# Conversion of *Xenopus* Ectoderm into Neurons by NeuroD, a Basic Helix-Loop-Helix Protein

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Basic helix-loop-helix (bHLH) proteins are instrumental in determining cell type during development. A bHLH protein, termed NeuroD, for neurogenic differentiation, has now been identified as a differentiation factor for neurogenesis because (i) it is expressed transiently in a subset of neurons in the central and peripheral nervous systems at the time of their terminal differentiation into mature neurons and (ii) ectopic expression of *neuroD* in *Xenopus* embryos causes premature differentiation of neuronal precursors. Furthermore, *neuroD* can convert presumptive epidermal cells into neurons and also act as a neuronal determination gene. However, unlike another previously identified proneural gene (XASH-3), *neuroD* seems competent to bypass the normal inhibitory influences that usually prevent neurogenesis in ventral and lateral ectoderm and is capable of converting most of the embryonic ectoderm into neurons. The data suggest that *neuroD* may participate in the terminal differentiation step during vertebrate neuronal development.

Much of our understanding of neural fate determination comes from studies of *Drosophila* where basic helix-loop-helix (bHLH) genes are required for proper neurogenesis. The bHLH proteins are transcription factors that participate in several aspects of development, particularly in cell-type determination, terminal differentiation, and sex determination (*1*). Biochemical and x-ray crystallographic studies have shown that the HLH domain is required for dimerization, whereas the basic region makes specific contacts with DNA (*2, 3*). In vertebrates, tissue specific bHLH proteins, such as those encoded by the *myoD* (myogenic determination) gene family, heterodimerize with members of the widely expressed E proteins (*2, 4*) to promote myogenesis (*4*). In *Drosophila*, the Achaete-Scute complex (AS-C) proteins heterodimerize with the widely expressed Daughterless (Da) protein, which bears sequence similarity to E proteins (*5*), to promote neurogenesis (*5, 6*). These bHLH heterodimers then bind to the appropriate DNA recognition sites termed E boxes (CANNTG) (*2*) and activate transcription of target genes that determine cell type. Other bHLH proteins such as stem cell leukemia (SCL) protein and the mammalian Achaete-Scute homolog 1 (MASH-1) have been implicated in hematopoiesis (*7*) and neurogenesis (*8*), respectively. Homodimers of bHLH proteins may also specify cell fate, as may be the case for the mammalian E proteins (E2A) that are required for B-cell development (*9*). Tissue specific bHLH proteins are often present as families of related genes. These families seem to (i) provide for overlapping or redundant functions (for example, MyoD and Myf-5) and (ii) to separate a determination step (MyoD or Myf-5) (*10*) from a differentiation step (myogenin) (*11*). In addition, associated *cis*-acting control sequences may respond to positional or temporal expression patterns for each member of the family.

In *Drosophila*, the genes of the AS-C (*achaete, scute, lethal of scute,* and *asense*) and *atonal* (*ato*) are known as proneural genes and their encoded proteins function as positive neural fate determination factors (*6, 12*), much like the function played by MyoD and Myf-5 in mammalian myogenesis. Thus far, there does not seem to be a functional equivalent to myogenin among the *Drosophila* proneural genes; that is, a gene specifically involved in terminal differentiation of neurons. The ability of the proneural genes to generate neurons is negatively regulated by a number of other proteins, such as Notch, Delta, and the inhibitory HLH proteins, including Hairy and Emc, and proteins of the Enhancer of the split complex (*6*).

Whereas *Drosophila* has been a crucial system for identifying genes controlling neurogenesis, the amphibian embryo has also been instrumental for the study of neurogenesis. During *Xenopus* development, ectoderm can develop into either epidermal or neural tissue (*13*). Neural induction has been considered an active process stimulated by inducing factors originating from the Spemann organizer region and the involuting axial mesodermal tissue (*13*). The epidermal fate is thought to be the default state that arises when inducers are absent. This concept emerged from experiments with animal cap (AC) explants derived from the dorsal ectoderm of blastula stage *Xenopus* embryos (*14*). In the absence of neural inducing factors, AC explants take on an epidermal fate. A candidate for such a neural inducer is Noggin (*15*). More recent experiments, however, have suggested that the default state may be the neural fate and that neural induction may result from the removal of endogenous inhibitors of neural differentiation that are usually present in AC explants (*16–18*). An initial indication came from experiments in which many dissociated single cells form *Xenopus* blastula stage embryos differentiated into neuronal cells when cultured in vitro (*16*). Recently, Hemmati-Brivanlou and Melton showed that a dominant negative activin receptor can also cause cells in the AC explants to differentiate into neural cells, as if the activin receptor and its ligands actively prevent neurogenesis (*17*). This view is supported by results with follistatin, which binds activin (*19*) and also has been shown to induce neurogenesis (*18*).

Vertebrate homologs of *Drosophila* proneural genes have been cloned and assayed in frogs by ectopic expression (*20*). Microinjection of RNA encoding *Xenopus* Achaete-Scute homolog-3 (XASH-3) into fertilized frog embryos causes a lateral expansion of the neural tube as presumptive epidermis and neural crest cells take on a neural fate (*20*). However, ventral and lateral ectoderm do not convert to nerve, which may be a result of specific inhibitors present in these regions in the developing embryo (*20*). MASH-1 has also been implicated in neural development (*8*). The elimination of MASH-1 by gene targeting leads to defects in the autonomic nervous system (a derivative of the neural crest) and in the olfactory system (*8*).

We now describe a bHLH gene that is transiently expressed in differentiating neurons in mice and frogs and displays several functional characteristics consistent with its role as a differentiation factor for neurogenesis. We have termed this gene *neuroD*.

**Cloning and expression analysis of *neuroD*.** As a means of exploring the role of bHLH proteins in cell-type specification during development, we adapted gene targeting techniques to the common HLH partner, E2A, to assess the cell types that are specified by heterodimers of tissue-specific bHLH and E proteins. Another way of approaching this problem is to use the yeast

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‡It is with sadness that we announce the death of Harold Weintraub on 28 March 1995.

two-hybrid system of Fields and Song (*21*) as modified by Hollenberg and co-workers (*22*) to identify additional tissue-specific HLH proteins. A fusion protein library of VP16 and complementary DNA (cDNA) was made from mouse embryonic stem cell tumors that contained many differentiated cell types (*23, 24*). The library was screened for cDNAs that interacted with LexA-Da, a fusion protein between the *Drosophila* Da bHLH domain and the LexA-DNA binding domain (*22, 25*). We screened more than $2 \times 10^7$ transformants and assayed the positive clones by pairing with LexA-lamin, which served as a negative control (*22*). Using the criteria that the clones from the cDNA library interact with LexA-Da and not with LexA-lamin (a non-HLH protein) (*22*), we isolated the cDNA encoding the bHLH protein that we call NeuroD.

The initial *VP16-neuroD* clone had approximately 450 base pairs (bp) that spanned the bHLH region. We used this clone to screen a cDNA library derived from a mouse at embryonic day 10.5 (e10.5) and a mouse genomic library to obtain the full-length coding sequence (*26*). Translation of the mouse *neuroD* coding region yielded a putative protein (40.1 kD) consisting of 358 amino acid residues (Fig. 1). The predicted NeuroD bHLH contained (i) the conserved residues that are characteristic of all members of the HLH protein family and (ii) several distinctive residues at the critical positions of the protein that make it different from previously identified HLH proteins (Fig. 2).

