon, or spinal cord levels. Because of this, we conclude that the notochord is not required to suppress *En-2* expression outside the mesencephalon and cranial metencephalon levels.

Spatial relationship between 4D9 expression and the position of the notochord

Serial sections of the 10 "good" operated embryos completely lacking notochord in the 4D9 expressing region were analyzed quantitatively to determine the rostrocaudal distance between the caudal end of the expressing region and the beginning of the notochord (Figure 3.3). Normally localized rostrocaudal expression of *En-2* could be detected when the distance between the 4D9 immunolabeled neuroectoderm and the nearest

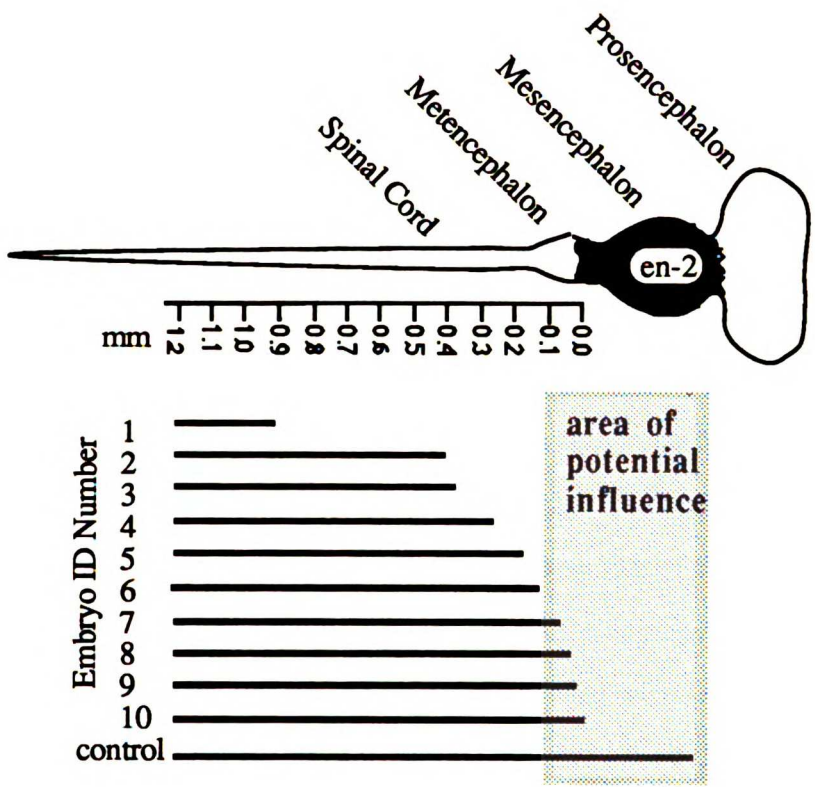


Figure 3.3 Spatial relationship between the position of the notochord and *En-2* expression in 10 experimental embryos after culture. Black lines indicate the rostrocaudal extent of the notochord. The shaded area covers the region where notochord would be expected to provide a short range vertical influence on patterning of the *En-2* expressing neuroectoderm (directly adjacent area plus 100 μm caudal) if induction occurs after convergence and extension of the neural plate and underlying ingressed mesoderm. Embryo numbers 2, 3, 5, 6, 8, and 10 were operated on prior to stage 4 (i.e., the stage when notochord formation begins; Hamburger and Hamilton, 1951). Therefore, rostral ectoderm was not exposed to cranial notochord prior to extirpation of Hensen's node. The remaining four embryos (numbers 1, 4, 7, and 9) were stage 4 or older at the time of Hensen's node extirpation and notochord had already begun to form. In all cases, *En-2* expression was localized to the appropriate mesencephalic and metencephalic levels. Planar induction between notochord precursor cells (in Hensen's node) and the adjacent neuroectoderm may have occurred at earlier stages prior to extirpation of Hensen's node.

notochord cells was as great as 900 μm. This relatively long distance makes its highly unlikely that remaining caudal levels of notochord could induce the rostrocaudally restricted gene expression occurring in the mesencephalon and cranial metencephalon levels.

En-2 expression in "inverted" embryos

Additional evidence that the cranial notochord is not required for regulated rostrocaudal expression of *En-2* was obtained from "inverted" embryos. In such embryos, the head bends backward into the hole left by surgery (Smith and Schoenwolf, 1991), and the two lateral halves of the mesencephalon form as separate open cups near the sides of the inverted head (Figure 3.2E). The two halves of the neural tube develop independently, although the relationship of each to other structures on the same side of the embryo appears normal. All "inverted" embryos immunolabeled with 4D9 in the appropriate restricted region of the neuroectoderm. Seventeen of the inverted embryos were sectioned (Figure 3.2G). Sections revealed that 29 of the 34 half-neural tubes from these 17 embryos developed without any notochord under the *En-2* expressing region. This higher rate of notochord absence (85% vs 20% for "good" and "fair" embryos) is consistent with prior observations that embryos that fail to heal properly after Hensen's node extirpation, also more frequently fail to regenerate the notochord (Grabowski, 1956). Because inverted embryos immunolabel with 4D9 and lack cranial notochord, these results show that neither adjacent notochord nor normal morphology at the mesencephalon and cranial metencephalon levels is required for regulated rostrocaudal expression of *En-2*.

Dorsoventral expression of *En-2*

Although a deficit in the adjacent notochord failed to influence the rostrocaudal expression of *En-2*, it did change patterning along the dorsoventral axis of the neural tube. In experimental embryos in which the notochord was largely intact, and in all but one control embryo, 4D9 immunolabeling was strong in the roof plate and lateral walls of the

neural tube but was weak or absent in the floor plate (Figure 3.1B). Observations by others indicate that *En-2* is lacking in the floor plate region at least until stage 13 (Gardner and Barald, in preparation). Because the floor plate is induced by the notochord (van Straaten et al., 1988; Jessell et al., 1989; Smith and Schoenwolf, 1989; Placzek et al., 1990a; Hirano et al., 1991; Yamada et al., 1991), in regions of experimental embryos in which notochord was absent, the floor plate failed to form. In these cases, 4D9 immunolabeling was observed throughout the entire dorsoventral extent of the neural tube, including its most ventral region (Figure 3.2F). This finding provides evidence that *En-2* is specifically suppressed in the floor plate owing to interaction between the notochord and overlying neuroectoderm.

DISCUSSION

Rostrocaudal pattern

The results presented here indicate that adjacent notochord is not required for the rostrocaudally restricted regulation of *En-2* expression in avian neuroectoderm. Also, notochord caudal to the mesencephalon and cranial metencephalon is not required to suppress *En-2* expression outside its normal rostrocaudal expression territory. It would seem at first paradoxical that previous experiments (Hemmati-Brivanlou et al., 1990) indicate that anterior notochord can induce a rostrocaudally restricted regulation of *En-2* expression, whereas we have shown in the present study that notochord is not required for expression. In the previous experiments, which used *Xenopus*, fertilized eggs were irradiated with ultraviolet light during the first cell cycle, and embryos were allowed to develop to the late blastula stage. Formation of dorsal mesoderm is inhibited in such embryos (i.e., they are ventralized), thereby reducing the chance that animal cap ectoderm, when isolated, would be contaminated with dorsal mesoderm. Isolated animal caps from irradiated embryos were wrapped around mesoderm obtained from unirradiated embryos, and the two tissues were co-cultured. The presence of anterior notochord resulted in the

strong expression of *En-2* in a high percentage of the recombinants (81%), but posterior notochord (or other types of mesoderm) did not (e.g., posterior notochord: 36% expressed *En-2* but at a lower level).

An obvious explanation for the discrepancy in our study and the previous one is the species difference between these two experiments (i.e., *Xenopus* and chick). Although species differences cannot be excluded, it is important to note that Hensen's node, the avian (and mammalian) equivalent (see Hara, 1978) of the amphibian dorsal lip of the blastoderm (i.e., the Spemann organizer), can evoke neural induction when co-cultured with a *Xenopus* animal cap (Kintner and Dodd, 1991). This implies that at least some of the induction signals and receptors are conserved between these organisms.

Another explanation for the discrepancy is that the notochord may be sufficient to induce regionally restricted *En-2* expression, but it may not be required for such expression, owing to the existence of both horizontal (planar) and vertical induction signals. As summarized in a recent paper (Keller et al., 1992a), planar induction of neuroectoderm is a concept that dates back to Spemann (1927; also see pp. 187-188, Spemann, 1938). Planar induction involves the passage of positional information through the plane of the ectoderm (Dixon and Kintner, 1989; Sharpe and Gurdon, 1990; Guthrie, 1991). Presumably, this patterning information could arise from prospective mesoderm (including prospective notochord) and endodermal cells contained in the dorsal lip of the blastopore (amphibians) or Hensen's node (birds and mammals). At the beginning of gastrulation, there is a close spatial relationship between cells of the prospective neuroectoderm and those of the dorsal lip of the blastopore (*Xenopus*: Keller et al., 1992b) or between cells of the prospective neuroectoderm and those of Hensen's node (chick: Schoenwolf and Alvarez, 1989; Schoenwolf et al., 1989). Also in both organisms, the prospective neuroectoderm is condensed around the area of mesoderm involution (lip) or ingression (node), and as a consequence, all rostrocaudal levels of the prospective neural tube are in close proximity to both one another and to the prospective endoderm and mesoderm. In short, because of

these complications of anatomy, the existence of planar inductions cannot be excluded in most experimental designs (see Keller et al., 1992a, b, for further discussion). Moreover, it has been suggested recently that planar induction alone is sufficient in *Xenopus* to regulate the rostrocaudal expression of *En-2* (Doniach et al., 1992). In these experiments, dorsal or ventral ectoderm was co-cultured with dorsal mesoderm in "Keller sandwiches." In both cases, ectoderm was induced to express three regionally distinct neural markers, including *En-2*, in an appropriate rostrocaudal pattern. However, as suggested elsewhere (Keller et al., 1992b), such sandwiches could have been contaminated with head mesoderm, allowing vertical induction to occur. Nevertheless, in another recent study where vertical induction was definitely ruled out and "Keller sandwiches" also were used, it was shown that planar contact alone is sufficient to induce the convergent-extension movements characteristic of many aspects of *Xenopus* gastrulation and neurulation (Keller et al., 1992a).

Taken collectively, the available data seem to suggest that both planar and vertical inductions act in regulating *En-2* expression in the neuroectoderm. One possible way this could occur is as follows. The organizer (lip or node) could pattern the adjacent neuroectoderm by releasing a morphogen that directly instructs the neuroectoderm in a planar fashion and also instructs the involution (or ingressing) mesoderm and endoderm as to their rostrocaudal positional pattern; secondarily, regionalized tissues underlying the neuroectoderm could reinforce the planar induction of the neuroectoderm by vertical induction. If this scenario is true, then it would explain how cranial notochord could be sufficient for rostrocaudal patterning of the neuroectoderm but may not be required for this event.

An ectodermal patterning gradient is consistent with the observed expression pattern of *en-2* in vertebrate embryos (i.e., decreasing from posterior to anterior) and with results obtained by grafting *en-2* expressing metencephalon into the prosencephalon (Martinez et al., 1991; Gardner and Barald, 1991). In the grafting experiments, the ectopic tissue

induced adjacent prosencephalon to express *en-2*, and in some cases (Martinez et al., 1991), to acquire a mesencephalic phenotype.

Two types of vertical induction signals in addition to those provided by the cranial notochord could be involved in reinforcing *En-2* expression. The first of these could be provided by the more caudal notochord, but this seems improbable. Posterior notochord can only weakly and infrequently induce *En-2* expression when cultured immediately adjacent to anterior ectoderm (Hemmati-Brivanlou et al., 1990). Furthermore, chick notochord is believed to be able to exert its other patterning influences on the neural tube across distances not greater than 100 μm (Smith and Schoenwolf, 1989; Placzek et al., 1990a). In the present study, the distance between the *En-2* expressing region and the beginning of the caudal notochord was as great as 900 μm in experimental embryos (Figure 3.3).

The second type of vertical induction signal could arise from other cranial tissues besides notochord, including ingressed cranial endoderm, head mesoderm, and somitic mesoderm. At stage 3, prospective endodermal cells are migrating from Hensen's node (Schoenwolf et al., 1992). Furthermore, head mesoderm and paraxial mesoderm begin their cranial migration from the streak at mid-stage 3; that is, near the time of Hensen's node extirpation in the present study. The fact that paraxial mesoderm can induce *en-2* expression, albeit at low levels (Hemmati-Brivanlou et al., 1990), implies that cranial notochord is not alone in its ability to reinforce a signal from the organizer. Thus, ingressed endoderm or mesoderm, acting through vertical reinforcement of a planar induction signal originating from Hensen's node, could also account for the rostrocaudal patterning observed in our experiments.

To summarize, in the study by Hemmati-Brivanlou et al. (1990), it was shown that *vertical* interactions increase the frequency of rostrocaudally restricted *En-2* expression, and in our study, it is shown that such expression still occurs in the absence of cranial notochord. In both cases, the existence of planar induction signals cannot be ruled out

entirely. Thus, the results of the two studies appear on further examination to be complementary rather than being mutually exclusive.

Dorsoventral Pattern

From the results of our observations and experiments, we conclude that vertical induction signals are important for the dorsoventral patterning of the cranial neural tube. When cranial notochord is present, *En-2* expression is lacking in the most ventral neural tube (i.e., floor plate), but when cranial notochord is absent, the floor plate fails to form and *En-2* expression occurs throughout the entire dorsoventral extent of the neural tube, including its most ventral aspect. Suppression of *En-2* expression in the most ventral neural tube by interaction with the notochord indicates that dorsoventral patterning overrides rostrocaudal patterning or that there is a different mode of rostrocaudal patterning for the floor plate as compared with that of the lateral walls. This latter possibility is consistent with the report that in the chick hindbrain, the lateral walls of the neural tube are segmented rostrocaudally, but the floor plate is not (Fraser et al., 1990).

Our observation that *En-2* is not expressed in the chick floor plate at stage 9, combined with our result that the notochord suppresses *En-2* expression in this region, reveals an early molecular difference between floor plate and lateral wall cells that is controlled by cell-cell interactions. A similar expression pattern for floor plate and lateral wall cells seems to hold for other homeobox genes as well (i.e., Hox 1.4, Toth et al., 1987; Hox 2.9, Frohman et al., 1990; Ghox-lab, Sundin and Eichele, 1990; *En-1*; Davis et al., 1991; Gardner and Barald, in preparation). However, in these cases it is unknown whether the notochord plays a role in dorsoventral patterning. Other molecular activities found later in floor plate, but not lateral wall, cells include: expression of FP1 antigen at stage 10 (Yamada et al., 1991), stronger retinoid production (Wagner et al., 1990), cellular retinoid binding protein production (Maden et al., 1989), the ability to induce laterality of spinal cord connections and influence motor neuron proliferation or differentiation (Clarke

et al., 1991; Yamada et al., 1991), and release of diffusible chemoattractants (Tessier-Lavigne et al., 1988; Placzek et al., 1990b). Whether the notochord establishes all these differences, or only some of them, is unknown.

Summary

In summary, our experiments show that although the notochord is required for dorsoventral patterning of the cranial neural tube, as assessed by *En-2* expression, it is not required for rostrocaudal patterning. What is required for this latter patterning remains to be determined experimentally.

CHAPTER 4

MAB-4D9 AS A MARKER FOR ANALYSING NEURAL PATTERN

INTRODUCTION

A general model currently being considered for the instigation of developmental processes is as follows: chemical or electrical gradients are established along dorsoventral and rostrocaudal axes. In response to these signals, master regulatory genes are activated and in turn regulate the activation and repression of a cascade of genes for both regulatory and structural proteins that ultimately give rise to regionally differentiated (patterned) tissues. Retinoic acid has been implicated as a morphogen in the development of the central nervous system (Mitrani and Shimoni, 1989). Homeodomain-containing proteins, which are putative transcription factors, have been implicated in the regulatory cascade and used as markers for regional differentiation. As I have previously shown, chick *En-2* expression can be used to elucidate both rostrocaudal and dorsoventral patterning of the cranial neuroectoderm in the presence of notochord (Chapter 3). Rostrocaudal pattern is revealed by *En-2* expression restricted to the mesencephalon and rostral metencephalon and a subset of the neural crest and overlying surface ectoderm of this region. Dorsoventral pattern is typically revealed by the expression of *En-2* in the roof plate and lateral walls and its suppression in the floor plate. In the absence of notochord, floor plate fails to form and *En-2* is expressed in the most ventral region of the neural tube as well.

These observations and those of others indicate that, in addition to the notochord, other mesoderm-ectoderm, endoderm-ectoderm and ectoderm-ectoderm interactions are probably required for the induction and patterning of the neural tube. To further address the relationship between the endoderm, mesoderm and ectoderm and to investigate the possible involvement of one potential morphogen in patterning the developing neural tube, four additional experiments were conducted using chick embryos manipulated in culture and the MAb-4D9 antibody as a marker for patterning of the mes-metencephalic region of the rudimentary CNS.

Transected Embryos

In *Drosophila,* the anterior half of the embryo appears to be organized rostrocaudally by the generation of a gradient of the Bicoid protein. Separation of the *Bicoid* mRNA in the anterior end of the embryo from the remainder of the embryo results in developmental defects in the patterning of the head and thorax (for review see St Johnston and Nüsslein-Volhard, 1992). Thus a continuity between the anterior pole of the embryo and the more caudal regions that will form the thorax is required for the patterning of the thorax.

Within the chick embryo, electrical currents have recently been implicated in the formation of the structures of the dorsal axis. Disruption of these currents results in abnormalities in cranial and caudal CNS development (Hotary and Robinson, 1992). Chemical gradients have also been proposed to explain patterning in chick limb (Thaller and Eichele, 1987; Summerbell and Maden, 1990; Tickle, 1991) and chick rostrocaudal axis (Mitrani and Shimoni, 1989). The ability of such a gradient to influence regions distal to the source may be dependent on physical continuity.

To ascertain whether the rostrocaudal continuity of any signal or mechanism, such as a morphogen gradient or electrochemical gradient, is required to establish and pattern the neural axis in chick, I chose to separate the rostral and caudal halves of the embryos from one another at stages 3c-5. In this experiment, embryos were transected perpendicular to the primitive streak and cultured for 24 hours. Half embryo development was evaluated by observing obvious morphology and patterning of the mesencephalon and rostral metencephalon was detected by observing the immunolabeling pattern of antibody 4D9.

Retinoic Acid Treatment

Retinoic acid is a known teratogen and embryonic exposure to RA results in cerebellar hypoplasia and aplasia, microcephaly and other cranial neural defects, craniofacial, heart and limb defects in humans and lower vertebrates (Lammer et al., 1985).

Treatment with all trans RA interferes with the development of the midbrain and rostral hindbrain and patterns of expression of some homeobox containing genes in chick, frog and fish. In mouse, RA apparently suppresses the development of the rostral hindbrain and interferes with the expression of two homeobox genes expressed in the region when administered during neurulation but before first somite formation (Morriss-Kay et al., 1991). Addition of exogenous RA to zebrafish embryos interfered with the development of the CNS by specifically inhibiting development of the caudal midbrain and rostral hindbrain and eliminating the expression of *En-2* in this region (Holder and Hill, 1991). Differences between the RA effects on amphibians, fish and mammals may be the result of differences in RA receptor and binding protein distribution. CRABP expression in mouse mesencephalon and rostral metencephalon correlates with *En-2* expression, with expression higher caudally than rostrally and lacking entirely in the floor plate (Ruberte et al., 1991). In this experiment, I sought to determine if retinoic acid (RA) could interfere with either *En-2* expression or the development of the mes-metencephalon region of the rudimentary chick CNS.

Separation of lateral ectoderm from medial mesoderm at stage 3b-d

Prospective midbrain ectoderm has been mapped to areas adjacent to Hensen's node (Schoenwolf and Sheard, 1990; also see other transection experiment, this chapter), and prospective notochord maps to the region within the streak and just caudal to Hensen's node at stage 3b and c, at Hensen's node at stage 3d and just rostral to Hensen's node at stage 4 (Hara, 1978). In this second transection experiment, the blastoderm laterally flanking the node at stage 3b-d (future midbrain neuroectoderm and surface ectoderm; referred to in this experiment as the ectoderm fragment) was surgically separated from the primitive streak and Hensen's node (future endoderm, notochord and other mesodermal tissues; referred to as the mesoderm fragment) and both fragments were cultured. The purpose of this experiment was to investigate the interdependence of the mesoderm and

ectoderm rudiments and the requirement for inductive or cell-cell interactions between the node/streak and future neuroectoderm in the patterning of the neuroectoderm. Induction of neuroectoderm and patterning of that tissue (detected by 4D9 immunolabeling) in ectoderm fragments separated from the notochord would be definitive evidence that the notochord itself is not required for the induction and patterning of the neural tube. This would confirm that either planar contact with the node or vertical contact with endoderm and non-notochordal mesoderm is sufficient for induction and patterning of the mesencephalon.

Expression in Ectopic Embryos

Several authors have proposed models in which multiple signals or competencies reinforce one another to achieve neurulation. Spemann's "double assurance" is a classic example of this. He proposes that both the mesoderm and ectoderm have some capacity to induce or self differentiate, respectively, and that these two processes reinforce one another to instigate the differentiation of the neural tube under normal circumstances. A question remains whether either source of inducing/differentiating and patterning information can act independently to generate a rostrocaudally patterned neural tube. To test one half of this hypothesis, one can ask whether the organizer can induce non-neural ectoderm to form neural plate/tube. In both frog and chick, the experiment can be done by explanting the organizer (in chick, Hensen's node), which is the source of rostral axial mesoderm, adjacent to non-neural ectoderm (surface or extra-embryonic) and evaluate the resulting structures after culture. If neuroectoderm develops in the ectopic embryo, one can then ascertain whether it is from induced host ectoderm or from 'contaminating' graft ectoderm. Differentiated graft ectoderm only would indicate that the organizer or axial mesoderm was not capable of inducing non-neural ectoderm to differentiate into neural structures. Differentiated host neuroectoderm, on the other hand, would indicate that the organizer or its progeny tissues contain sufficient information to induce neurulation. It is, in fact, the

definition of the organizer that it can induce a new axis and neurulation in such a graft experiment (Spemann, 1927, as cited in Hamburger, 1988).

In avian chimera experiments, quail Hensen's node can be explanted under germinal crescent ectoderm of a chick host and because of a quail specific nucleolar marker (Le Douarin, 1969), the differentiating ectopic neuroectoderm can be distinguished as chick or quail. In this way, induction can clearly be distinguished from self-differentiation. In a definitive experiment of this type, neuroectoderm was induced most frequently in young (3a-3c) or intermediate (3d-4+) stage hosts with young or intermediate stage grafts. Older hosts or grafts (stage 5-8) tended to result in self differentiated graft neuroectoderm rather than induction (Dias and Schoenwolf, 1990; Storey et al., 1992). In addition, the Dias and Schoenwolf observed that, irrespective of graft age, neural induction always resulted in the formation of prosencephalon. That is, neuroectoderm arising as a result of neural induction always began, by structural criteria, at the rostral extreme of the neural plate. In self-differentiated neuroectoderm, on the other hand, older grafts appeared to give rise to more posterior neural structures without being preceded in the graft by rostral neuroectoderm. In either case, however, regional specification of the neural axis was generated in the ectopic embryo. These observations raise questions about the patterning mechanisms involved in neurulation as well as the inductive competencies of the grafted organizer.

In this experiment, I attempted to determine whether self-differentiated and induced neuroectoderm in ectopic, chimaeric embryos expressed *En-2* as a marker of midbrain differentiation. Previous patterning information on ectopic embryos was dependent on morphological criteria for determining the positional character of the developed neuroepithelium. Frequently, however, ectopic embryos contain vesicles or structurally ambiguous neuroepithelium. Using the *En-2* antibody as a marker for midbrain, I was able to show that both induced and self-differentiating ectopic neuroectoderm can express *En-2*, and that that expression is regionally localized to positionally appropriate regions, when these can be ascertained.

MATERIALS AND METHODS

Transected Embryos

Embryos were isolated and cultured according to Spratt (Spratt, 1947) In brief, blastoderms of stage 3 or 4 embryos (exp. 1) or stage 3b-d (exp. 2) were isolated with the vitelline membrane into a dish of 123mM NaCl, freed of the vitelline membrane and cultured on a plate containing 123mM NaCl mixed 1:1 with thin albumen. On the plate, blastoderms were bisected above, through or below Hensen's node using a glass needle. The two halves were floated apart, the dish was drained of excess saline and the halves were cultured for 24 hours at 37°C in a humid environment. Embryo halves were stained with MAb-4D9 as specified previously (Chapters 2 and 3). Embryos in which the precursor of the mesencephalic ectoderm was dissected away from the chordamesoderm were treated similarly.

Retinoic Acid Treatment

Embryos treated with RA were isolated for New culture as mentioned above except, for the first 4 hours they were cultured with 0, 6 or 10 μM all-*trans* retinoic acid (RA) both in the plates and diluted in 123mM salt (1:1000) in 4 drops of liquid over the top of the culture (Olof Sundin and Gregor Eichele, manuscript in preparation). After 4 hours, New cultures were washed with RA-free 123mM salt solution and transferred to a RA-free plate for 24 hours culture as above. Embryos were photographed, immunolabeled with MAb-4D9, embedded and paraffin sectioned.

Ectopic Embryos

Chicken eggs were incubated for 16-24 hours. Vitelline membranes with attached blastoderms were explanted to New culture (New, 1955) as modified by Dias and Schoenwolf (Dias and Schoenwolf, 1990). Quail graft embryos were removed from their vitelline membranes and placed dorsal side up in Spratt culture (Spratt, 1947). Embryos

were staged by traditional criteria (Hamburger and Hamilton, 1951) with stage 3 sub-divided into four categories dependent on the length and structure of the primitive streak (Schoenwolf, 1988). Hensen's nodes were cut from quail blastoderms with a glass needle and transferred to the chick host blastoderm in a capillary pipette. Nodes were inserted beneath the surface ectoderm within the germinal crescent. Embryos were cultured for 24-30 hours, fixed in 10% formaldehyde and immunolabeled with MAb-4D9 to detect midbrain En-2 immunolabelling in the ectopic embryos. Formaldehyde is not the standard fixative for Fuelgen stain but was required for MAb-4D9 immunolabeling. Fuelgen stained nucleoli could be detected in quail cells under the microscope. They were, however, less distinctive than in embryos fixed with Carnoy's fixative. Nine ectopic embryos were sectioned and Fuelgen stained to determine whether the immunolabelled cells were host or graft neuroectoderm.

RESULTS AND DISSCUSSION

Transected embryos

In the first experiment, embryos between stages 3b and 5 were transected at varying rostrocaudal levels before culture (Figure 4.1). Overall developmental morphology and the *En-2* immunolabeling pattern was reviewed after culture. Expression of *En-2* was used as a molecular marker of mesencephalic/rostral metencephalic development. I inferred from the independent development of the separated halves of the embryos, that a continuous chemical or electrical gradient or signal was not required for the induction and patterning of embryo axis between stage 3 and 10 (approximate stage after 24 hours culture). The location of the *En-2* labeling region after transection confirmed previous fate maps for various rostrocaudal levels of the future neuroectoderm.

Twenty-two of 31 embryos bisected at the level of the node between stages between 3b and 5 developed independent halves, with prosencephalon (and heart) development rostrally and somites and spinal cord development caudally, indicating that a continuous

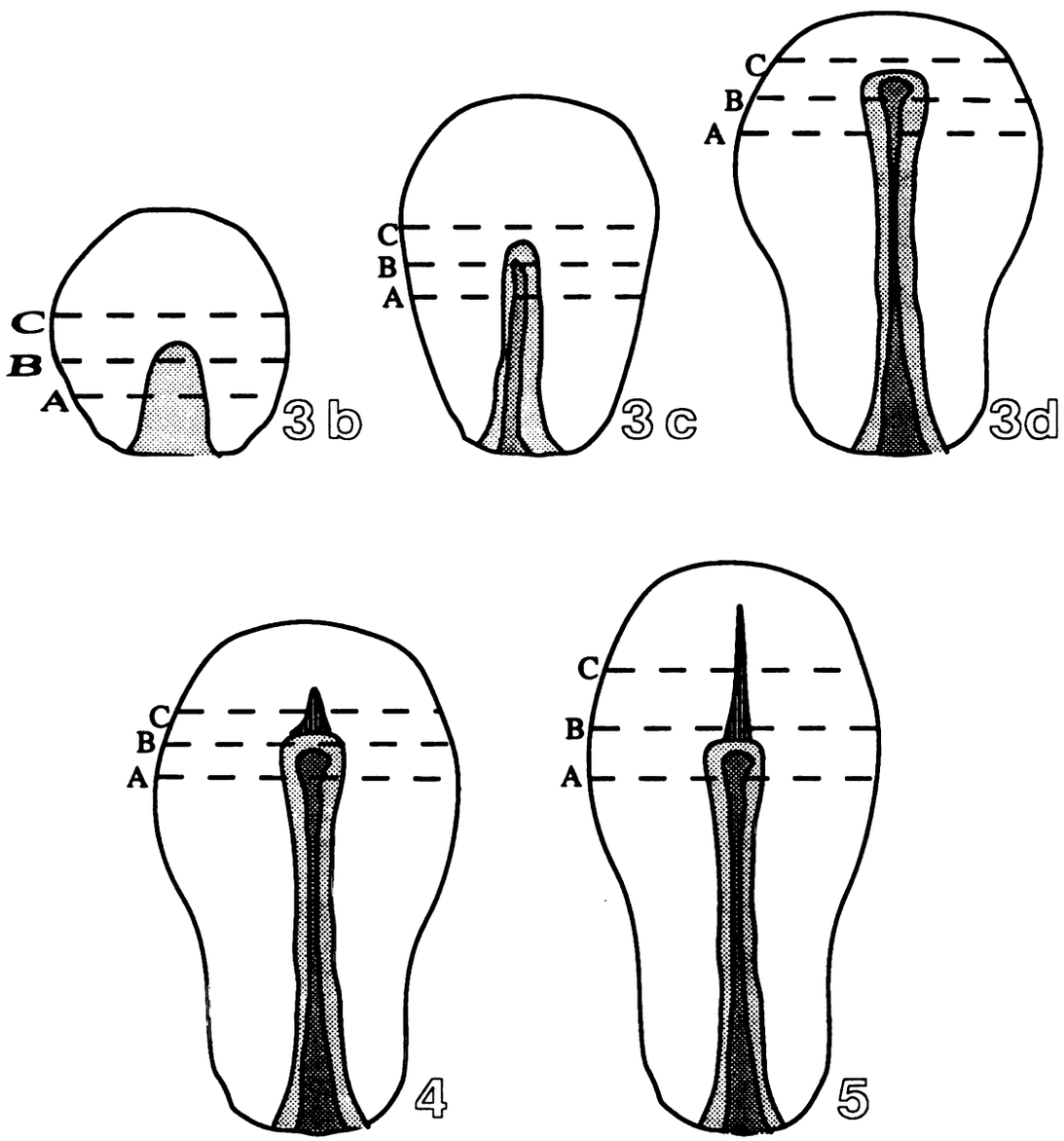


Figure 4.1 Cartoon of transected embryos prior to culture. Embryos at stages 3b-5 (labeled lower right of each cartoon) were transected at on of three levels: A) below Hensen's node, B) at the node or C) above the node. Transection at B divided the developing embryo through the mesencephalon region. Transection at A resulted in mesencephalon development in the rostral half embryo whereas transection at C resulted in mesencephalon development in the caudal half.

gradient between the rostral and caudal ends of the embryonic disc was not required beyond stage 3b to specify positional information for the developing neural tube. This morphological result is identical to the result achieved by Schoenwolf and co-workers (1989), who were looking at neural structure in bisected embryos. However, their conclusions were dependent on the interpretation of morphological characteristics to identify regionalization, whereas I also used a regional molecular marker, *En-2*. Since morphology was frequently abnormal at the level of the cut, this was helpful to identify convincingly the mes/metencephalon in embryos cut at the level of the node. Immunolabeling for *En-2* expression as a marker of midbrain/ rostral hindbrain differentiation indicated that stage 3c-d embryos cut just below the level of Hensen's node (Figure 4.1, cut A; Figure 4.2A and 4.3A), 4D9 labeled midbrain and attached prosencephalon and diencephalon (by morphology) developed in the rostral half of the embryo, whereas rhombencephalon and spinal cord developed in the caudal half. Similar results were achieved for stage 4 embryos cut just below the level of the node, and stage 5 embryos cut at the level of the node (Figure 4.1, cut A; data not shown). For stage 3c-4 embryos cut through the level of the node (cut B), the *En-2* expressing region could be split and MAb-4D9 immunolabeling could be seen in both rostral and caudal halves of the cultured embryos (stage 3d; Figure 4.2B and 4.3B). Stage 3c-d embryos cut just rostral to the node (cut C) most often had immunolabeling against the *En-2* protein in the caudal half of the embryo, with the cranial part developing only apparent prosencephalon (stage 3d; Figure 4.2C and 4.3C). Embryos transected significantly rostral to or caudal to the node resulted in one piece (node containing) which formed a nearly complete embryo and one piece (nodeless) which frequently failed to develop any obvious axial structures (data not shown). This is also consistent with the similar experiments mentioned above (Schoenwolf et al., 1989). However, this result needs to be confirmed with markers to more rostral and caudal structures, such as antibodies against other regionally restricted homeodomain proteins, so that identification of positional development can be made definitively.