Expression of *neuroD* in mice was assayed by in situ hybridization to mouse embryo sections (*28, 29*). The *neuroD* RNA was first detected in embryos at e9.0 to e9.5 in the developing trigeminal ganglia (*30*). At e11.5, stronger expression was detected in all of the cranial ganglia V to XI and dorsal root ganglia (DRG) (Fig. 3, A and B). In both the cerebral cortex and spinal cord, regions outside, but not within, the mitotically active ventricular zone showed strong hybridization signals for *neuroD* expression (Fig. 3, D and E). Expression of *neuroD* was also detected in sensory organs where active neuronal differentiation was occurring; the nasal epithelium and the retina of the eye expressed *neuroD* in the regions that contain differentiating neurons (*30*). By e14.5, *neuroD* expression disappeared in the cranial ganglia and DRG (where active neuronal differentiation had ceased), but the expression persisted in the brain and sensory organs (where active neuronal differentiation still occurred). We conclude from this analysis that *neuroD* is expressed transiently in differentiating neurons during mouse embryonic development (*31*). In contrast, *MASH-1* seems to be expressed in mitotic neural precursor cells in the central nervous system (CNS) (*32*). For example, in situ hybridization on e11.5 embryo sections through the cerebral cortex showed that *MASH-1* was expressed in the mitotic ventricular zone of the cortex whereas *neuroD* was expressed in the cells adjacent to this region containing a population of postmitotic differentiating neurons (Fig. 3, C and D). Although *neuroD* expression was high in the developing sensory ganglia derived from the neural crest, its expression was not observed in sympathetic and enteric ganglia (*30*) that are also derived from the migrating neurogenic neural crest that requires *MASH-1* for complete differentiation (*32*).

We used microinjection into *Xenopus* embryos (*33*) to examine the role of *neuroD* during vertebrate neural development. We isolated several cDNAs encoding the *Xenopus* homolog of *neuroD* (*34*) and compared predicted protein sequence to those of mouse *neuroD* (Fig. 1). Sequencing of the frog cDNA revealed high conservation of the predicted NeuroD protein between mouse and frog (*35*). We also identified, through a computer search, a *neuroD* counterpart in *Caenorhabditis elegans* (*36*). All the predicted critical residues in the basic domain are also found in *C. elegans* (Fig. 2).

The *neuroD* expression pattern in *Xenopus* at different stages (*37*) was examined by in situ hybridization to whole embryo (*38*) that showed similar expression patterns to those observed in mouse embryos. Expression was first detected at stage 14 (neural

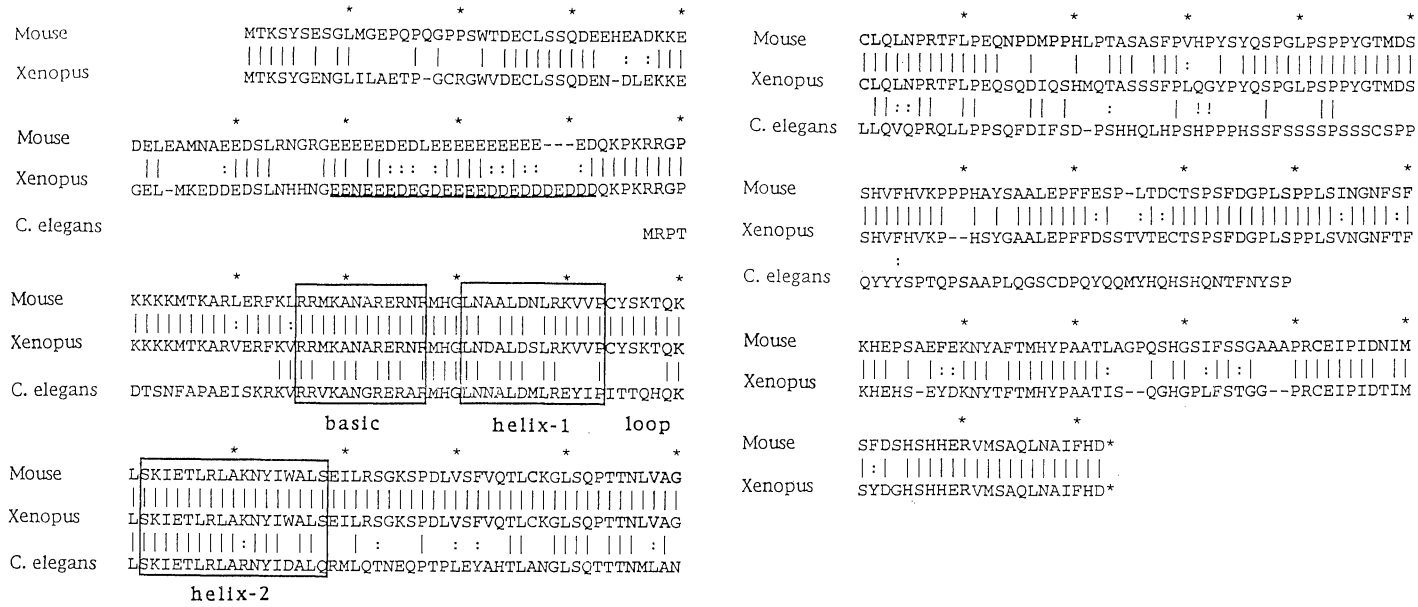


**Fig. 1.** Alignment of the predicted protein sequences of mouse, *Xenopus*, and *C. elegans* NeuroD. The mouse coding sequence is based on the sequences obtained from cDNA and genomic clones. The *Xenopus* sequence is derived from several cDNA clones. The *C. elegans* sequence represents the predicted coding sequence from computer analysis of the *C. elegans* cosmid C34E10, which was sequenced during the sequencing project of *C. elegans* chromosome III (*38*). The predicted first methionine represents the first methionine after an upstream termination codon in all three sequences. The sequences outside the predicted coding sequences diverge between mouse and frog. The basic, helix-1, loop, and helix-2 domains are boxed, and the preceding acidic domain in mouse and *Xenopus* sequences is underlined. A solid line between the aligned sequences represents identity and a colon (:) represents conserved change or similarity. The *C. elegans* residues that are identical to mouse sequence but not to *Xenopus* sequence are marked with (!). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

plate stage) in the trigeminal placodes in the head (Fig. 3G). At the same time, we detected expression in rows of primary neurons adjacent to the midline of the spinal cord and the lateral edges of the neural plate, representing primary motor neurons and Rohon-Beard cells (primary mechanosensory neurons), respectively, as assessed by their positions in the embryo (Fig. 3, F and G) (*39*). Primary neurons are the first neurons to differentiate and are responsible for establishing the escape response during early stages of *Xenopus* development (*39*). By the tail bud stages all the cranial ganglia showed strong expression patterns (Fig. 3H), as observed in mouse embryos. In *Xenopus*, as in other vertebrate organisms, neural crest cells give rise to skeletal components of the head, all ganglia of the peripheral nervous system (PNS), and pigment cells (*40*). Among these derivatives the cranial sensory ganglia, which are of mixed neural crest and placode origin, appears to be the only group of cells that express *neuroD*. Expression in the eye could be correlated with active neuronal differentiation in the retina at this stage (Fig. 3H) (*37, 41*). Expression observed in the developing olfactory placodes and otic vesicles was similar to that in mice (Fig. 3H). The pineal gland also expressed *neuroD*. Expression of *neuroD* in *Xenopus* was also transient, suggesting that *neuroD* functions during the neuronal differentiation process and is not required for maintenance of these differentiated cell types. However, *neuroD* expression in the DRG, which was quite high in developing mouse embryos, was not observed in *Xenopus* embryos at the stages examined probably because the few DRG cells in *Xenopus* develop late, during the tadpole state (after stage 39) (*37, 39*). *Xenopus* embryos show stronger *neuroD* expression in the eye than do mouse embryos of comparable developmental stage, a result that perhaps could be due to the more rapid neuronal differentiation in the *Xenopus* eye (*41*). Thus, in both mice and frogs, *neuroD* expression shows a temporal and spatial correlation with neuronal differentiation in a subset of neural tissues. The populations of neurons that express *neuroD* are derivatives of the neural crest, various placodes, and the neural tube.

**Conversion of both nonneural ectodermal and neural crest cells into neurons by ectopic expression of *neuroD*.** We injected in vitro generated transcripts (*42*) encoding a fusion protein of a Myc-epitope-tag (Myc-tag) and NeuroD (*43*) into one cell of two-cell stage frog embryos in which the uninjected side of the embryo serves as an internal control. The effects of misexpression of *neuroD* were assayed with specific antibodies to neuronal cells (*44*) and digoxigenin-labeled RNA probes (Figs. 4 and 5) (*38*). The ventral and lateral epidermis on the side of the embryos injected with *neuroD* RNA showed ectopic expression of neural cell adhesion molecule (N-CAM), a neural-specific marker in *Xenopus* (*45*) (Fig. 4B). We stained more than 150 *neuroD* RNA injected embryos, and more than 98 percent of these embryos showed ectopic N-CAM staining on the injected side. We used double-label immunostaining with antibodies to the Myc tag and N-CAM to ensure that the side of the embryo that was N-CAM–stained was the injected side (Fig. 6E). Ectopic N-CAM immunostaining was first observed at late neurula (stage 19) (Fig. 6D) when N-CAM staining in the epidermis showed no overt neuronal differentiation, such as the formation of nerve processes. These cells expressing N-CAM subsequently differentiated into morphologically typical N-CAM positive neural cells with processes by tail bud stage (stage 24) (Fig. 4B). Initial ectopic N-CAM staining at stage 19 was somewhat delayed and patterned in that individual N-CAM positive cells were surrounded by nonstaining neighbors (Fig. 7D). That the ectopic N-CAM expression resulted from a direct effect on the presumptive epidermal cells, and not from aberrant neural cell migration into the lateral and ventral epidermis, was confirmed when we injected *neuroD* RNA into a single blastomere of the top tier of 32-cell stage embryos, which targeted the injection into cells destined to become epidermis, and obtained ectopic neuronal cells (*46*) (Fig. 6, C and D). Ectopic generation of neurons by *neuroD* was confirmed with other neural specific markers, such as a neural-specific class II β-*tubulin*, acetylated α-tubulin, *tanabin*, neurofilament-M (NF-M), Xen-1, and Xen-2 (Fig. 5, A to H) (*47*). These markers displayed ectopic staining on the side injected with *neuroD* RNA. Injection of *neuroD* mRNA into vegetal cells did not yield any ectopic expression of neural markers, suggesting the absence of cofactors or the presence of inhibitors in vegetal cells.

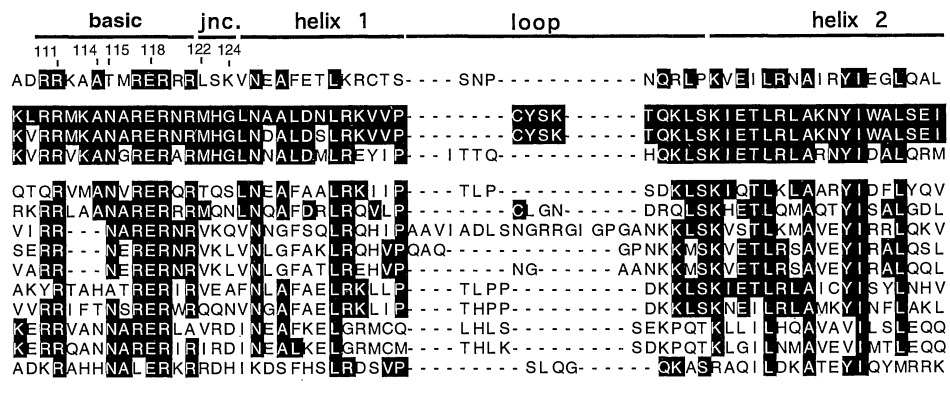


**Fig. 2.** Alignment of the bHLH domains of NeuroD and other bHLH protein family members. The three-dimensional structures of bHLH proteins are highly conserved (*3*), making it possible to compare residues at specific positions. With the MyoD sequence as a reference, $E^{118}$ is conserved in all bHLH proteins as well as in NeuroD; this critical residue makes contacts with C and A on one strand of the consensus E box (CANNTG) and the corresponding T and G on the opposite strand. $L^{122}$ in MyoD (which is an M in NeuroD, a V in E12, and an R in Max) is critical in determining the central NN residues of the E box. Each R residue at this position in Max homodimer specifies the central symmetrical CG residues by a direct contact with a G on the corresponding DNA strand. For MyoD and E12 heterodimer, this residue is small and does not contact DNA, and the central residues (GC) may result from indirect effects. NeuroD, with its M in this position, could also be expected to fall into this class. Positions 114, 115, and 124, when exchanged from MyoD into E12, allow E12 to activate myogenesis (*27*). Positions 114 and 115 are crucial residues for transcriptional activation; they are highly conserved within families, but differ between families. At both of these positions, NeuroD shares the identical residues with the Twist and Atonal proteins. Both residues face the DNA bases, and $T^{115}$ of MyoD makes a direct contact with the T in the consensus. Many mutations in $A^{114}$ in MyoD inhibit transcriptional activation without affecting DNA binding. Therefore, $A^{114}$ is crucial for the positive control activity. The MyoD crystal structure has suggested that $A^{114}$ and $R^{111}$ (which are also present in NeuroD and Atonal) affect (communicate with) each other. Usually the $R^{111}$ side chain makes a direct contact with the last G of the consensus DNA sequence. However, when there is a bulky side chain at position 114 (as in the case of the N in E12 or the H in Max), the $R^{111}$ side chain is constrained and flips out, pointing away from the DNA, thus possibly causing a conformational change that can alter interactions with basal transcription factors. In MyoD, $K^{124}$ is on the outside of the basic region α helix and may be recognized by a cofactor involved in transcription. Although this position is conserved in individual bHLH proteins (and their families), it is variable between families. The G at this position occurs only in NeuroD.

We used several markers (including HNK-1, *shaker-1*, and islet-1) that detect Rohon-Beard cells (*48*) to test whether primary neurons are generated by ectopic *neuroD* expression because *neuroD* is normally expressed in a subset of developing primary neurons (such as primary motor neurons and Rohon-Beard cells). More cells were stained with these markers on the injected side than on the other (Fig. 6, E to H), indicating that ectopically expressed *neuroD* can convert nonneuronal cells (that is, presumptive epidermis) to neurons, including those with primary neuron characteristics.