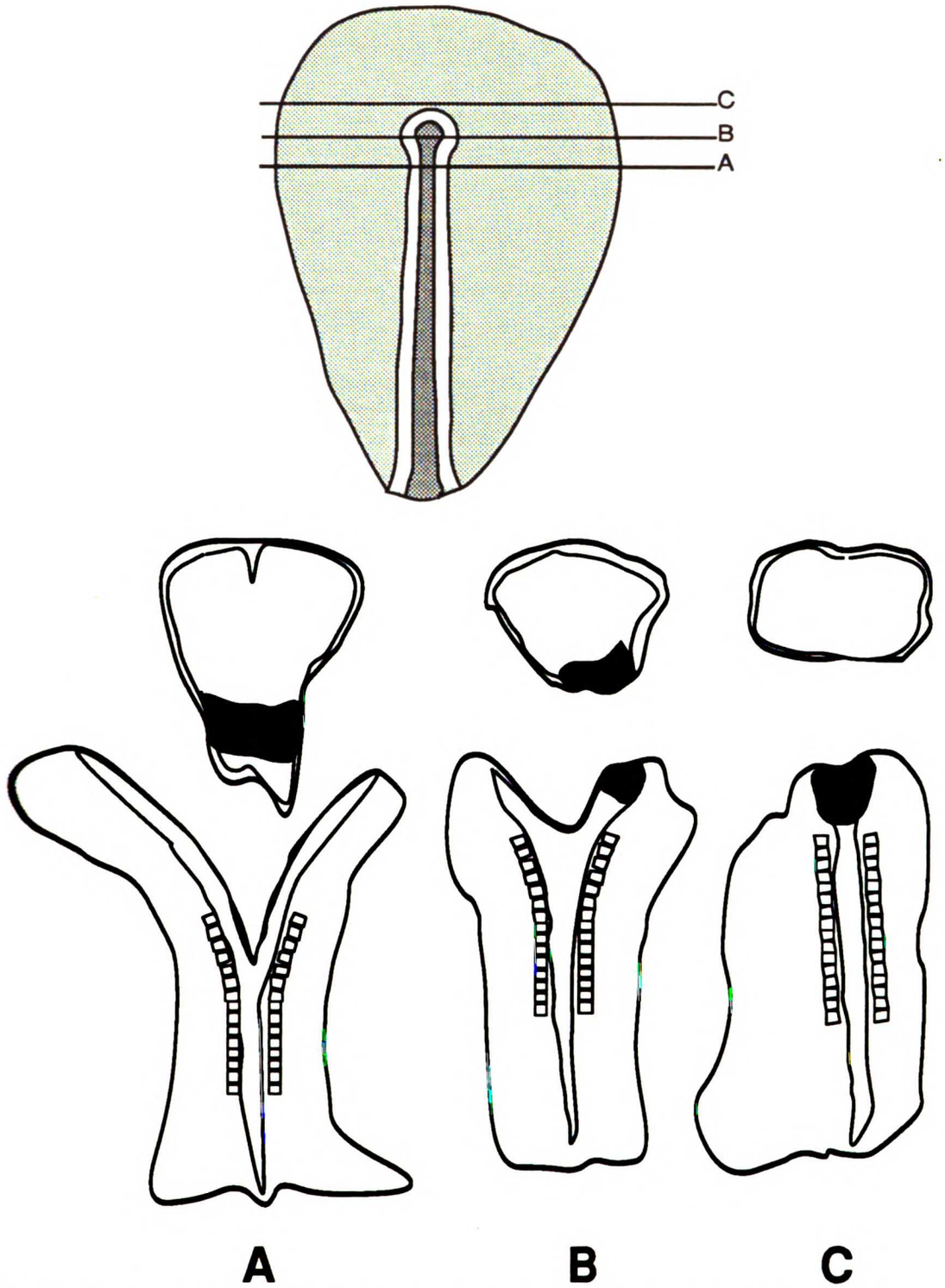


Figure 4.2 Cartoon of stage 3d embryo transection experiment and the results of **monoclonal** antibody 4D9 labeling of transected embryos after 24 hours in culture. See **legend** and photos in Figure 4.3.

Figure 4.3 Photographs of embryos transected at stage 3d, cultured for 24 hrs and immunolabeled with 4D9. (Cartooned in figure 4.2) Photos show "half" embryos immunolabeled with MAb-4D9. Embryos cut caudal to the node at stage 3d result in half embryos as shown in A that express *En-2* in the rostral half. Embryos cut through the node at stage 3d result in half embryos as shown in B that express *En-2* in both rostral and caudal halves indicating that the node lies at the level of the future mesencephalon/metencephalon at this stage. Embryos cut rostral to the node at stage 3d result in half embryos as shown in C that express *En-2* in the caudal half of the embryo.

A
A
B
B
C
C

A morphogen gradient has been proposed as one model for establishing positional information and pattern in the developing embryo (for review see Wolpert, 1989). Two gradient models are consistent with the observations made in this experiment; a double gradient, organized in both rostral and caudal directions from the central node and a stabile gradient already established before the stage of transection. Considering the first model, cuts through the node region would separate the two halves of the double gradient but leave each half to influence the respective half embryo's development resulting in half embryos that develop normally. In this model, either the source or sink of the gradient (the highest or lowest concentration/intensity of the signal) might occur at the node. Whether the chemical or electrical gradient that influences the patterning of the neuroectoderm is a morphogen gradient cannot be determined by this experiment. The second model calls for a totally stabile gradient (any orientation). This does not account for the observation that transections at a distance from the node do result in a loss of development in the distal fragment,. However, these observations have not yet been supported by markers for rostral or caudal positional genes and, therefore, this model cannot be eliminated. It is clear, however, that no continuous, unstable rostrocaudal signal is required beyond stage 3c to pattern the neuroectoderm.

A model with Hensen's node as a transition point between two gradients of morphogen (the conjoined-gradient model; Figure 4.4) is consistent with the regular initiation of *Antennapedia* and *Bithorax*-like homeobox (Hox) genes caudal to the midbrain in mouse and other vertebrates. Many of these Hox genes are expressed in patterns that begin abruptly at anterior segment boundaries (a threshold response?; for a recent review see McGinnis and Krumlauf, 1992). Another group of homeobox genes is expressed in a similar but mirror-image pattern rostral from the midbrain into the forebrain (Edoardo Boncinelli, personal communication).

Between these two sets of homeobox genes, and expressed at the level of Hensen's node prior to neurulation, are the *engrailed* genes. The *En-2* gene is expressed in a discrete

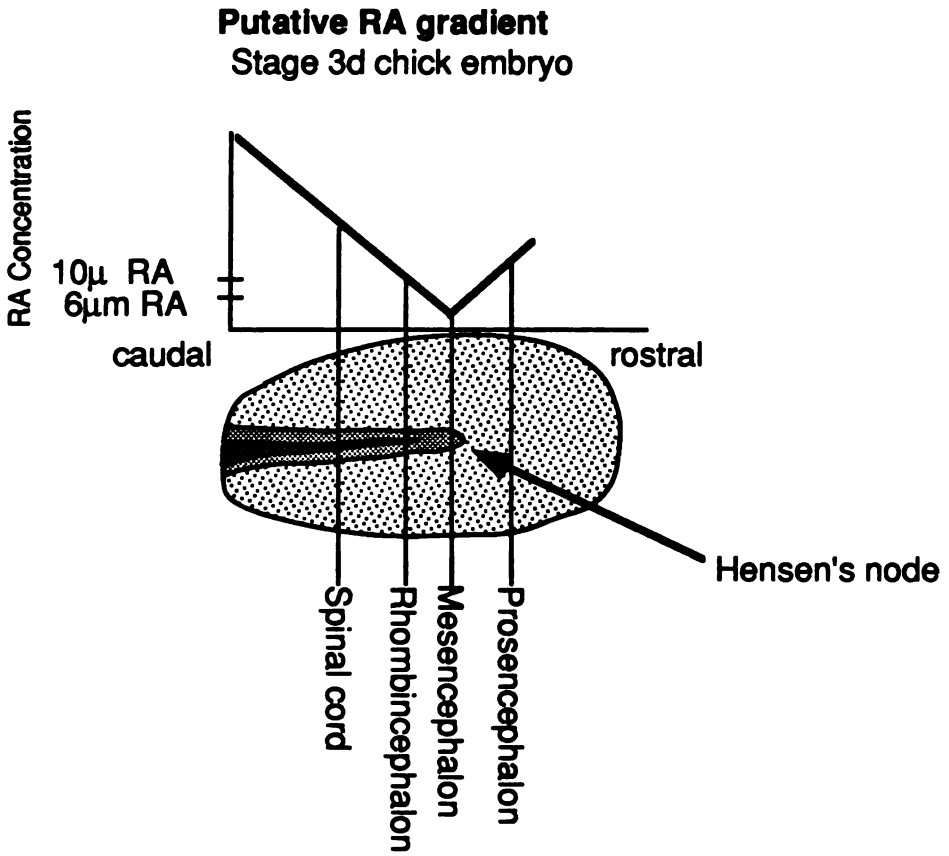


Figure 4.4 A conjoined-gradient model of retinoic acid concentration in the stage 3d chick embryo. The chart at the top of the figure shows putative RA concentrations across the length of the stage 3d chick embryo diagramed below. Hensen's node and the level of the mesencephalon fall at the low point of the proposed RA gradient. The concentration increases along the Y-axis.

band across the mesencephalon and rostral metencephalon which diminishes rostrally. *En-1* overlaps with *En-2* but its expression also extends a bit more rostrally, and caudally into the hindbrain and spinal cord like other homeobox genes expressed caudal to the mesencephalon (Davis and Joyner, 1988; Gardner and Barald, in prep). Thus, many homeobox genes follow an expression pattern consistent with two conjoined gradients of a patterning morphogen: one that extends from the node/midbrain rostrally, and another that extents from the node/midbrain caudally. This evidence does not specifically address whether the gradients increase or decrease in the proximity of the node, nor whether the node makes the morphogen or some regulator of it.

Another piece of evidence is consistent with the conjoined-gradient model in which the node is the lowest point in both gradients and involves retinoic acid regulation of Hox gene expression. The sensitivity to regulation by RA of the Hox genes expressed caudal to the mesencephalon has been determined in embryonal carcinoma cells, with anterior genes having high RA responsiveness (needing lower concentrations of RA to become activated) and posterior genes having low responsiveness (requiring high concentrations of RA for activation; Papalopulu et al., 1991; Simeone et al., 1990). A conjoined-gradient of RA with Hensen's node as the low point in both gradients could be generated by an up-regulation of the cellular retinoic acid binding protein (CRABP) in the vicinity of the node. CRABP has been shown to regulate RA availability by sequestering free RA and reducing the level of RA available for other uses. A high concentration of CRABP at the level of the node, decreasing both rostrally and caudally, in the presence of a consistent original concentration of free RA would generate a gradient like the one modeled above, with the lowest free RA concentration at the node and increasing concentrations distally.

If a dearth of RA is the hallmark of the node and, at the stages used in this experiment the future mesencephalic ectoderm is fate mapped to lie immediately adjacent to the node, then perhaps the cells forming the mesencephalon have the lowest threshold of

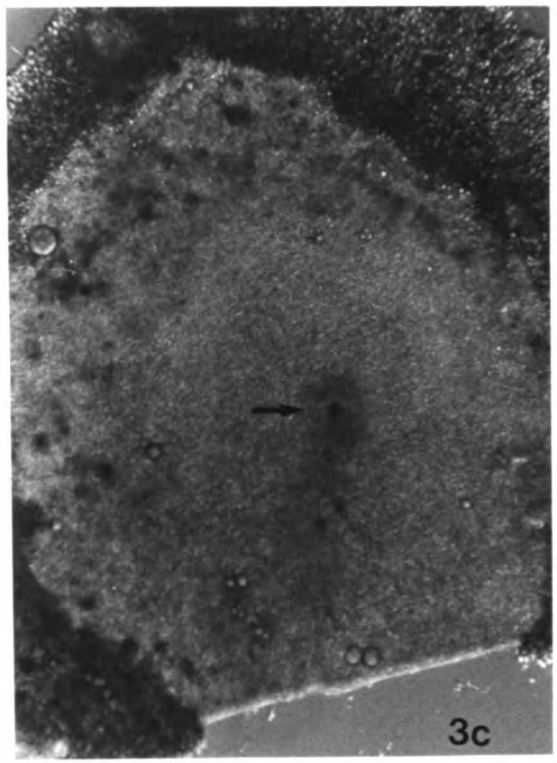
response, ie. the greatest sensitivity, to RA. If this is true, then increasing the concentration of RA these cells are exposed to might alter their developmental fate.

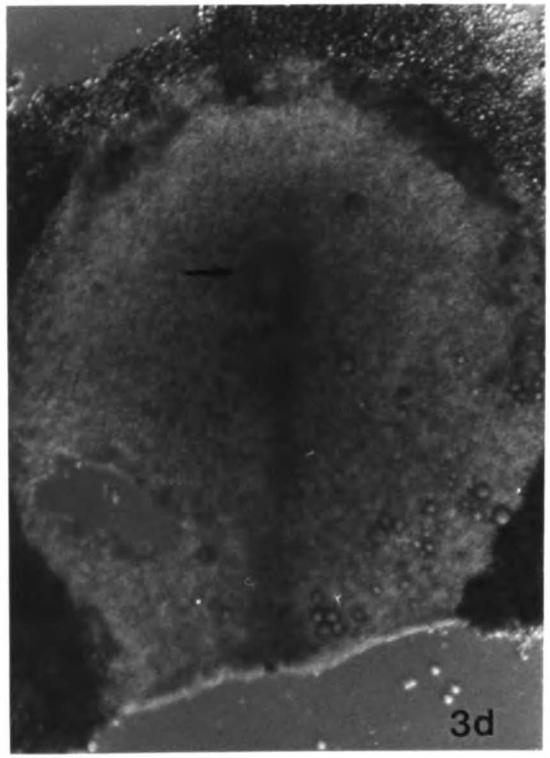
Retinoic Acid treatment of early chick embryos.

To determine if all *trans* -retinoic acid, a developmental morphogen/teratogen, was influencing development of the mesencephalon and rostral metencephalon region in chick, stage 3b-5 chick embryos were cultured in New culture in the presence of 0, 6 or 10 μm RA. After 4 hrs of exposure, embryos were rinsed and transferred to New culture, free from RA. After 24 hours, embryos were fixed and immunolabeled with MAb-4D9. Embryos at stages 3c, 3d and 4 (Figure 4.5) gave results that varied by the age at the time of treatment. Embryos treated with RA at stages 3b and c generally failed to develop or were severely retarded, with development arrested prior to or during early neurulation (Figures 4.6 and 7). None of these embryos expressed *En-2* , but this could be expected since normal expression does not begin until the embryos develop to the 4 somite stage. In previous experiments (Chapter 3), severely abnormal embryos, but not severely retarded embryos have expressed *En-2* .

Embryos treated at stage 3d, when the primitive streak is fully extended but just before exiting notochord mesoderm is visible rostral to Hensen's node, developed good axes with increasingly diminished rostral/midbrain structures as RA concentrations were increased (Figures 4.8-9). MAb-4D9 immunolabeling of these embryos indicated that the size of the *En-2* expressing region and the intensity of *En-2* expression was diminished in 6 μm RA treated embryos (Figure 4.8B and 4.9B) and absent or severely diminished in 10μm treated embryos (Figure 4.8C and 4.9C). Sections of these embryos revealed that the rostral neuroectoderm was frequently a neural plate or cord rather than a tube with a lumen, and that it was rarely covered with surface ectoderm (Figure 4.10). In sections, small numbers of lightly labeling neuroectoderm cells could be seen in the 10μM treatment that were not visible in whole mount embryos (Figure 4.10 C and D).

Figure 4.5 Photographs of three chick blastoderms at stage 3c, 3d and 4. (A) shows a chick blastoderm at approximately 16 hours after laying; stage 3c. The primitive streak is the vertical structure extending from the caudal margin of the disk. Hensen's node at the rostral margin of the streak is marked with an arrow. (B) shows a chick blastoderm at approximately 18 hours after laying; stage 3d. Hensen's node is labeled (arrow) and the primitive streak is fully extended. (C) shows a chick blastoderm at approximately 20 hours after laying; stage 4. Hensen's node (arrow) has begun to regress and the notochord cells involuting through it are migrating rostrally underneath the ectoderm.


3c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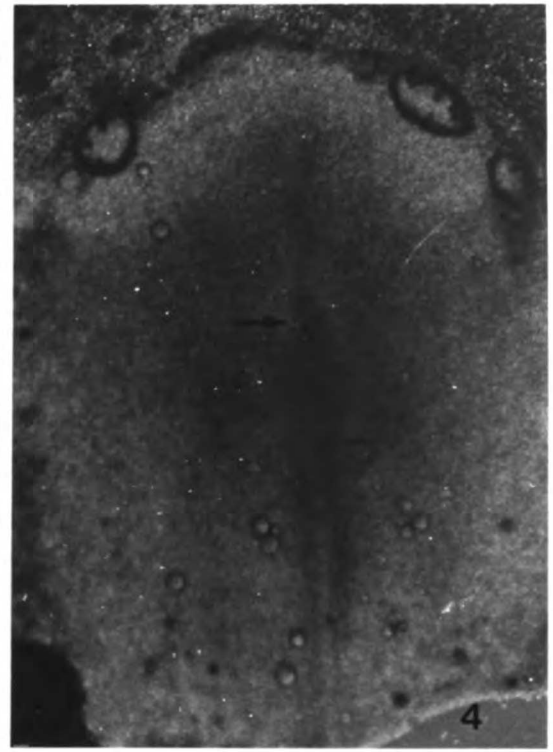
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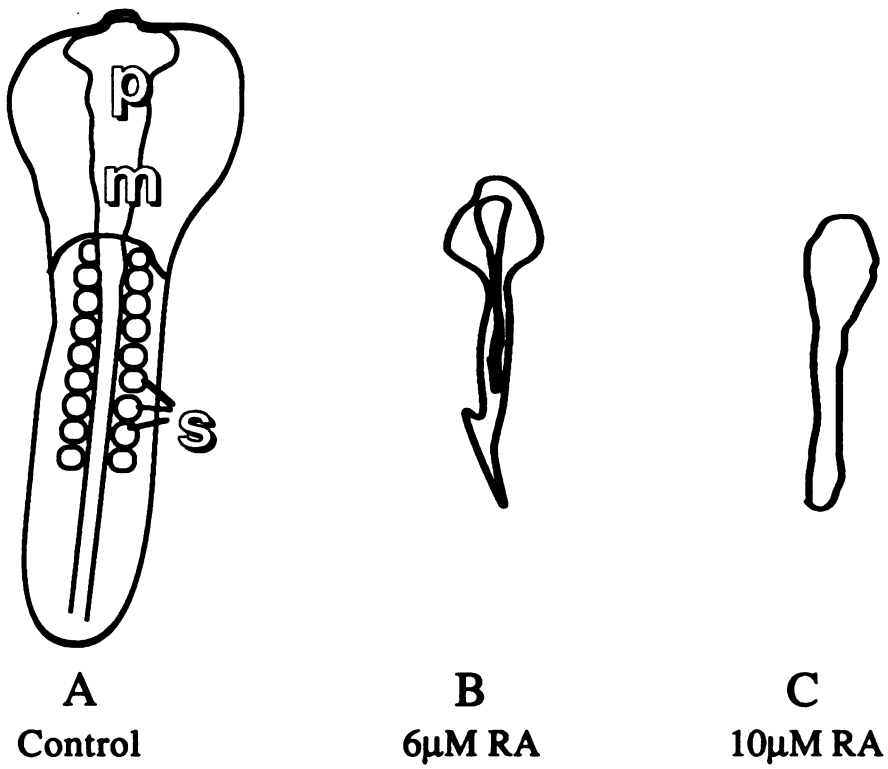


Figure 4.6 A cartoon of embryos treated with RA at stage 3c and cultured for 24 hours (see figure 4.7 for actual photos). Control embryos (A) developed normally, including the differentiation of prosencephalon (p), mesencephalon (m) and somites (s), whereas embryos treated with 6μM RA (B) or 10μM RA (C) failed to develop or developed only to the early neural plate stage.

Figure 4.7 Photographs of embryos treated with RA for 4 hours beginning at stage 3c and followed by 24 hours in culture. Panels A and A' show control embryos, panels B and B' show embryos exposed to 6μM RA and C and C' to 10μM RA. Embryos in panels B and B' developed very limited axes and some neural plate ectoderm. Embryos in panels C and C' failed to develop except for minor axial extension.

A'
A
B'
B
C'
C

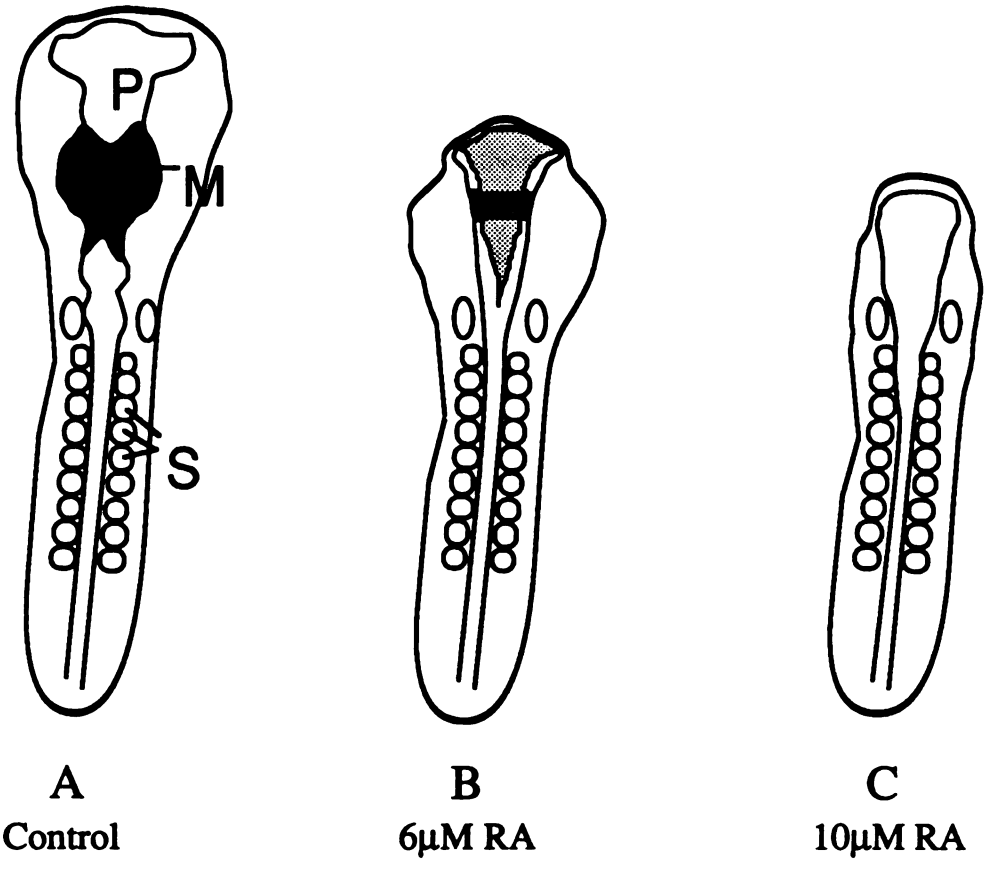


Figure 4.8 A cartoon representation of embryos treated with RA at stage 3d and cultured for 24 hours. (For actual photographs see figure 4.9) Control embryos (A) develop normal prosencephalon (P), mesencephalon (M) and somites (S) and express *En-2* in their mesencephalon and rostral metencephalon (shown as a solid black area). Embryos treated with 6μM RA (B) express *En-2* in a smaller area, and at lower levels (black and dark stippling). Some of these embryos also had dysraphic (open) rostral neural tubes (stippled area). Embryos treated with 10μM RA (C) failed to express *En-2* or expressed it at levels undetectable in whole mount embryos. These embryos also frequently had dysraphic rostral neural tubes and poor development of structures rostral to the mesencephalon..

Figure 4.9 Photographs of embryos treated with RA at stage 3d and cultured for 24 hours. Embryos in column A are controls treated with 0μM RA; these display a normal En-2 expression pattern. Embryos in column B were treated with 6μM RA and have diminished En-2 expression and dysraphic rostral neural tubes in some cases. Embryos in column C were treated with 10μM RA and do not appear to express En-2. These embryos also had frequent dysraphic rostral neural tubes and abnormal anterior head structures. For all three treatments, the spinal cord and somites were normal.

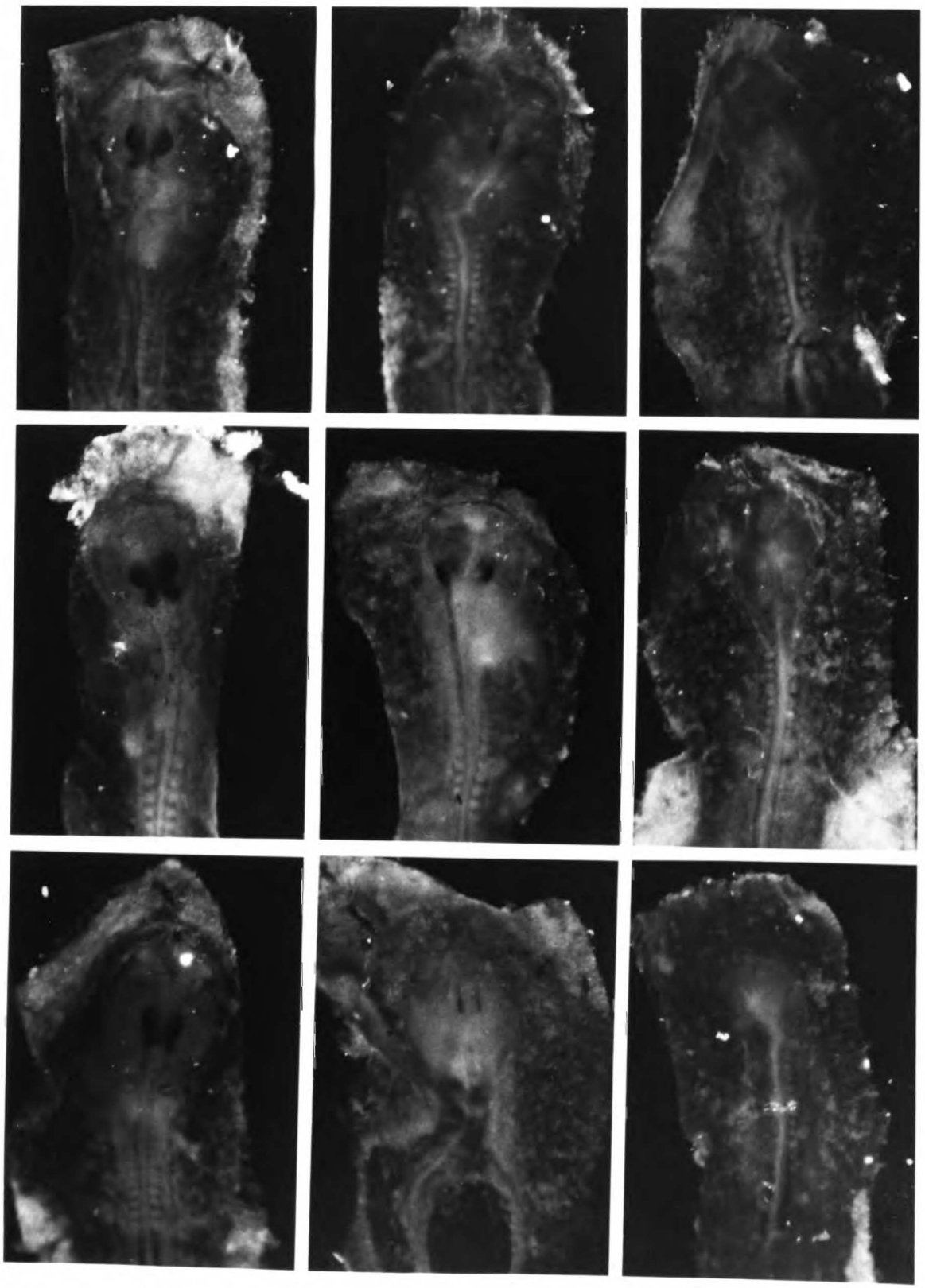

A
B
C

Figure 4.10 Photographs of cross sections from embryos treated with RA at stage 3d and cultured for 24 hours. Embryos treated with 0μM RA (A) show normal morphology and immunolabeled with 4D9 (arrowhead), whereas those treated with 6μM RA (B) showed reduced rostrocaudal En-2 expression (arrowhead) and dysraphic neural tube in some cases (star). Embryos treated with 10μM RA (C and D) had no expression of En-2 or significantly reduced expression in a few neuroectodermal cells (arrowheads). The rostral neural tubes of these embryos lacked a central cavity (C) or were dysraphic (D).

A
B
C
D

Embryos at stage 4 or older treated with RA were most likely to have nearly normal morphology and express *En-2* in a rostrocaudal pattern similar to control embryos (Figures 4.11-13). RA did appear to affect head shape, reducing the size of the region surrounding the prosencephalon. The severity of this result was dependant on the concentration of RA (Figures 4.12-15). In cross section (Figure 4.16) it appeared that anterior mesoderm was present and surface ectoderm volume was diminished. This may be due to a reduced fluid pressure within the embryo or simply a growth retardation in the part of the embryo developing most rapidly during the four hour RA treatment (the region rostral to the node, the prosencephalon region).

In retinoic acid treated embryos, the loss of *En-2* expression in stage 3d but not stage 4 embryos indicates that there is a fairly narrow window (2-3 hours) during which exogenous RA can influence the development of the chick mesencephalon and rostral metencephalon in this way. Later exposure (stage 4) appears to influence mesoderm migration in the rostral head and surface ectoderm volume and, therefore, head shape, but has no effect on the regionalization of the mesencephalon as assayed by En-2 expression in a distinct rostrocaudally defined band. Most teratogens are know to exert their influence during a limited period due to the interruption of a specific developmental pathway or function. It is possible in this case that exogenous RA is interfering with an endogenous RA gradient, perhaps by overwhelming the cellular RA binding proteins or RA receptors (CRABPs or RARs), therefore, disrupting the normal quantities of free RA available, and altering the regionalization within the neuroectoderm. This is easiest to imagine if the RA gradient appropriate for pattern induction is high distal to the node and low proximal to the node, as in the conjoined gradient model described in the previous transection experiment. The addition of exogenous RA would fill in the trough in the conjoined-gradient and one would see loss of the structures (midbrain) that require the lowest threshold of RA (Figure 4.4).

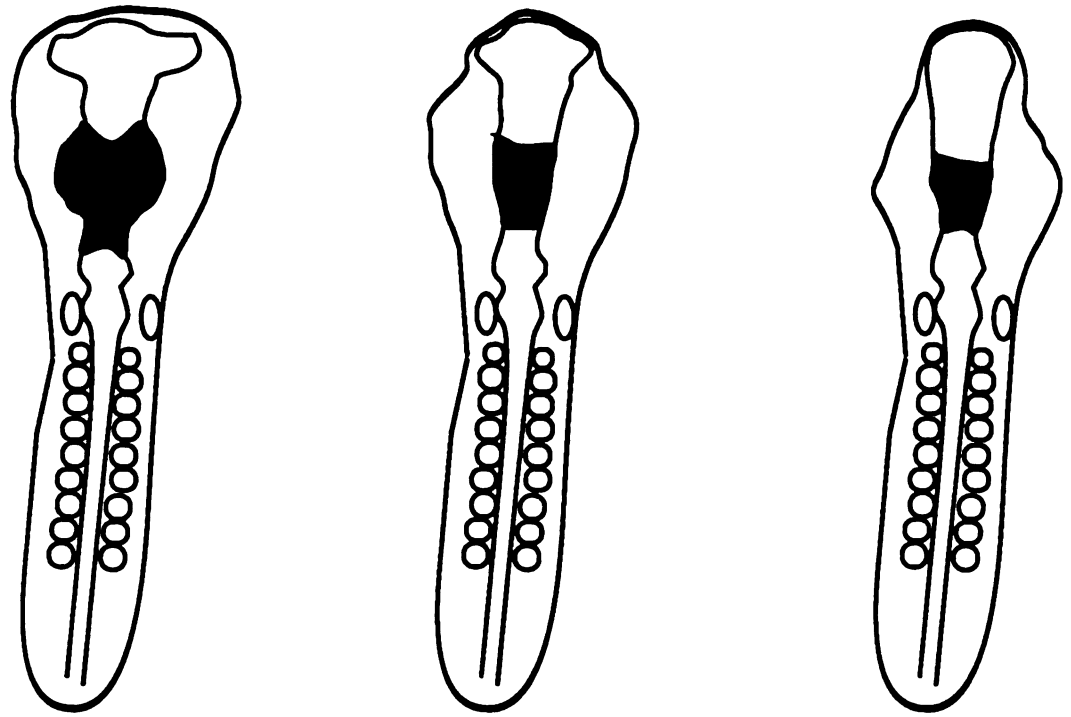


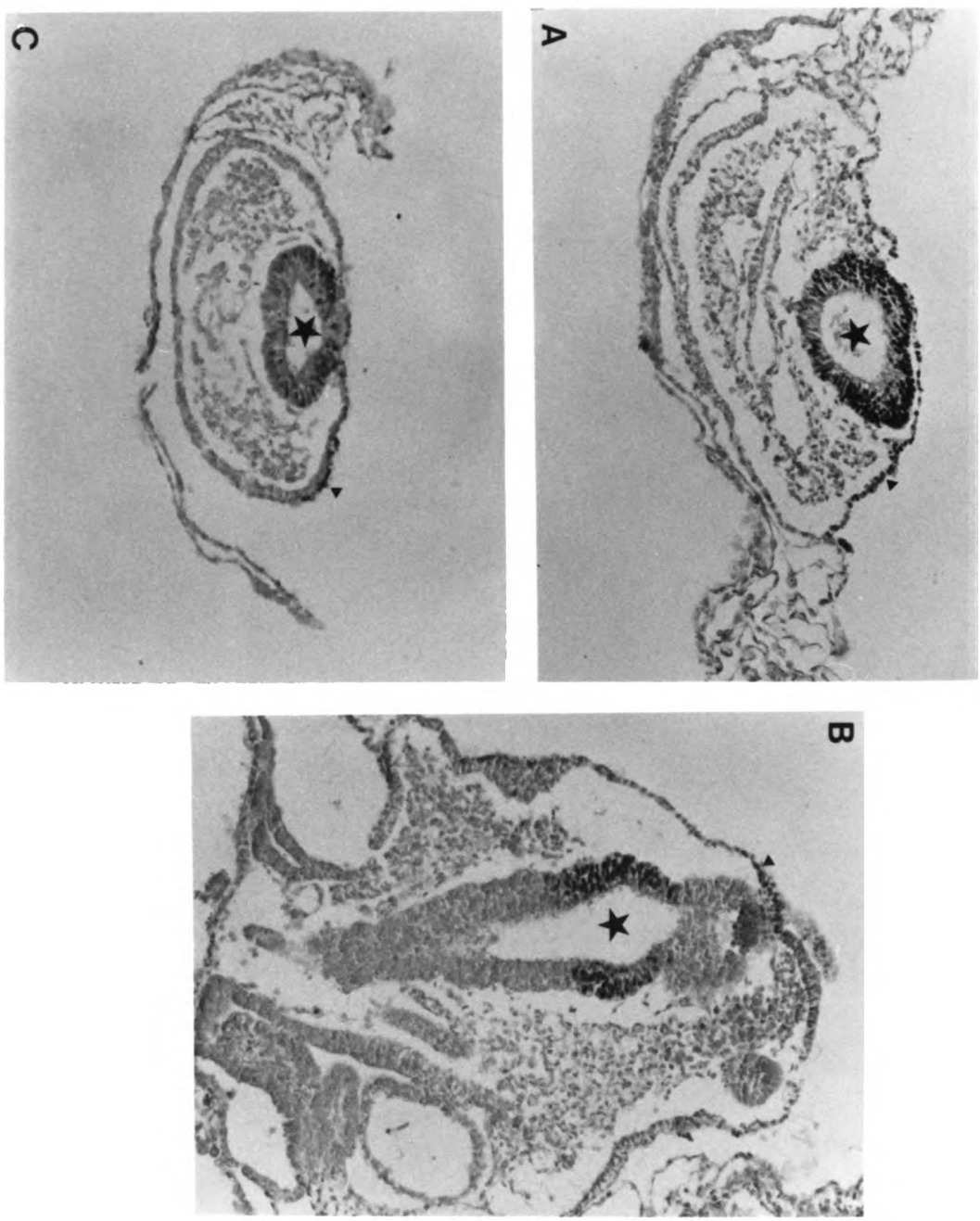
Figure 4.11 A cartoon representation of embryos treated with RA at stage 4 for 4 hours and cultured for 24 hours. (For actual photographs see figure 4.12.) All embryos immunolabeled with 4D9 (black) in the mesencephalon and rostral metencephalon although embryos treated with RA (B and C) showed a diminished size of the labeling region and other changes in the rostral head (see figures 4.12-15).