In order to confirm that NeuroD can convert uninduced ectodermal tissue into neurons, we injected *neuroD* RNA bilaterally in two-cell stage *Xenopus* embryos, isolated animal cap explants at mid-blastula stage, and cultured these until their uninjected sibling embryos reached tail bud stage (stages 26 to 34) (*49*). We observed N-CAM staining only in the animal cap explants that were injected with *neuroD* (54 percent of these showed N-CAM staining, $N = 50$) (Fig. 6, A and B). The staining in the animal cap cells seemed stable and persisted at least for 64 hours of incubation, an indication that *neuroD* could convert uninduced animal cap cells into neural cells in the absence of neural induction.

In wild-type embryos, the cells of the cephalic neural crest migrate and form most of the head structure, including cranial cartilage, muscle tissue, and nerve cells (*40*). In embryos injected with *neuroD*, there was often an increased cell mass in the region from which the cranial neural crest and its derivatives originate (Figs. 4B, 5, B, D, and F, and 7D). To examine whether *neuroD* converted the nonneural components of neural crest cells into the neural lineage, we used *neuroD* injected embryos to assay for alterations in the expression of *Xtwi*, the *Xenopus* homolog of *Drosophila twist* (*50*). In wild-type embryos, *Xtwi* is expressed in the nonneural population of cephalic neural crest cells that give rise to the connective tissue and skeleton of the head (Fig. 8A) (*50*). At stage 16, embryos injected with *neuroD* gave no evidence of *Xtwi* expression in the migrating cranial neural crest cells on the injected side (Fig. 8B). The failure to generate sufficient cranial mesenchymal neural crest precursors in embryos injected with *neuroD* was evident morphologically because branchial arch development in the head was deficient in many of the injected embryos (*51*). Furthermore, the increased mass of cells in the cephalic region stained for N-CAM, β-*tubulin*, and Xen-1 (Figs. 4B, 5, B and D, and 6C), an indication that these cells were neural in character. The converse experiment in which frog embryos were injected with *Xtwi* RNA (*52*, *53*) revealed that ectopic expression of *Xtwi*

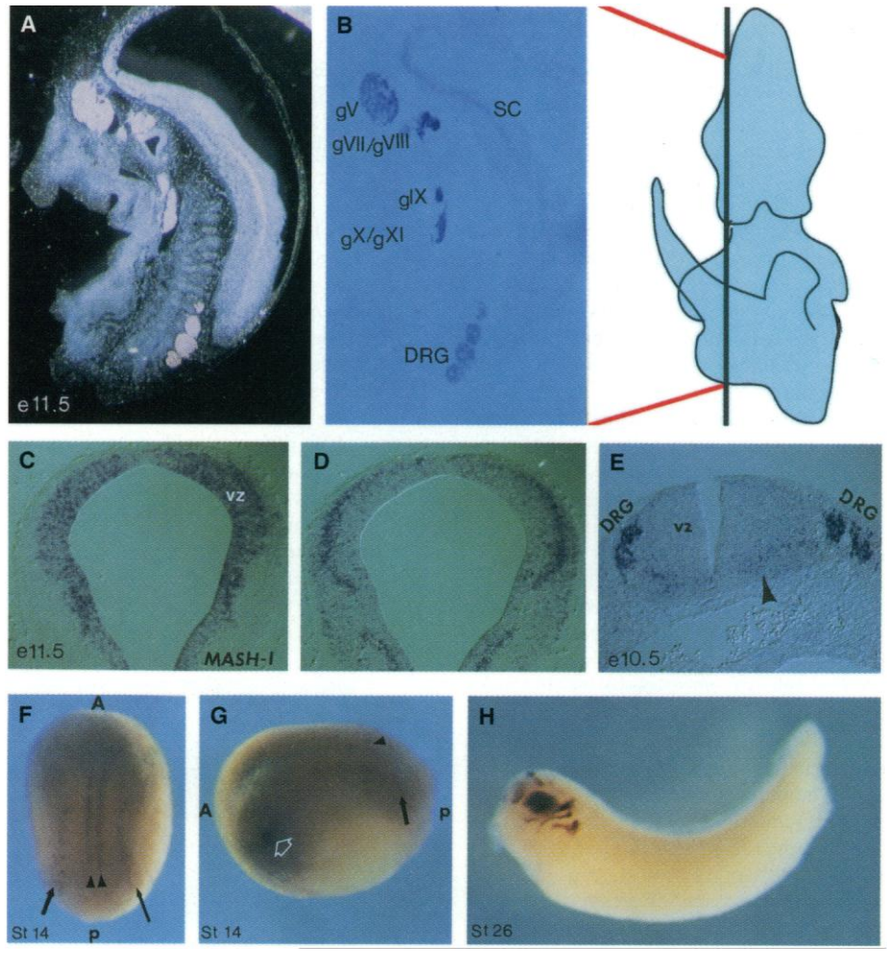


**Fig. 3.** Expression patterns of *neuroD* in mouse and frog embryos. In situ hybridization on mouse embryo sections (e11.5, **A** to **D**; e10.5, **E**) and whole *Xenopus* embryos (**F** to **H**) with digoxigenin-labeled RNA probes. (A) and (B) are parasagittal sections, as shown on the diagram: (A) and (B) correspond to close serial sections photographed in dark-field and bright-field, respectively. Expression of *neuroD* is detected in the cranial ganglia ($g^{V-XI}$), dorsal root ganglia (DRG), and spinal cord (sc), as labeled in (B). (C and D) Close serial frontal sections of midbrain hybridized to *MASH-1* and *neuroD* probes, respectively. *MASH-1* is expressed in the mitotically active ventricular zone (vz) in the cortex, whereas *neuroD* is expressed mostly outside the vz. (E) A bright-field picture of an e10.5 sc section hybridized to *neuroD* probe. Expression is visible in the DRGs next to the sc. Within the sc, *neuroD* is expressed more intensely in the regions outside the vz (arrowhead). (F to H) Whole mount in situ hybridization on *Xenopus* embryos. At stage 14, *neuroD* is expressed in the primary neurons in the sc (F and G), the primary motor neurons (arrowheads), the Rohon-Beard cells (black arrows), and in the trigeminal placode (G, white arrow); in tail bud stage embryos (stage 24) (H), *neuroD* is expressed in all the cranial ganglia, eye, olfactory placode, pineal gland, and several places in the CNS including the mid- and hindbrain boundary and rhombomeres.

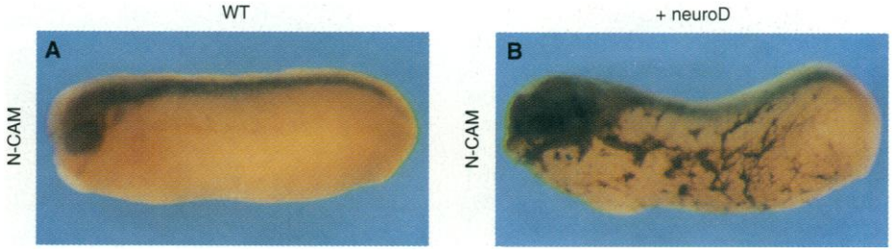


**Fig. 4.** Ectopic N-CAM expression in frog embryos injected with *neuroD* RNA. Immunostaining with an antibody to N-CAM on a wild-type (WT) embryo at the tail-bud stage (stage 24) (**A**), and on a *neuroD* injected embryo (**B**) that showed ectopic conversion of neural crest and epidermal cells into neurons. Ectopic neurons displayed terminally differentiated neuronal morphology with processes. Microinjection of RNA was routinely performed by injecting approximately 4 to 5 nl of RNA (100 pg/nl, in water) into one cell of two-cell stage *Xenopus* embryos at two positions in the animal hemisphere.

decreased *neuroD* expression on the injected side (Fig. 8, E and F). Thus, two members of the bHLH family, *neuroD* and *Xtwi*, may compete for defining the identity of different cell types derived from the neural crest. In the embryos injected with *neuroD*, exogenous *neuroD* may have induced premigratory neural crest cells to differentiate into neurons in situ, and consequently these cells do not migrate to their normal positions. However, injections of *neuroD* RNA did not affect all neural crest cells because even in the most severely affected animals, the head structure remained partially formed.