Figure 4.12 Photographs of embryos treated with RA at stage 4 and cultured for 24 hours. All embryos labeled with 4D9 (black) in the mes- and metencephalon although embryos treated with RA (6μM: B and B'; 10μM C and C') showed a diminished size of the labeling region and other changes to the rostral head (see Figures 4.13-15).

A.
B.
C.

A
B
C

Figure 4.13 Photographs of sections through embryos treated with RA at stage 4 and cultured for 24 hours. A cross section through the mesencephalon of a control embryo (A) shows normal morphology and 4D9 labeling in the neural tube (star). A coronal section through an embryo treated with 6μM RA (B) shows *En-2* expression is restricted to a limited band within the neuroectoderm at the level of the mesencephalon (star). Expression of *En-2* can also be seen (star) in mesencephalon level cross sections of embryos treated with 10μM RA (C). In all three cases, 4D9 labeling is also seen in the overlying surface ectoderm (arrowheads).


C
A
B

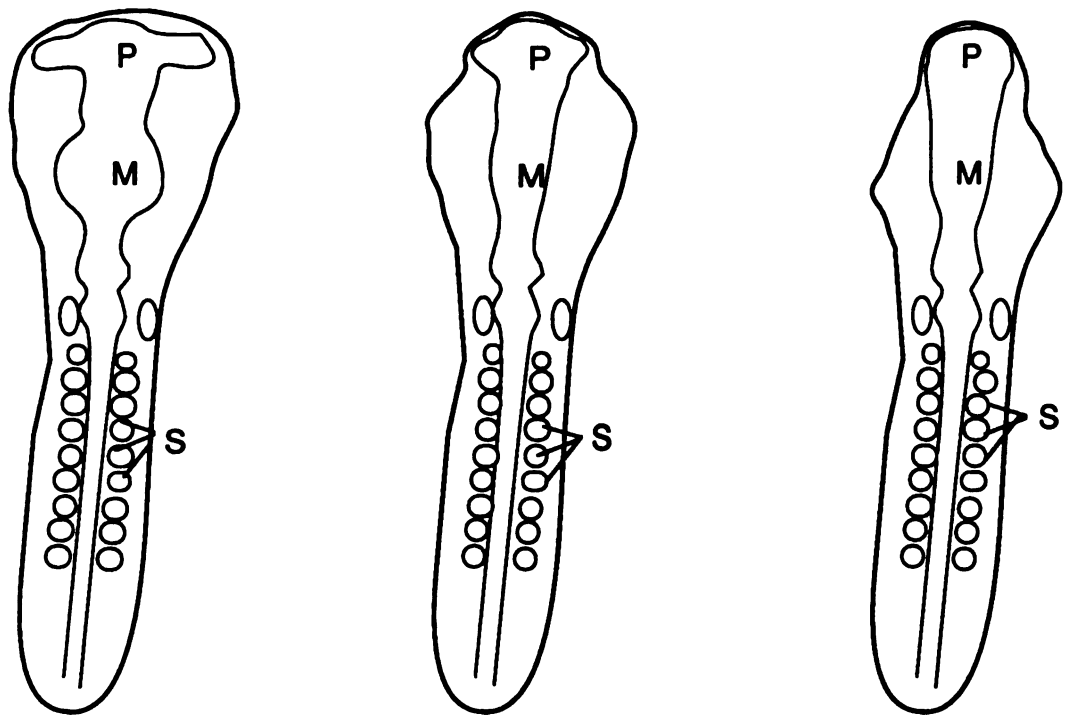
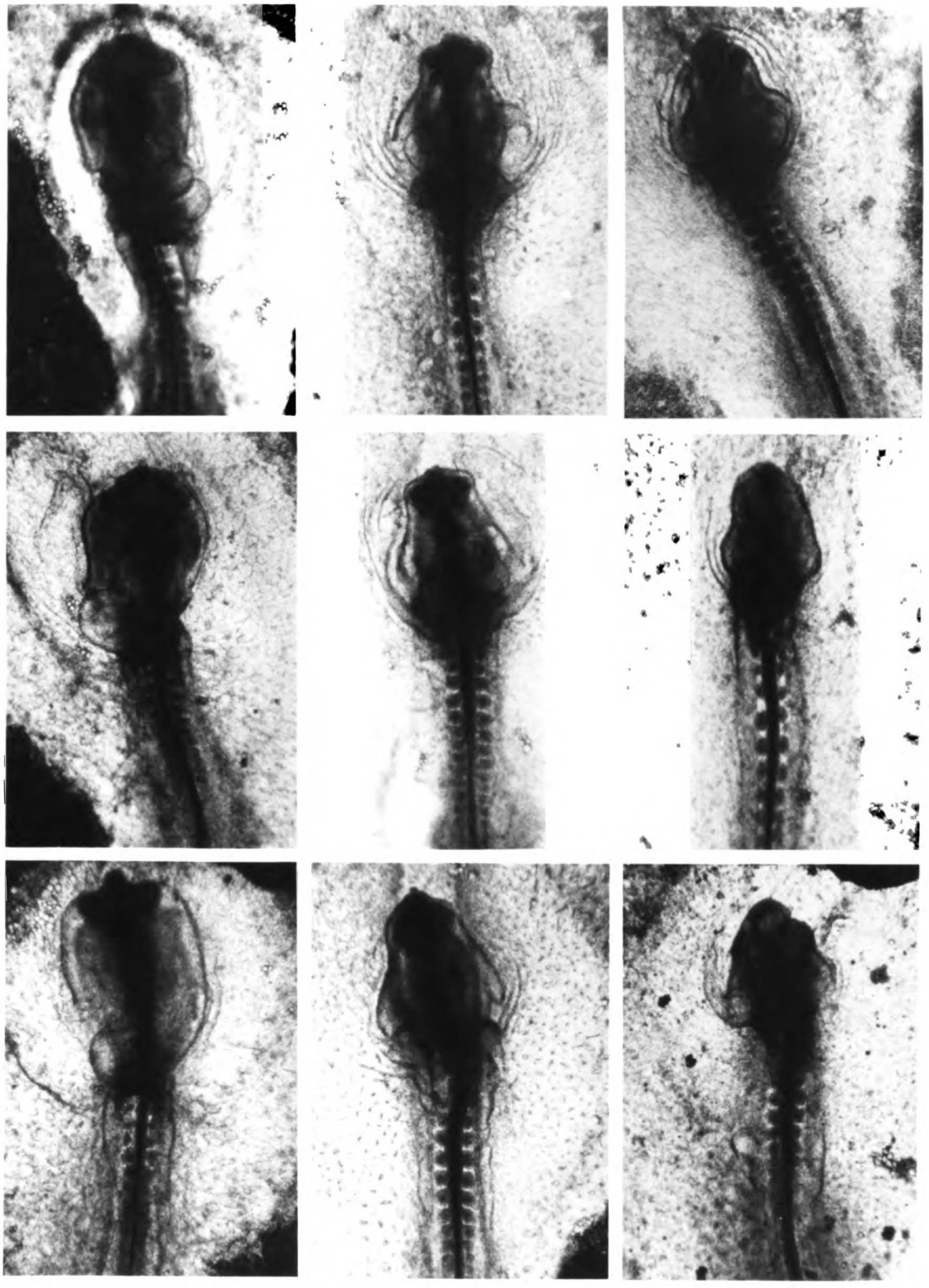


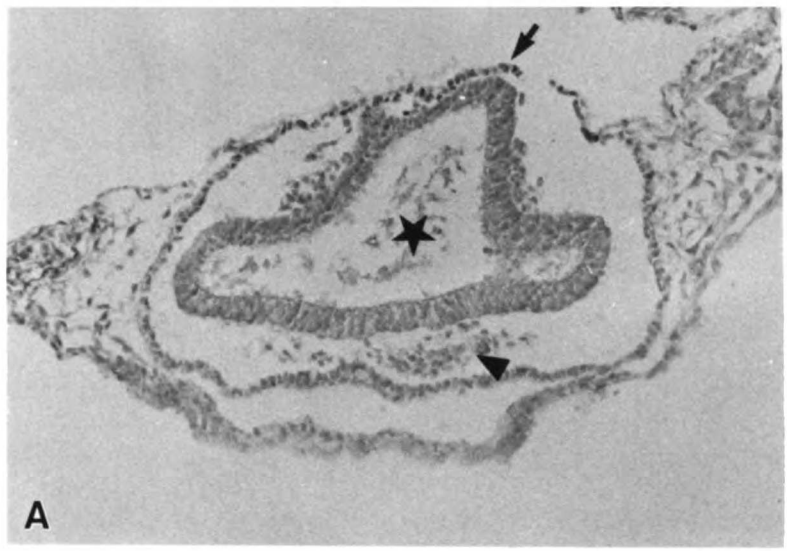
Figure 4.14 A cartoon representation of embryos treated with RA for 4 hours at stage 4 and cultured for 24 hours. Prosencephalon (P), mesencephalon (M) and somites (S) appear normal or nearly normal for control embyros (A), embryos treated with 6μM RA (B) and embryos treated with 10μM RA (C). Rostral structures were diminished in width. (For photographs of embryos from these treatment groups see figure 4.15.)

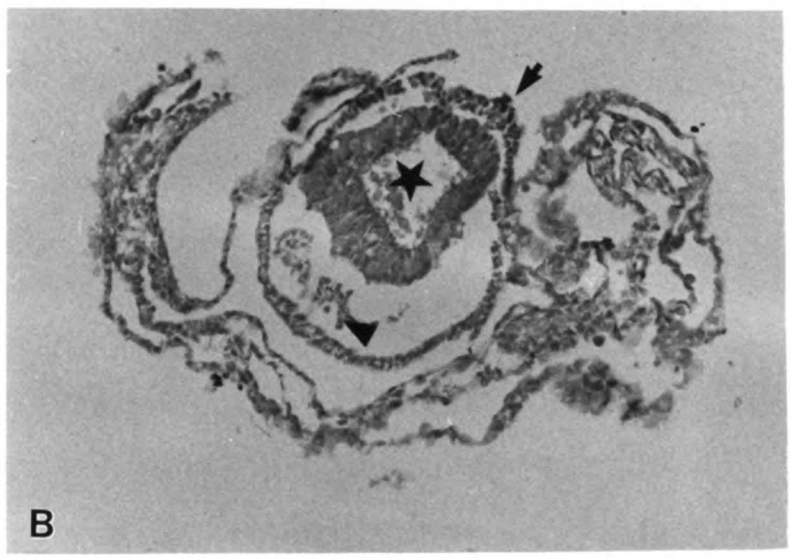
Figure 4.15 Photographs of whole, unstained embryos (see cartoon Figure 4.14) treated at stage 4 with RA and cultured for 24 hours. Control embryos in column A were treated with 0μM RA, embryos in column B were treated with 6μM RA and those in column C with 10μM RA. Rostral head shape is increasingly altered with increasing concentrations of RA. Prosencephalon is present but reduced in size (see also Figures 4.20 and 4.21) as is the amount of surface ectoderm around the rostral head.



A B C

Figure 4.16 Photographs of cross sections through the prosencephalon region of control embryos treated with 0μM RA (A), and experimental embryos treated with 6μM RA (B) or 10μM RA at stage 4 followed by 24 hours of culture. All three show a clear neural tube (star) and overlying surface ectoderm *En-2* expression (arrows). All three also show the presence of some paraxial mesoderm (arrowhead), indicating that RA does not completely inhibit the migration of rostral mesoderm, or that this mesoderm had migrated prior to stage 4.


A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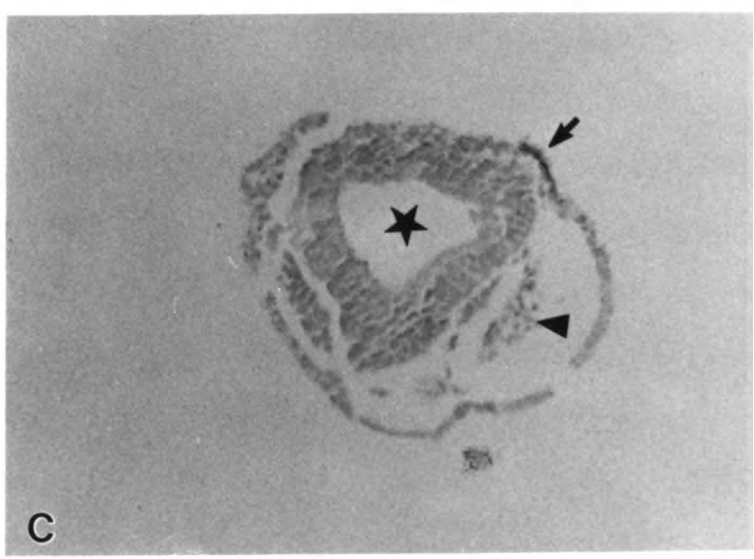
C

The caudal gradient half of this model is consistent with data that show that RA induces nine genes of the Hox2 gene cluster sequentially in embryonal carcinoma cells with cranially expressed genes induced at low levels of RA ($5x10^{-8}M$) and caudally expressed genes induced at higher levels of RA ($5x10^{-6}M$; Simeone et al., 1990). In addition, these researchers demonstrated temporal regulation in the expression of these Hox2 genes with anteriorly expressed genes requiring the shortest exposure to RA and posteriorly expressed genes the longest. This result is also consistent with the conjoined-gradient model because the node regresses caudally during neurulation so that anterior RA would be suppressed early (short exposure to low levels of RA for anterior genes) and posterior RA would not be suppressed until significantly later (long exposure to higher levels for posterior genes). The complicated nature of the RA/CRABP/RAR interactions make it unlikely that any simple RA concentration measurements will confirm or refute the proposed model.

If a RA gradient exists, its could influence development via either the mesoderm or the ectoderm. The apparent decrease in head mesoderm in stage 3d embryos treated with RA concomitant with the loss of some anterior head structures and *En-2* expression might indicate that RA acts by inhibiting mesoderm migration. If there is a requirement for appropriate mesoderm to underlie appropriate ectoderm in order for normal rostral CNS to be formed and abnormally high levels of RA at the node prohibits mesoderm from migrating anteriorly then induction of anterior neuroectoderm may fail to occur. The fact that neuroectoderm forms anterior to the *En-2* expressing region in RA treated embryos would seem to contradict this conclusion. Perhaps little or no underlying mesoderm is required for the regionalization of the prosencephalon This is consistent with one model of neural induction and patterning in which induced neuroectoderm has a prosencephalic character and regionalization is required for caudalization (Nieuwkoop, 1955).

Development of the anterior CNS may depend on gradient of RA that is lowest at the node (mesencephalon) and higher rostrally (prosencephalon). This RA gradient may act directly on homeobox-containing genes and other transcription factors expressed in

specific regions of the head or, alternatively, the rostral CNS may require stimuli from migrating mesoderm in order to become patterned and exogenous RA may effect this mechanism. Further experiments are required to asses the mechanism by which RA exposure during stage 3d inhibits the development of the mesencephalon in the chick. Regardless of the precise mechanism, it is likely that the patterning of the future neuroectoderm involves a planar interaction between the cells of the node and the rostral ectoderm prior to gastrulation. Experiments to separate the node/streak from prospective mesencephalic neuroectoderm prior to stage 4 might increase our understanding of the mechanisms behind neuroectodermal patterning.

Ectoderm/mesoderm separation

Prospective midbrain ectoderm ('ectoderm') was dissected away from the streak in three types of dissection. In the first, the lateral midbrain ectoderm and the area rostral to Hensen's node were dissected away from the streak and remaining epiblast (Figure 4.17A; 9 embryos); in the second, prospective midbrain ectoderm and the epiblast and hypoblast lateral to it were dissected away from the remaining parts of the embryo (Figure 4.17B; 7 embryos); and in the third, the entire streak was excised away from the remaining blastoderm (Figure 4.17C; 4 embryos). In all three cases, according to the fate map, the mesencephalic neuroectodermal rudiment was separated from the notochord rudiment before stage 4. For this reason, "ectoderm" fragments were pooled from all three surgeries, as were "mesoderm" fragments. After 24 hours in culture, embryo fragments were immunolabeled with 4D9, the antibody specific for the *En-2* nuclear protein in midbrain neuroectoderm and neighboring surface ectoderm and neural crest, and Not-1, an antibody that binds a notochord cytoplasmic protein. With these two markers, I was able to make a preliminary assessment of whether midbrain could develop in the total absence of notochord.

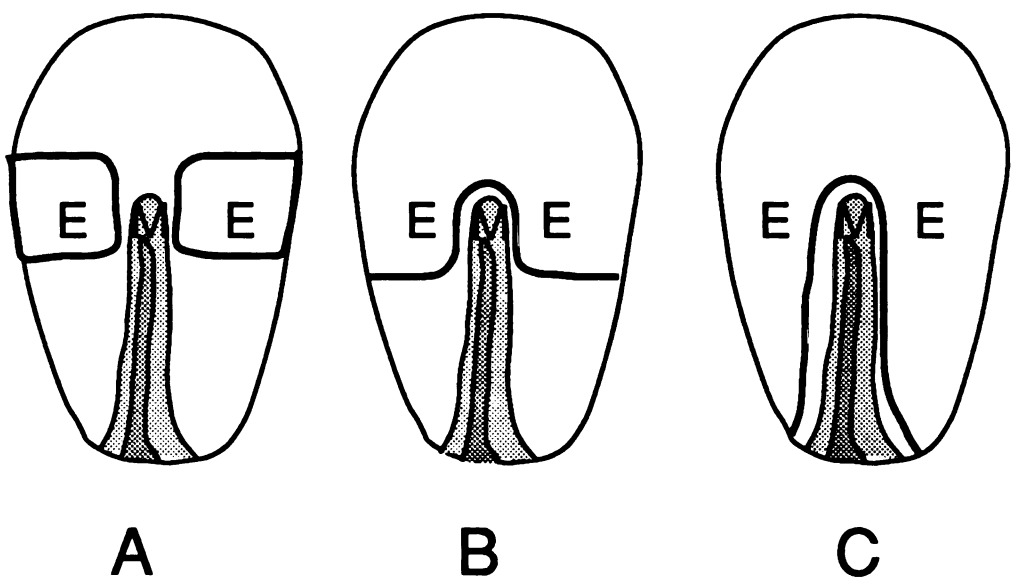

Figure 4.17 A cartoon representation of three transections of stage 3c embryos that separate the mesencephalic level ectoderm rudiment (E) from the notochord and rostral mesoderm rudiment (M). Fragments labeled E were cut apart from fragments labeled M and cultured separately. Both types of fragments were immunolabeled with antibody 4D9 against the *En-2* protein and antibody Not-1 against a notochord protein.

Each embryo contributed what were considered an 'ectoderm' fragment and a 'mesoderm' fragment. Due to small size and delicacy of isolated fragments, pieces were sometimes lost or destroyed during the staining or sectioning. Twelve mesoderm fragments survive, as did ten ectoderm fragments. In 11 of 12 of the surviving mesoderm fragments the notochord antigen was expressed (Figure 4.18A). In each case, Not-1 was expressed in cells that appeared to form a discrete bundle (although not always a cylindrical cord) and immunolabeling was strong. In three of 11 cases, the mesoderm fragment also expressed the midbrain *En-2* antigen in neuroectoderm overlying the notochord (Figure 4.18C). This is expected since the ectoderm remaining in the mesoderm fragments could be induced to form mesencephalon. The ectoderm may have previously been fated to be non-mesencephalic ectoderm and been recruited to form mesencephalon as ectoderm is in ectopic embryos, or, the normal floor plate ectoderm from this region may have altered its mediolateral fate to become lateral wall neuroectoderm. Another possibility is that perhaps I was unable to completely separate the normal precursor cells for the lateral wall of the neuroectoderm from the notochord precursors in all surgeries. In none of the 12 cases did the mesoderm fragment express *En-2* in the absence of the Not-1 antigen. In the 12th case, neither antibody labeled cells from the fragment. In no case did expression of the antigens seem to be stage dependant, occurring in stage 3b-d embryos.

'Ectoderm' fragments also expressed the notochord antigen in the majority of cases (7/10), although sometimes less intensely and in a less well defined structure than for mesoderm fragments (Figure 4.18B). Expression may indicate that the distribution of prospective notochord cells is broader than originally concluded, even at early stages. Alternatively, the dissections may have inadvertently included a few cells from the primitive streak in the "ectoderm" fragments. Within these seven fragments, four expressed the Not-1 antigen only, indicating that even though by mapping they should contain prospective mesencephalic ectoderm, the ectodermal rudiments present in the fragments did not become positionally identified as mesencephalon, even in the presence of notochord. The other

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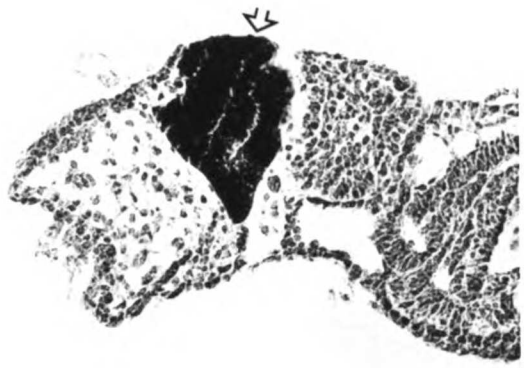
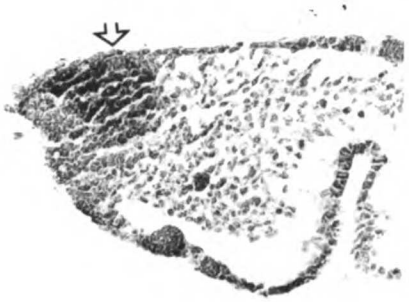
Figure 4.18 MAb-4D9 and Not-1 immunolabeling of mesencephalon and notochord, respectively, from transected embryo fragments. Panel A shows Not-1 label (open arrow) in notochord developing in the mesoderm fragment of an embryo represented in Figure 4.4a. Panel B shows Not-1 label (open arrow) in notochord from an ectoderm fragment from a similar embryo. Panel C shows 4D9 label in the neuroectoderm (arrow) and surface ectoderm (small arrow) and Not-1 label in the notochord (open arrow) from a mesoderm fragment of an embryo represented in Figure 4.17B. Panels D and E show 4D9 label in neuroectoderm (arrow) and surface ectoderm (small arrow) for ectoderm fragments represented in Figure 4.17B and A, respectively.

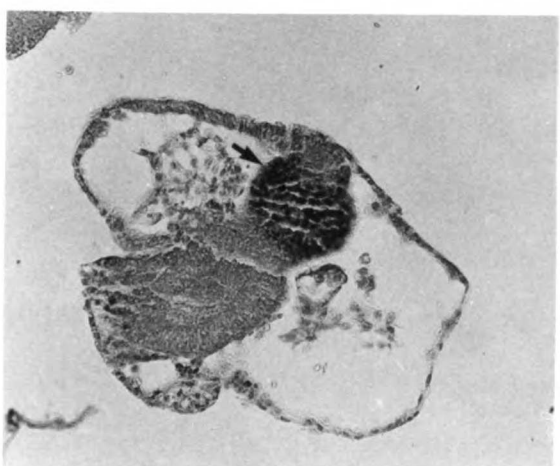


A

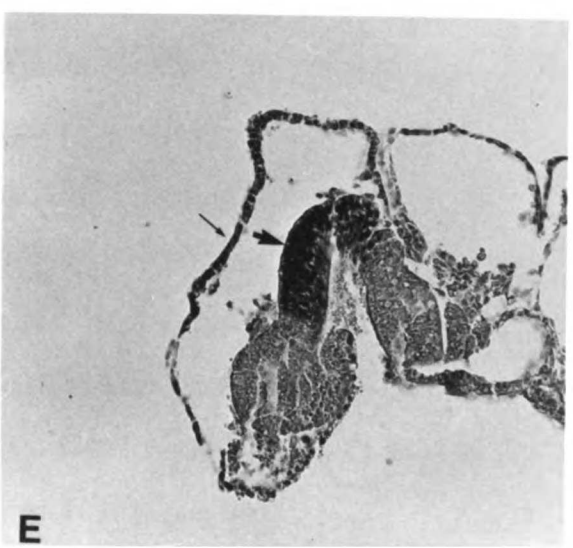


B

C



D



E

three of the seven expressed both Not 1 and 4D9 (data not shown). The three remaining ectoderm fragments were from stage 3c and 3d embryos and expressed only the midbrain *En-2* antigen (fig 4.18D and E). In these three cases, serial sections revealed that neural plate had formed in the total absence of Not-1 immunolabeling notochord cells. Overlying surface ectoderm was also observed to label with 4D9, as it does in control embryos. No neural crest labeling was observed, however, the sample size was small, as were the explants.

Several general observations can be made regarding the embryos that were dissected into midbrain ectoderm and mesoderm fragments. First, almost every fragment expressed the Not-1 antibody epitope, indicating that notochord is very self-contained in its ability to differentiate under adverse conditions. This is consistent with the early organizer experiments where notochord was determined to "self differentiate" with respect to its migration through the organizer and its differentiation (Hamburger, 1988). Second, most mapping studies describe the notochord cells as exiting the node beginning at stage 4. The Not-1 expression in the ectodermal fragments can, therefore, be interpreted in several ways. Perhaps migration through the streak is not required for the formation of notochord in chick; or, perhaps notochord cells migrate through and out of the streak earlier than previously thought; or, perhaps cells that would normally form paraxial mesoderm can differentiate into notochord in the absence of adjacent notochord. Heterotopic and homotopic transplants between chick and quail mesoderm from the node and paraxial region might help to resolve the questions raised by this observation.

The other interesting result from this experiment was the expression of *En-2* in ectoderm fragments separated from the node and streak as early as stage 3c. One interpretation of this result is that both induction and patterning of the neuroepithelium occur before stage 3c. This interpretation is consistent with observations by Kintner and Dodd, who were recently able to induce ectopic neuroectoderm from *Xenopus* animal caps with Hensen's node in the absence of the migration of the rudimentary notochord cells,

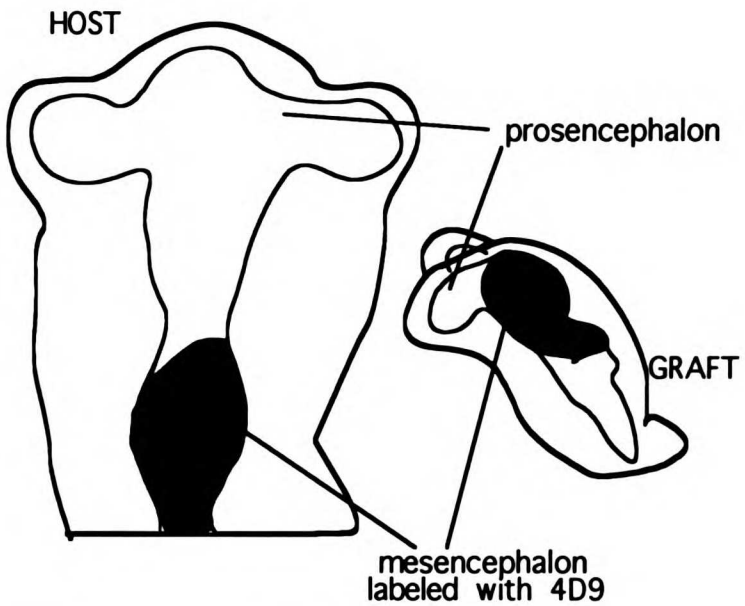
indicating that the node contains inducing signals very early (Kintner and Dodd, 1991). An alternate interpretation is that the underlying endoderm and paraxial mesoderm are sufficient to induce and or pattern the midbrain ectoderm. Because the numbers of embryos considered was so small, repetition of these experiments and more restrictive isolation experiments are required to establish the probability of these interpretations.

Ectopic Embryos

Sixty stage 3b-4+ quail (or chick) Hensen's nodes were transplanted to the germinal crescent of host chick embryos between stages 3c-4+; 53 developed into ectopic embryos. Forty of the 53 ectopic embryos immunolabelled with 4D9. The frequency of neural induction for embryos at these stages was expected to range from a high of about 90% for stage 3b/c grafts into stage 3c hosts to a low of about 40% for the stage 4+ grafts into stage 4+ hosts. The frequency of self-differentiation of graft ectoderm into neuroectoderm was expected to range from a low of 29% for stage 3b/c grafts and stage 3c hosts to a high of approximately 80% for the oldest hosts and grafts (Dias and Schoenwolf, 1990). Given these ranges, it seemed likely that the ectopic embryos in this experiment that had labeled with the *En-2* antibody would include representatives of both induced and self-differentiated neuroectoderm.

Nine chick/quail chimaeric ectopic embryos that had been exposed to the 4D9 antibody were sectioned and Feulgen stained (to detect the quail nucleolar marker) so that induced and self-differentiated neuroectoderm could be definitively distinguished. Of these, eight immunolabeled with 4D9. Antibody binding was observed in whole mount (Figure 4.19 and 4.20) and sectioned embryos (Figure 4.21A, 4.22A and B and 4.23A). In two of these embryos, Feulgen staining revealed quail nucleolar markers in the neuroectoderm as well as the underlying mesoderm of the graft (Figures 4.21 B-D and 4.22B-D). In fact, sections from one of these self-differentiating ectopic embryos revealed that the neuroectoderm had inserted into the host endoderm rather than the surface

Figure 4.19 Cartoon and photo of whole mount host and ectopic embryo generated by transplanting Hensen's node from a quail donor into the germinal crescent of a chick host embryo and culturing for 24 hours. Host and graft were immunolabeled with MAb-4D9 against the En-2 protein in the mesencephalon and metencephalon. Neural tube in the graft embryo is, in this case, derived from self-differentiated quail cells. Cross sections can be seen in Figures 4.21 and 4.22.