**Premature differentiation of neural precursors in the CNS by exogenous *neuroD*.** The effect of the introduced *neuroD* on the fate of (i) cells that normally express *neuroD*, such as cranial ganglia, eye, otic vesicle, olfactory organs, and primary neurons, and (ii) other CNS cells that normally do not express *neuroD* was then examined. When the cranial region of the embryo was severely affected by ectopic *neuroD*, the injected side of these embryos displayed either small eyes or none at all, and poorly organized brains, otic vesicles, and olfactory organs (*54*) (Figs. 4B and 5, B, D, and F). As the embryos grew, the spinal cord was retarded, remaining thinner and shorter on the side injected with *neuroD*. Several differentiation markers were then used to examine whether these phenotypes resulted from premature differentiation and growth arrest of neural precursor cells.

The first indication that *neuroD* might cause premature differentiation in the CNS came from N-CAM staining in neurula stage embryos (stage 19). In the normal embryo at early stages, N-CAM staining is not uniform throughout the entire neural plate, but is more prominent in the medial region of the neural plate (*45*). When injected embryos were analyzed for N-CAM expression, the neural plate on the injected side of early stage embryos stained more intensely and more laterally (Fig. 7D). This increase in N-CAM staining was not associated with lateral expansion (*54*), in contrast to what had been seen with *XASH-3* injection that caused neural plate expansion (*20*). These observations suggest that *neuroD* causes neural precursors in the neural plate to differentiate prematurely. This hypothesis was tested with two neural markers, neural specific β-*tubulin* and *tanabin*, which are expressed in differentiated neurons (*47*) Ectopic β-*tubulin* positive cells were already detected on the injected side at the end of gastrulation (stage 12) when the control side did not yet show any β-*tubulin* positive cells (Fig. 7A). At stage 14, overexpression of *neuroD* increased the β-*tubulin* signals in the region of the neural plate containing primary neurons (Fig. 7, B and C). In addition, *tanabin* was expressed in more cells in the spinal cord on the *neuroD*-injected side of these embryos at stage 14 (*51*). Thus, overexpressed *neuroD* resulted in an initial expansion of the neuronal population in the CNS, possibly due to the premature differentiation of neural precursors. Subsequently, cells may withdraw from the cell cycle, depleting the mitotic neural precursor population and resulting in the poor eye and spinal cord development observed later. Another possibility (one that does not explain the phenotypic effects seen at later stages) is that the extra cells that express these neural markers in the CNS may not represent prematurely differentiated neural precursors, but rather precursors of nonneuronal cell types, such as glial cells, that are converted to neurons (*55*).

In summary, *neuroD* is transiently expressed in the embryonic tissues that contain terminally differentiating neurons in both mice and frogs. These neurons arise from different ectodermal derivatives such as the neural crest, placodes, and the CNS, and are not obviously related by functional properties, position, lineage, time of differentiation, or the expression of any known set of molecular markers. The expression pattern of *neuroD* is, therefore, consistent with its participation in the differentiation process of many neuronal cell types, which is now being tested by gene targeting. Other factors would have to interact with NeuroD to define precisely the type of neuron being generated at any particular time and place. Proteins that contain the LIM homeodomain motif might form a class of such factors (*56*).

The ectopic expression experiments suggest that (i) *neuroD* expression is sufficient to convert both nonneural populations of neural crest and presumptive epidermal

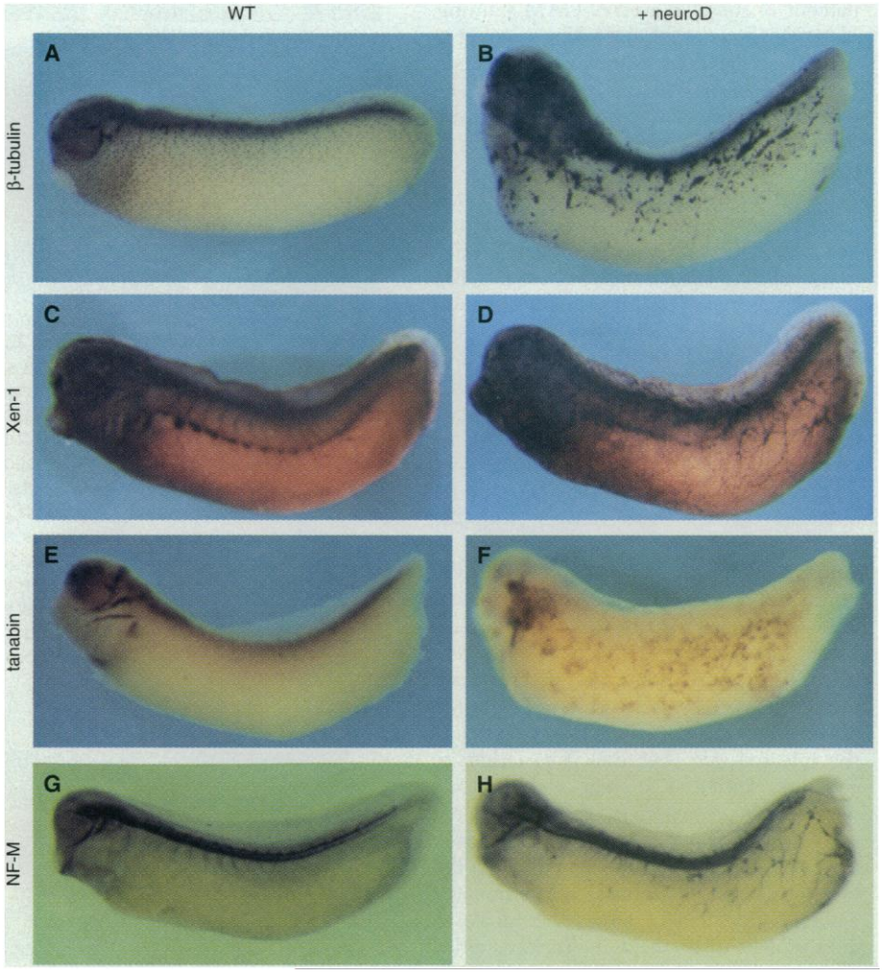


**Fig. 5.** Ectopic expression of other neural markers in *neuroD* RNA–injected frog embryos. (**A** and **B**) β-*tubulin* expression and (**E** and **F**) *tanabin* expression were detected by in situ hybridization. (**C** and **D**) Xen-1 and (**G** and **H**) NF-M proteins were detected by immunostaining. Wild-type embryos were either uninjected embryos (A, E, and G) or the uninjected side of an injected embryo (C). Embryos stained for NF-M (G and H) have been cleared (*47*). In situ hybridization with a β-*tubulin* probe without the ribonuclease treatment step detects ciliated epidermal cells (*47*) (as in A, dots over the epidermis).

cells or AC cells into neurons. This cell fate conversion can occur in the apparent absence of neural induction. Moreover, the capacity of *neuroD* to activate neuronal differentiation seems to override any inhibitors present in the ectoderm. (ii) *neuroD* causes neuronal precursors within the CNS to terminally differentiate before and simultaneously with endogenously differentiating neurons. We interpret this as premature differentiation of neural precursor cells, which is consistent with expression data indicating that *neuroD* is expressed only in cells undergoing neuronal differentiation. These observations suggest that *neuroD* may control the initiation of terminal differentiation in a subset of neurons during vertebrate development. (iii) The capacity for *neuroD* to generate ectopic neurons seems limited to ectodermal derivatives (*57*). We detected ectopic neuron formation or premature neuronal differentiation only in the CNS, neural crest derivatives, and epidermal cells. It is possible that *neuroD* requires an ectoderm-specific factor or cofactor to induce neuronal differentiation or to escape endogenous inhibitors. While the capacity of *neuroD* to convert ectodermal cells into neurons might suggest a determination function, we prefer the hypothesis that *neuroD* is more likely to function as a differentiation factor during normal embryogenesis, on the basis of its expression pattern and the earlier expression of *XASH-3*.

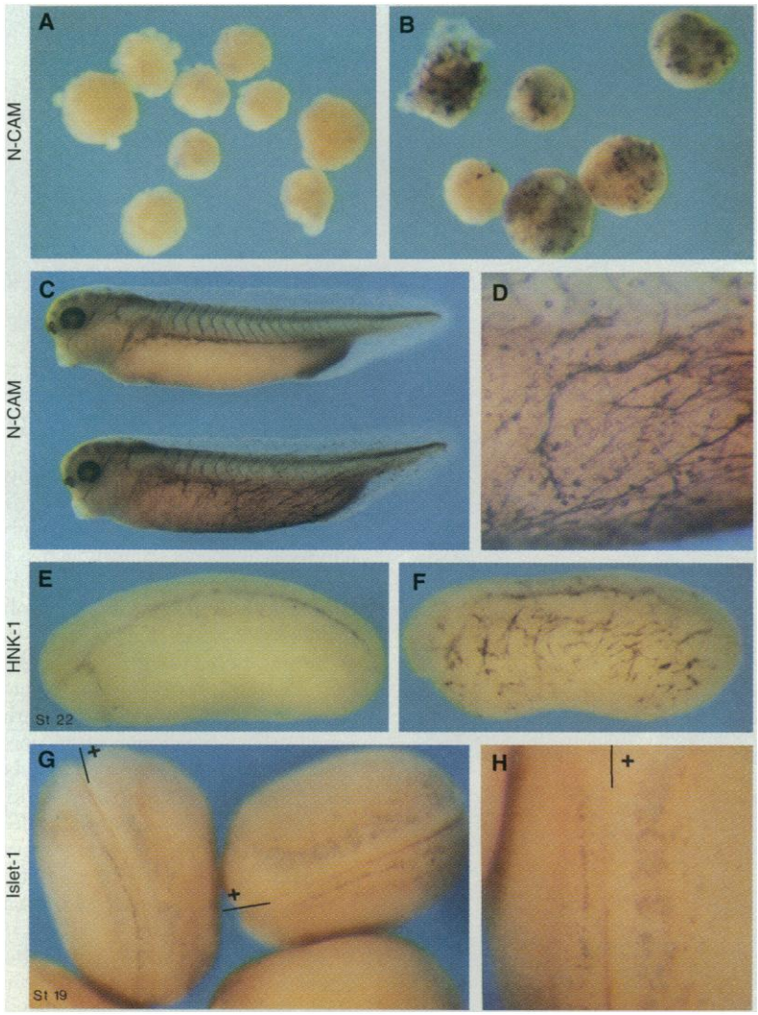


**Fig. 6.** Conversion of AC explants and epidermal precursors into neurons and generation of ectopic primary neurons by *neuroD*. (**A** and **B**) N-CAM expression in uninjected and *neuroD*-injected AC explants, respectively. (**C**) N-CAM expression in a wild-type control embryo (top) and in an embryo that had been injected with *neuroD* RNA into a single blastomere in the top tier at the 32-cell stage (bottom). In the injected embryo, extensive N-CAM staining is visible in the lateral and ventral epidermis without any effect on the nervous system. (**D**) A high magnification of the epidermis of the injected embryo in (C). The N-CAM–positive cells display morphological phenotype of neurons with processes. Generation of ectopic primary neurons was detected with antibodies to HNK-1 (**E** and **F**) and islet-1 (**G** and **H**). (E) HNK-1 immunostaining of a wild-type embryo and (F) a *neuroD* RNA–injected embryo. (G) Islet-1 staining of the neurula stage (stage 19) embryos that have been injected with *neuroD* on one side at the two-cell stage. The injected sides are marked as "+" on the cranial side of each embryo. (H) High magnification of the dorsal region of an embryo shown in (G). The wild-type side shows islet-1 expressing Rohon-Beard (R-B) cells as a single row of cells near the midline on the dorsal surface. The injected side shows more islet-1–positive cells in a wider area and deeper layers.

Our results with ectopic expression of *neuroD* independently raise an intriguing issue with regard to pattern formation. The early pattern of ectopic neurons generated by *neuroD* at the neurula stage (stage 19), as assayed by N-CAM staining, shows neurons surrounded by cells that do not express this marker, even though the ectopically injected *neuroD* RNA should be present in most cells in the injected region of the embryo (Fig. 6E). This pattern is reminiscent of the pattern generated by lateral inhibition during *Drosophila* neurogenesis, and thus raises the following possibilities: (i) *neuroD* not only activates a terminal neurogenic pathway, but also a lateral inhibition (or something like lateral inhibition) pathway (*6*), or (ii) there is already such a pre-pattern in the ectoderm. Some evidence for the latter comes from the fact that there is a regular pattern of ciliated epidermal cells on frog skin that can be detected by *tubulin* markers (Fig. 5, A and B) (*58*). Whatever generates such a pattern might interact with *neuroD* so that *neuroD* activity mirrors the underlying patterning of epidermis. Evidence that proneural genes can activate some lateral inhibition genes is also available (*20*, *59*).

In mice, ectopic expression and gene targeting experiments have led to the conclusion that MyoD and Myf-5 are redundant myogenic determination factors (*10*) and that myogenin is a differentiation factor (*11*). Our ectopic expression studies with *NeuroD* cannot readily distinguish whether *neuroD* is a determination (proneural) or differentiation factor. However, the fact that *neuroD* is expressed in differentiating cells and not in mitotic neural precursors and that it causes premature differentiation of neural precursors suggest a role as a differentiation factor, perhaps similar to myogenin. If true, it is clear that NeuroD must be just one of the factors in specifying the vast array of neuronal cell types.