HOST
prosencephalon
GRAFT
mesencephalon
labeled with 4D9

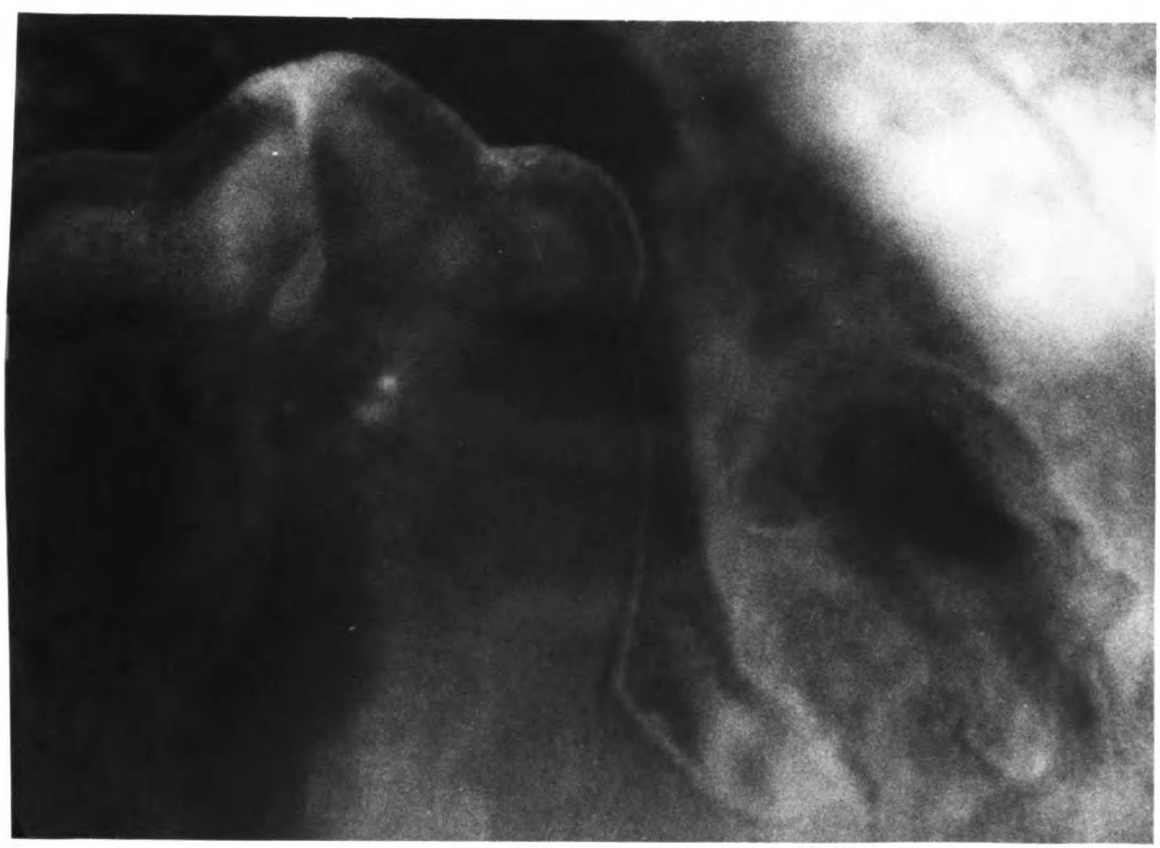
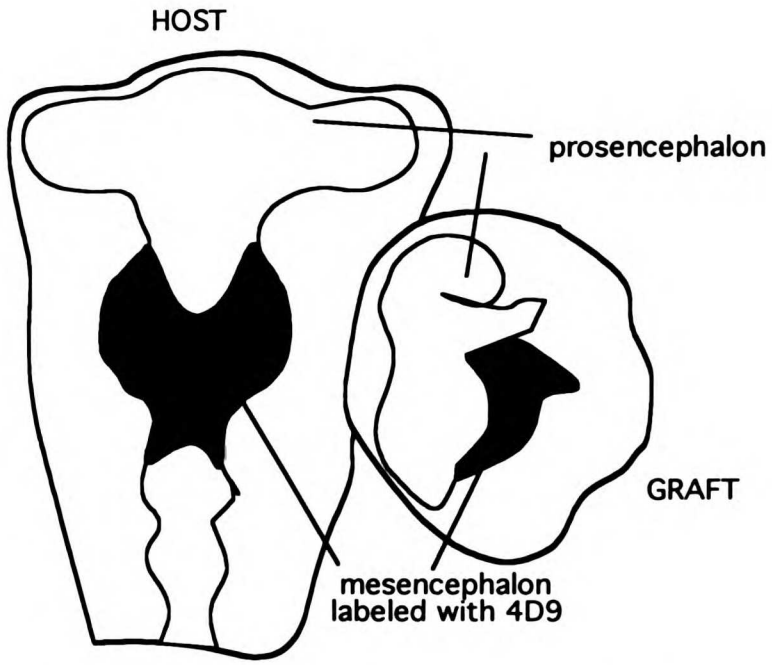


Figure 4.20 Cartoon and photograph of whole mount host and ectopic embryo generated by transplanting Hensen's node from a quail donor into the germinal crescent of a chick host embryo and culturing for 24 hours. Host and graft were immunolabeled with MAb-4D9 against the En-2 protein in the mesencephalon and metencephalon. The neural tube in the graft is, in this case, induced from chick tissue by underlying quail mesoderm or through planar signals from the original node graft. Cross sections of induced ectopics can be seen in Figures 4.23-24.


HOST
prosencephalon
GRAFT
mesencephalon
labeled with 4D9

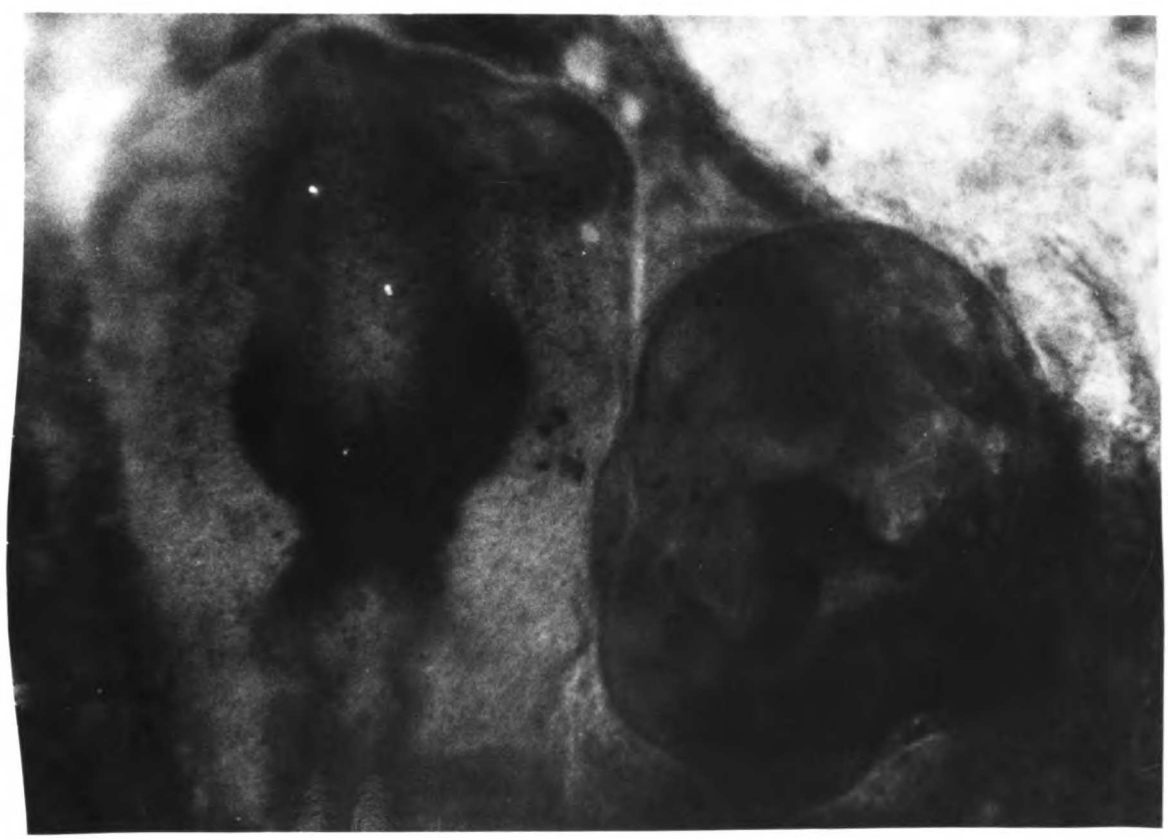


Figure 4.21 Photographs of cross sections through ectopic embryos showing self-differentiated quail neuroectoderm. Panels A and B show a cross section through both host (H) and ectopic graft (G) embryos. Panel A shows 4D9 immunolabelling of the mesmetencephalon (arrow) and panel B shows regions of the ectopic embryo that were photographed at higher magnification to reveal quail nucleolar marker in Feulgen stained regions of the mesoderm (panel C) and neuroectoderm (panel D). In panels C and D, small arrows point out quail nucleolar marker evident in some cells in this plain of focus.

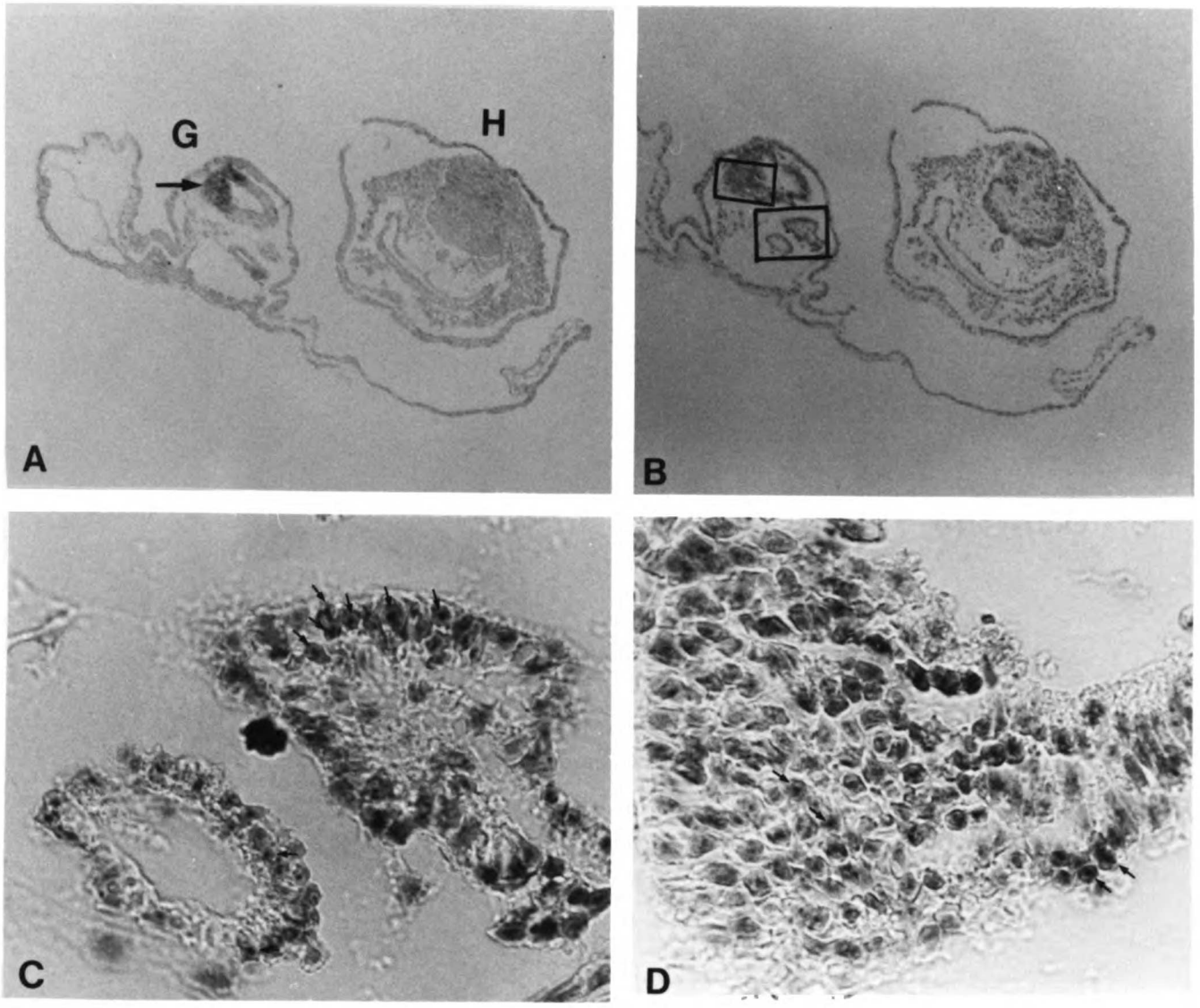
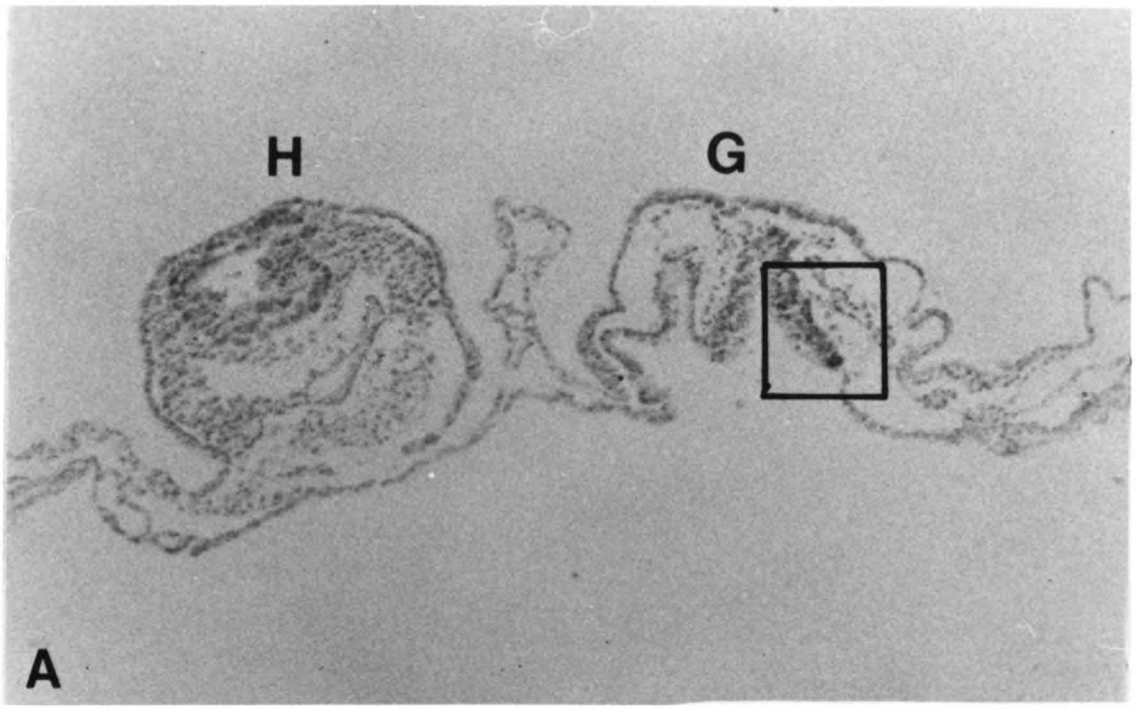

G
H
A
B
C
D

Figure 4.22 Photographs of cross sections through A) a second self-differentiating quail ectopic embryo (G) and its chick host (H). In this instance, the dorsal quail neuroectoderm differentiated attached to the chick endoderm. The region of the quail neuroectoderm boxed in A is shown at higher magnification in B. Quail nucleoli can be observed as dark dots in several cells in this plain of focus (small arrows) and the very tip of the neuroectoderm can still be seen to immunolabel with 4D9.


H
G
A

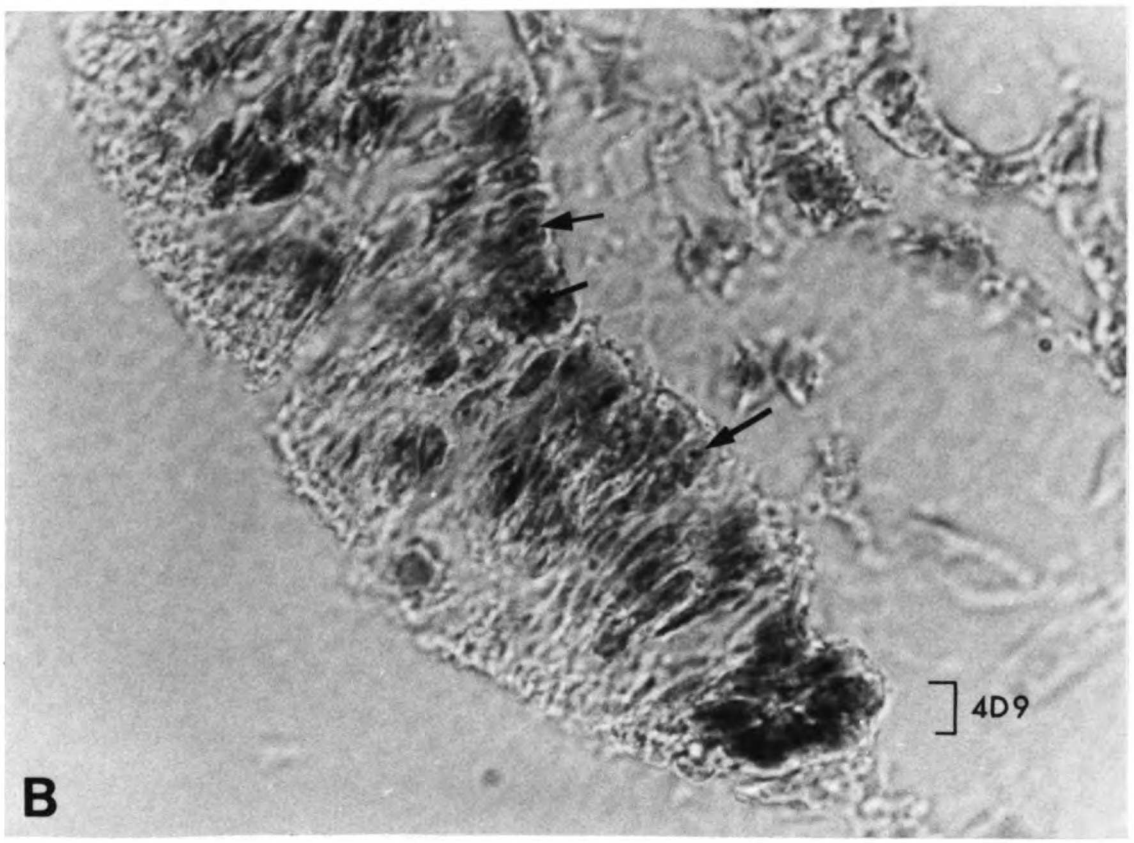

4D9
B

Figure 4.23 Photographs of cross sections through ectopic embryos with chick neuroectoderm induced after transplant of quail Hensen's node into germinal crescent of chick during stage 3. A) shows immunolabeling of mesencephalon/metencephalon with 4D9. B) shows neighboring section stained for quail nucleolar marker. Boxed regions from B are shown enlarged in C and D. C shows quail marker in the mesoderm of this ectopic embryo whereas D shows no quail marker in the 4D9 immunolabeling region of the neuroectoderm, indicating that it is induced chick ectoderm.

C
A
D
B

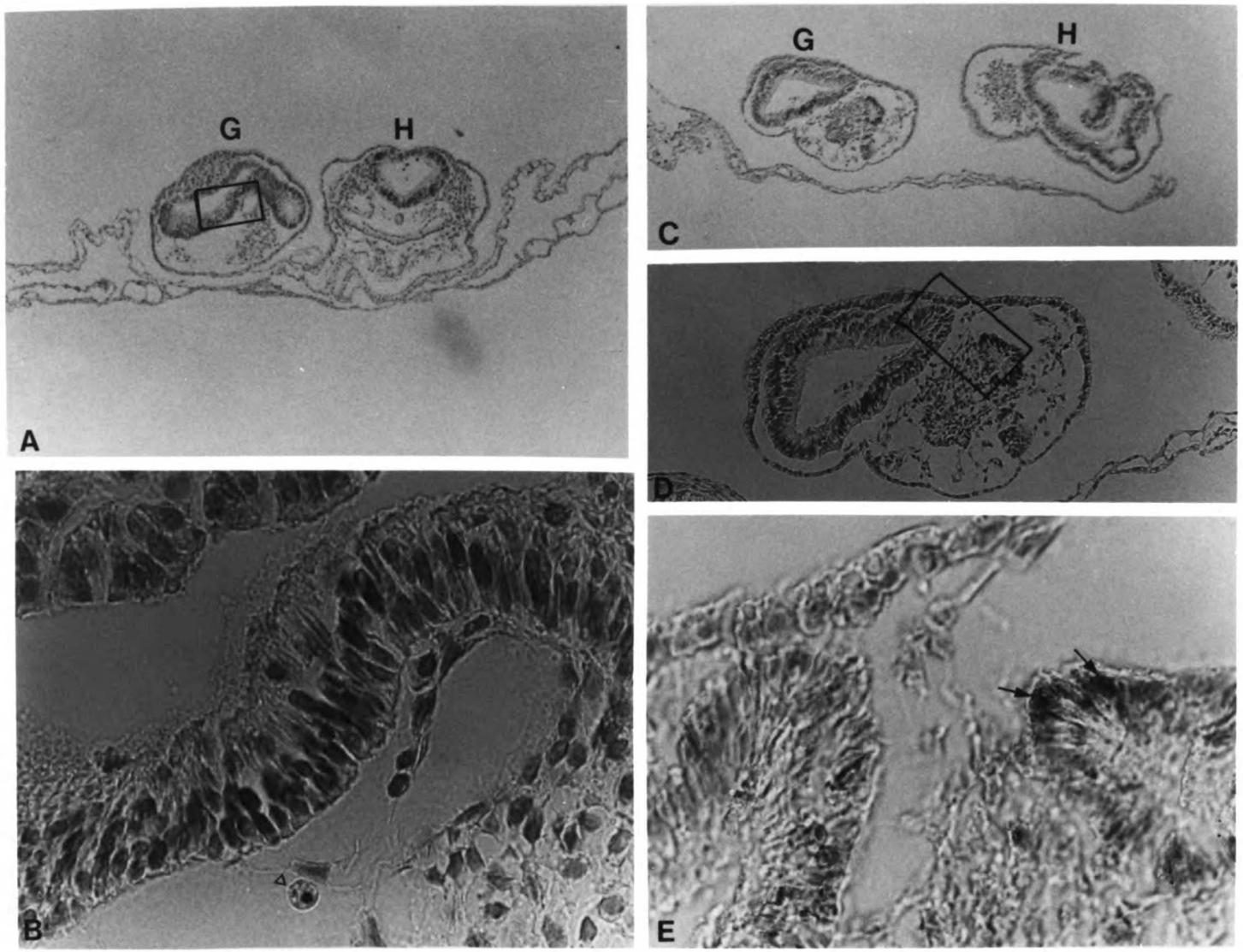
ectoderm (the transplanted node was probably inverted). This embryo, nonetheless expressed *En-2* and formed an open neural tube (Figure 4.22). These two ectopic embryos represent self-differentiating, graft neuroectoderm and show that grafted ectoderm can become patterned to express a mesencephalic marker in an ectopic environment, and that it does not require the normal continuity of spatial relationships between surface ectoderm, endoderm and neuroectoderm.

Of the nine ectopic embryos sectioned, six of those remaining had neural plates or tubes that immunolabeled with 4D9 but failed to exhibit the nucleolar markers associated with quail cells, and were, therefore, determined to be induced chick neuroectoderm (Figure 4.23-4.24). Immunolabeled cells were caudal to neural tube which morphologically resembled prosencephalon, indicating that the *En-2* expression in the ectopic embryos was positionally correct in this respect and consistent with the conclusions of Dias and Schoenwolf (1990) that induction always includes the rostral neuroectoderm when any positional differentiation can be detected. Quail cells were identified in other structures of all the ectopic embryos including vesicles, gut endoderm, notochord mesoderm and paraxial mesoderm (Figure 4.20C, 4.23E).

Dias and Schoenwolf also identified a population of ectopic embryos in which the induced or self-differentiated neuroectoderm could be classified only as neural tube or plate, without obvious regional character (11-100%, depending on the graft and host age). Embryos in this category may account for some or all of the 16 ectopic embryos (35%) that failed to immunolabel with 4D9. Of the nine embryos sectioned, one failed to immunolabel with 4D9 and cross sections of this ectopic embryo revealed small cysts or vesicles made up of either chick or quail cells that may have been ectodermal, mesodermal or endodermal in character (data not shown). A sectioned sample size of one is clearly insufficient to determine the developmental fate of the 16 embryos that failed to immunolabel with 4D9.

In this experiment I was able to demonstrate that both self-differentiating and induced neuroectoderm of ectopic embryos have the capacity to express *En-2* in a

Figure 4.24 Photographs of cross sections through ectopic embryos induced after transplantation of quail Hensen's node into chick host germinal crescent. A&C show two sets of host (H) and graft (G) embryos. B is a higher magnification view of the boxed region in A showing a blood cell (open arrowhead) with a dark nucleus that might be mistaken for a quail nucleolar marker. The neuroectoderm of this ectopic embryo is of chick origen. C shows another example of both host (H) and graft (G) embryos in cross section. D) An enlargement of the graft embryo shows a boxed area enlarged in E. In this panel, the nucleolar markers for quail are obvious in the mesoderm (small arrows) but not in the neuroectoderm (left).


G
H
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regionally appropriate and restricted manner. Thus, the epiblast/ectoderm overlying Hensen's node contains or receives sufficient information to self-differentiate into positionally-specified, midbrain neuroectoderm. In addition, non-neural ectoderm can be respecified to form patterned neural ectoderm in the presence of Hensen's node.

Under normal circumstances, the midbrain is patterned not only rostrocaudally, but also dorsoventrally. In the presence of notochord, a floor plate develops in the midline of the neural plate and this region fails to express En-2. The question of floor plate suppression in ectopic neuroectoderm could not be adequately addressed in this experiment because definitive floor plates were not identified in any of the ectopic embryos. Morphology was frequently abnormal and ectopic embryos, being very small, were often not successfully cross-sectioned. Thus, the question of floor plate suppression in ectopics remains to be addressed.

The observations made in this experiment are similar or identical to those of Dias and Schoenwolf (1990) and Storey and co-workers (1992). In all three experiments, the neuroectoderm of ectopic embryos, whether self-differentiated or induced, can be patterned to form mesencephalon. However, whereas Dias and Schoenwolf were dependent on morphological characteristics alone to identify positional identity of the neuroectoderm, and Storey and her collaborators were unable to show both *En-2* expression and the quail nucleolar markers in the same section due to fixative incompatibilities, my results directly support the conclusion that self-differentiating quail and induced chick neuroepithelium express *En-2* in the mesencephalon and rostral metencephalon. Also, Storey and co-workers conclude that the presumptive notochord is responsible for regionalizing the neuroectoderm, whereas my previous experiments (Chapter 3 and Chapter 4) indicate that the notochord is not required for patterning of the neuroectoderm.

Another possible discrepancy between our results involves the ability of caudal neural structures to be induced in the absence of attached rostral structures. Kintner and Dodd were able to demonstrate that the regionalization of *Xenopus* neuroectoderm is

dependent on the age of the grafted node (Kintner and Dodd, 1991). This observation is currently a point of debate for the chick embryo. Dias and Schoenwolf (1990) concluded that induced neuroectoderm always proceeded from prosencephalon caudally and never initiated with a more caudal structure. In the Storey paper they conclude that induced tissues can begin at more posterior levels. This discrepancy may be due to the site of transplant. Spemann demonstrated that the rostrocaudal level of the graft site influenced the level of induced neuroectoderm in salamander. Perhaps the more caudal sites selected by Storey and her collaborators or the fact that they transplanted to the extraembryonic area opaca rather than the rostral area pellucida can account for the different observations of these two groups. The embryos sectioned in this experiment appeared to support the claim by Dias and Schoenwolf that mesencephalon only develops caudally to prosencephalon. However, some of the 15 embryos that did not immunolabel with 4D9 may have contained patterned, induced neuroectoderm of regional character caudal to the mesencephalon. Unlabeled ectopic embryos in this experiment may also represent induced neuroectoderm that failed to pattern or to extend caudally into the midbrain region or self-differentiated neuroectoderm that failed to form midbrain either rostrally or caudally to the remaining neuroectoderm.

The issue of whether prosencephalon always forms in induced neuroectoderm (or not) needs to be resolved because it is key to evaluating the various models of induction and patterning. For example, if rostral structures are required for more caudal regionalization, then the activation-transformation model of Nieuwkoop (1955) would be supported. In this two-step model, ectoderm initially becomes specified (activated) as rostral neuroectoderm. In a second step, this neuroectoderm is caudalized (transformed) to acquire additional caudal regional character. If caudal structures can be induced in the absence of rostral structures, this model would not be supported. The evaluation of ectopic embryos in various host sites by numerous regional markers will be required to resolve this question.

One cannot necessarily conclude from these ectopic embryo experiments, as Spemann and others have through the years for organizer experiments in amphibians, that the rostral axial mesoderm which exits Hensen's node and comes to lie underneath the induced ectoderm is responsible for the induction and patterning of the neuroectoderm. As mentioned in a previous section, several studies indicate that the induction may be planar in nature rather than vertical and may occur in the absence of mesodermal migration. For example, in a recent paper by Kintner and Dodd (1991), Hensen's node of the chick was grafted into a *Xenopus* ectodermal cap. Due to treatment of the node, its development was arrested at stage 3, and no exodus of mesodermal cells was observed during the culture period. In spite of the lack of mesoderm migration and the cross-species nature of the host and graft tissues, Xenopus neuroectoderm was induced. In addition, it may not even be mesodermal precursor cells that are responsible for the planar induction event. Dias and Schoenwolf (1990) show a strong correlation between the quantity of graft endodermal cells incorporated with the host's tissues and the vigor of the induction response. They infer that prospective endodermal cells of Hensen's node likely have a paramount role in neural induction in avian embryos. The notions of planar induction and patterning before stage 3c or through the endoderm or paraxial mesoderm are supported by experiments in Chapters 3 and 4 of this dissertation.

CONCLUSIONS

In chick, the mesencephalon and rostral metencephalon, the rudiment of the cerebellum, develop at the craniocaudal level of Hensen's node, the recognized "organizer" of avian embryos. The organizer directs the development of the dorsal structures, including the neural tube, in both cranial and caudal directions through planar induction, the rostral migration of endoderm and mesoderm and the caudal regression of the node itself. Blastoderms transected at the node and cultured become embryos transected at the mesencephalon. Evolutionarily, the mesencephalon and rostral metencephalon form the most advanced component of the ancient CNS, the more rostral structures having evolved more recently as an appreciated accessory. In this sense, the mesencephalon and rostral metencephalon are temporally central, between the old and the new CNS. Morphologically, the notochord ends in this region, it is the location of the major flexure of the CNS during development and it forms the junction between the apparently 'segmented' and 'unsegmented' components of the CNS. The observations presented in this dissertation and those of other authors published over the past century lead me to conclude that the region of the midbrain and rostral hindbrain, which express *En-2*, represents the "center" of the central nervous system, and that the patterning of the CNS is established well before the notochord cells migrate out of the node to take their axial position beneath the neuroectoderm.

I have considered a model with the node as the center of a conjoined gradient for the morphogen, retinoic acid (RA). Small increases in RA at the stage just prior to neurulation results in the loss of the mesencephalon and anterior metencephalon; the entire *En-2* expressing region. The loss of this region after treatment with RA has also been recently documented by others in the zebrafish, *Xenopus*, and mouse, and in the chick using more caudal homeobox genes as probes (Clarke et al., 1991; Holder and Hill, 1991; Morriss-Kay et al., 1991; Sunden and Eichele, 1992). Many other homeobox-containing genes, transcription factors to which the patterning of the CNS is often attributed, are located in an

array that flanks the mesencephalon and rostral metencephalon, with some genes extending caudally, and others extending rostrally. Only the other *engrailed*-like gene, *En-1*, is known to cross this central boundary. If RA regulates these genes as a pathway to controlling the cascade of patterning events in CNS development, then it may do so with increasing concentrations as one moves distally from the node/mesencephalon. This model is consistent with evidence in mouse embryonal carcinoma cells that Hox genes proximal to the node are activated by short exposures to low concentrations of RA whereas those Hox genes distal to the node require higher concentrations and longer exposures to RA for expression to be induced (Simeone et al., 1990). It is not clear whether the influence of the node on the patterning of the CNS is due to a RA gradient, nor whether that gradient might be expected to extend across the whole embryo, or over just a short region adjacent to the node. It does appear from my experiments, however, that the rostral and caudal halves of the embryo require no continuity after stage 3c to become patterned, implying that the rostral and caudal halves of the embryo are responding to independent information, like two gradients, conjoined at the node.

It has been proposed that positional information is established/transmitted by the cells of the node after migration. In fact, many authors since the time of Spemann have proposed that the notochord is the mesodermal structure that carries positional information to the overlying neuroectoderm. In the experiment in Chapter 3, in which embryos lacking a rostral notochord were generated by removing Hensen's node, no rostrocaudal positional deficit was observed in the rostral neural tube as assayed by *En-2* expression and morphological criteria. In addition, Doniach and her co-workers have shown planar induction from the organizer results in appropriate regional gene expression in neuroectoderm from *Xenopus* (Doniach et al., 1992). Also, I have tentatively shown that ectoderm can be separated from the node at stage 3c and neuroectoderm will still form and become patterned to express *En-2* in the absence of notochord cells. Therefore, I concluded that it is not the notochord that establishes the rostrocaudal patterning of the

array that flanks the mesencephalon and rostral metencephalon, with some genes extending caudally, and others extending rostrally. Only the other *engrailed*-like gene, *En-1*, is known to cross this central boundary. If RA regulates these genes as a pathway to controlling the cascade of patterning events in CNS development, then it may do so with increasing concentrations as one moves distally from the node/mesencephalon. This model is consistent with evidence in mouse embryonal carcinoma cells that Hox genes proximal to the node are activated by short exposures to low concentrations of RA whereas those Hox genes distal to the node require higher concentrations and longer exposures to RA for expression to be induced (Simeone et al., 1990). It is not clear whether the influence of the node on the patterning of the CNS is due to a RA gradient, nor whether that gradient might be expected to extend across the whole embryo, or over just a short region adjacent to the node. It does appear from my experiments, however, that the rostral and caudal halves of the embryo require no continuity after stage 3c to become patterned, implying that the rostral and caudal halves of the embryo are responding to independent information, like two gradients, conjoined at the node.

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neuroectoderm, but rather, the rudiment within the node before stage 3c. The fact that the node at this stage contains a large percentage of endoderm cells, and that grafted endoderm in ectopic embryos seems to be highly correlated with neural induction and differentiation (Dias and Schoenwolf, 1990) is consistent with the conclusion that it is the endoderm, not the mesoderm cells of the node that are responsible for the patterning of the neuroectoderm. This conclusion is also consistent with the observation from transected embryo fragments that ectoderm completely separated from the node at stage 3c can form patterned neuroectoderm. At this stage, some endoderm has already migrated beneath the ectoderm and away from the node

I also believe that the node retains this ability to pattern the neuroectoderm through stage 4 or 5, but that this ability diminishes at these later stages, perhaps because the endoderm cells are totally absent from the node at these later stages. This conclusion explains the results of Storey and her co-workers (1992), Dias and Schoenwolf (1990), and myself, who have shown that the node is capable of initiating a new axis in an ectopic site between stages 3b/c-4/5, although the frequency of this initiation diminishes with age. Storey and her co-workers and I have also shown that the new axis expresses the positional marker *En-2*, indicating that exposure to the node is sufficient to induce appropriate neural markers in previously non-neural ectoderm. This conclusion, that the node can initiate positional information acquisition by the adjacent ectoderm between stages 3b/c and 4/5 resolves the apparent paradox in the role of the node raised by the node removal and node transplant experiments.

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