We believe that there is an important distinction between determination and differentiation factors in regard to their response to inhibitory factors. In *myogenin* mutant mice, muscle precursors (myoblasts) are formed at the right place, time, and number (under the determinative influence of MyoD or Myf-5), but they fail to differentiate in situ; however, when placed in culture, they differentiate normally into myotubes (*11*). These results suggest that inhibitors present in the embryo prevent MyoD or Myf-5 from activating the terminal differentiation pathway in the embryo while myo-

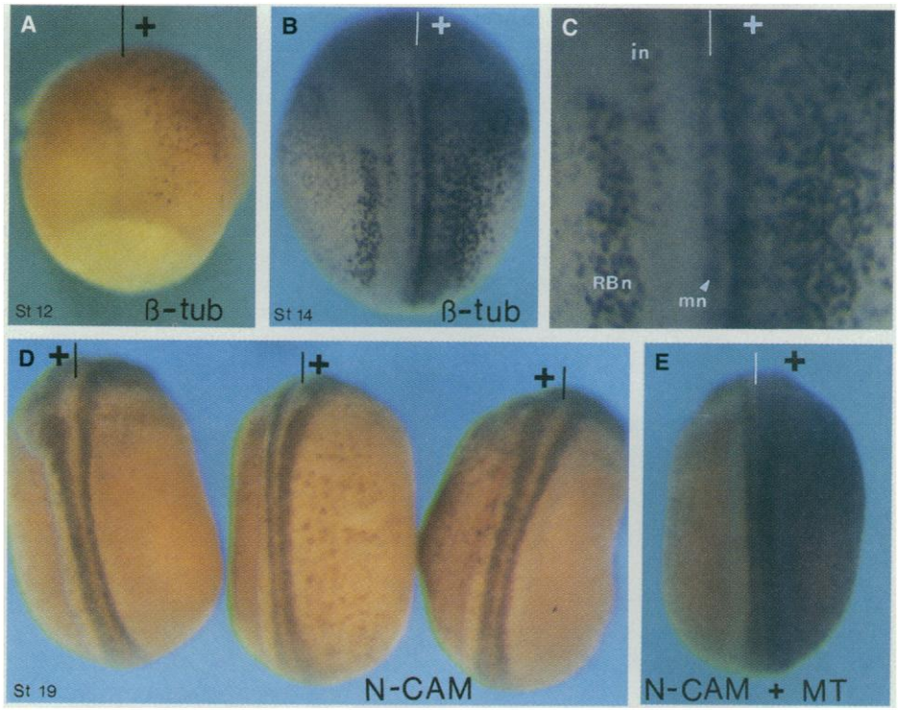


**Fig. 7.** Premature differentiation of neural precursors by ectopically expressed *neuroD*. The injected sides are marked as "+" on the cranial side of each embryo. (**A** to **C**) In situ hybridization on unilaterally injected embryos at the neural plate stage with β-*tubulin* (β-tub). (A) The earliest ectopic β-*tubulin*–positive cells in the neuroectoderm generated by ectopically expressed *neuroD* are observed at the medium yolk plug stage (stage 12) during gastrulation, when the wild-type side does not yet show any β-*tubulin–positive* cells. (B) The injected side displays extra β-*tubulin*–positive cells within the neural plate, in addition to ectopic β-*tubulin*– positive cells in the epidermis. A high magnification picture (C) shows formation of extra neurons at the positions where interneurons (in), primary motor neurons (mn), and Rohon Beard neurons (RBn) arise in the spinal cord. (**D**) N-CAM staining of *neuroD* RNA–injected embryos at stage 19. On the injected side, the staining in the neural tube is wide, but the neural tube itself is of normal size, unlike XASH-3–injected embryos that display expanded neural tube (*20*). In addition, ectopic, but isolated, N-CAM–staining pattern can be seen in the epidermis. At this stage, N-CAM–staining cells have not yet formed neuronal processes. (**E**) Double staining of one of the embryos shown in (D) with an antibody to Myc-epitope tag (MT), which is shown in purple staining, indicating that the side of the embryo with wider neural tube and ectopic epidermal staining for N-CAM is indeed the injected side.

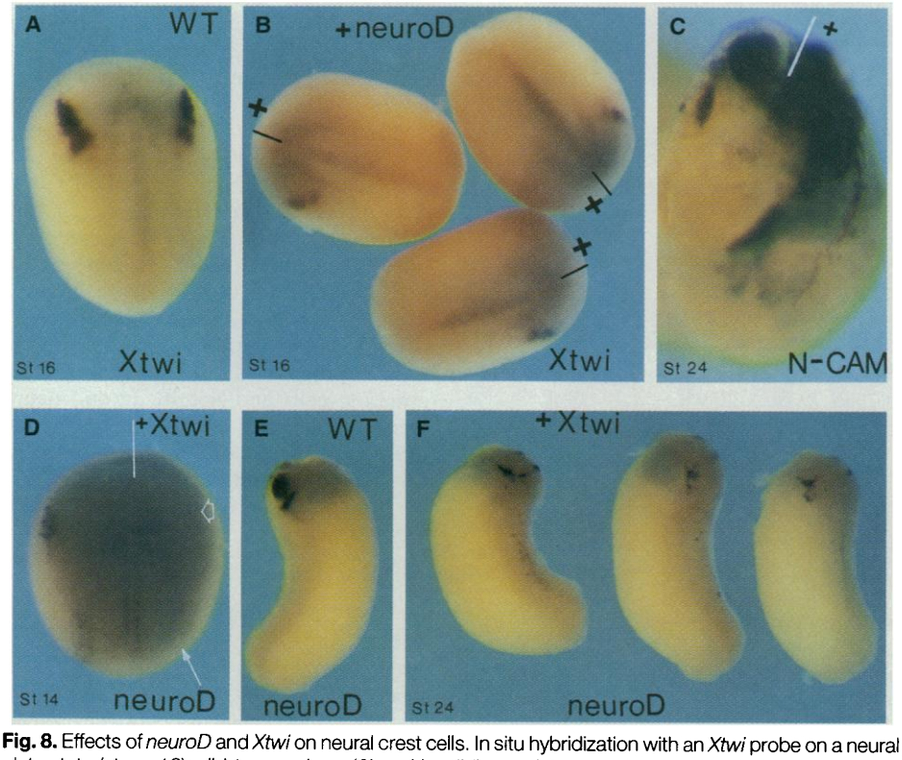


**Fig. 8.** Effects of *neuroD* and *Xtwi* on neural crest cells. In situ hybridization with an *Xtwi* probe on a neural plate state (stage 16) wild-type embryo (**A**) and its sibling embryos that had been injected with *neuroD* RNA on one side (**B**). The injected sides are marked as "+" on the cranial side of each embryo; *neuroD* causes suppression of *Xtwi* expression on the injected side. (**C**) A frontal cut (at the cephalic neural crest) of a *neuroD*-injected tail bud stage (stage 24) embryo that was immunostained with an antibody to N-CAM. Most of the cephalic neural crest cells on the injected side stained with N-CAM. (**D** to **F**) In situ hybridization with *neuroD* probe on *Xtwi*-injected embryos. (D) Early neural plate state (stage 14) embryo is missing *neuroD* expression in the trigeminal placode (unfilled white arrow) and R-B cells (filled white arrow) on the injected side. (E) Wild-type *neuroD* expression at tail bud stage (stage 24). (F) Reduced *neuroD* expression in *Xtwi*-injected embryos at stage 24.

genin can bypass the effects of these embryonic inhibitors, perhaps because it does not contain a target site for such inhibitors. Therefore, in the absence of myogenin, MyoD or Myf-5 are competent to induce terminal differentiation only if inhibitors are removed, as seems to occur when cells are placed in culture (*11*). In frog embryos, related results are seen with frog MyoD, which contains a domain that responds to endogenous inhibitors that block nuclear entry (*53*). Similarly during *Xenopus* neural development, *XASH-3* is expressed earlier than *neuroD*, but its ectopic expression, which causes expansion of the neural tube in the dorsal part of the embryo, cannot force ventral and lateral ectoderm to differentiate into nerve (*20*). In contrast, *neuroD* can readily do this, possibly because it does not harbor targets for endogenous inhibitors possibly present in *XASH-3*.

In *Drosophila*, the distinction between a determination and a differentiation step is not apparent during neurogenesis. Although the *asense* (*ase*) gene seems to be involved in events downstream of the proneural determination step (*60*), there are qualitative functional and sequence differences between *ase* and *neuroD*: (i) *ase* is expressed in cells that are still dividing, whereas *neuroD* is expressed in postmitotic cells; (ii) misexpression or overexpression of *ase* does not result in premature differentiation as observed with *neuroD*; (iii) ectopic expression of *ase* causes formation of ectopic sensory organs only in very restricted

regions of the epidermis. In contrast, misexpression of *neuroD* in *Xenopus* can form ectopic neurons in most of the epidermis, again suggesting that *neuroD* may not be sensitive to inhibitors (for example, Emc or Id) to which *ase* may be (*6*, *60*).

The lack of a clear distinction between determination and differentiation genes in *Drosophila* may be due to the existence of a complex dorso-ventral (D-V) and anterior-posterior (A-P) coordinate system that allows a precise temporal and spatial regulation of the terminal differentiation of different types of neuron or muscle cell. In vertebrates, specification may be more contextual, depending on where and when a precursor of a specific cell type (for example, myoblast or neuroblast) finds itself. Determination genes, such as MyoD, Myf-5, or XASH-3 proteins, might have originally been differentiation competent. However, a need for additional and new types of muscle and nerve cells may require a mechanism to cease differentiation and to increase cell number so that already specified myoblasts and neuroblasts could migrate to the appropriate place and differentiate at the appropriate time. One adaptation might have been the addition of inhibitory domains to these genes, thus making these domains responsive to new and, perhaps, more regulatable environment cues.

Therefore, there may be a reason for the evolution of separate determination and differentiation genes; namely, that one (determination) is responsive to inhibitory signals and the other (differentiation) is not. Removal of the inhibitory effects for determination genes and their activation of downstream differentiation genes can then be controlled by the environmental cues and generally available signal transduction cassettes (*61*). In the cases of neurogenesis and myogenesis, this may involve complex signals from the neural tube or notocord (*62*). For neurogenesis, removal of negative factors might occur by classical neural induction (*13*). Proposed differentiation genes such as *myogenin* or *neuroD* may not contain such inhibitory domains and hence, when expressed, can bypass negative signals and rapidly program a terminally differentiated state.

## REFERENCES AND NOTES


1. H. Weintraub *et al.*, *Science* **251**, 761 (1991); H. Weintraub, *Cell* **75**, 1241 (1993); E. Olson and W. Klein, *Genes Dev.* **8**, 1 (1994); Y. N. Jan and L. Y. Jan, *Cell* **75**, 827 (1993); S. M. Parkhurst, D. Bopp, D. Ish-Horowicz, *ibid.* **63**, 1179 (1990).
2. C. Murre, P. S. McCaw, D. Baltimore, *Cell* **56**, 777 (1989); K. T. Blackwell and H. Weintraub, *Science* **250**, 1104 (1990).
3. A. R. Ferre-D'Amare, G. C. Prendergast, E. B. Ziff, S. K. Burley, *Nature* **363**, 38 (1993); T. Ellenberger, D. Fass, M. Arnaud, S. C. Harrison, *Genes Dev.* **8**, 970 (1994); P. C. Ma, M. A. Rould, H. Weintraub, C. O. Pabo, *Cell* **77**, 451 (1994).
4. C. Murre *et al.*, *Cell* **58**, 537 (1989); A. B. Lassar *et al.*, *ibid.* **66**, 305 (1991).
5. C. V. Cabera and M. C. Alonso, *EMBO J.* **10**, 2965 (1991).
6. J. A. Ghysen and C. Dambly-Chaudiere, *Trends Genet.* **5**, 251 (1989); S. Campuzano and J. Modolell, *ibid.* **8**, 202 (1992); C. V. Cabera, *Development* **115**, 893 (1992); F. Jimenez and J. Modolell, *Curr. Opin. Genet. Dev.* **3**, 626 (1993); J. A. Campos-Ortega, *J. Neurobiol.* **24**, 1305 (1993); *Cell* **77**, 969 (1994).
7. C. G. Begley *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2031 (1989); C. G. Begley *et al.*, *ibid.*, p. 10128.
8. F. Guillemot *et al.*, *Cell* **75**, 463 (1993).
9. Y. Zhuang, P. Soriano, H. Weintraub, *ibid.* **79**, 875 (1994).
10. M. A. Rudnicki, P. N. J. Schnegelsberg, T. Braun, H.-H. Arnold, R. Jaenisch, *ibid.* **75**, 1351 (1993).
11. P. Hasty *et al.*, *Nature* **364**, 501 (1993); Y. Nabeshima *et al.*, *ibid.*, p. 532.
12. A. P. Jarman, Y. Grau, L. Y. Jan, Y. N. Jan, *Cell* **73**, 1307 (1993).
13. H. Spemann, *Embryonic Development and Induction* (Yale Univ. Press, New Haven, CT, 1938), reprinted in *Great Books in Experimental Biology* (Garland, New York, 1988), vol. 10; J. M. Slack and D. Tannahill, *Development* **114**, 285 (1992); J. E. Dixon and C. R. Kintner, *ibid.* **106**, 749 (1989); T. Doniach, *J. Neurobiol.* **24**, 1256 (1993); A. Ruiz i Altaba, *ibid.*, p. 1276; R. Harland, *Curr. Opin. Genet. Dev.* **4**, 543 (1994).
14. S. Sudarvati and P. D. Nieuwkoop, *Wihelm Roux's Arch. Dev. Biol.* **166**, 189 (1971).
15. T. M. Lamb *et al.*, *Science* **262**, 713 (1993).
16. S. F. Godsave and J. M. W. Slack, *Development* **111**, 523 (1991); H. Grunz and L. Tacke, *Cell Differ. Dev.* **28**, 211 (1989).
17. A. Hemmati-Brivanlou and D. A. Melton, *Nature* **359**, 609 (1992); *Cell* **77**, 273 (1994).
18. A. Hemmati-Brivanlou, O. G. Kelly, D. Melton, *Cell* **77**, 283 (1994).
19. K. Kogawa *et al.*, *Endocrinology* **128**, 1434 (1991).
20. D. L. Turner and H. Weintraub, *Genes Dev.* **8**, 1434 (1994).
21. S. Fields and O. Song, *Nature* **340**, 245 (1989).
22. S. Hollenberg, R. Sternglanz, H. Weintraub, in preparation.
23. Y. Zhuang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 12132 (1992).
24. RNA for making the cDNA library was obtained from tumors made with the mouse embryonic stem cells in which both alleles of the E2A gene have been targeted (*23*). The VP-16 fusion cDNA library was made with random primers for first strand synthesis to normalize representation of different cDNA fragments and to facilitate interactions by providing minimal interaction domains. Double-stranded cDNA inserts (400 to 800-bp) were then ligated onto a pVP-16 vector (*22*). The ligated plasmids were transformed into electroporation-competent DH10B cells as described [D. Hanahan, J. Jessee, F. R. Bloom, *Methods Enzymol.* **204**, 63 (1991)]. The initial library size was $8.1 \times 10^6$.
25. The cDNA fusion library was paired with the Da HLH domain because initial screening showed that it displays a stronger and broader spectrum of interaction with known HLH proteins compared with other HLH proteins tested (*22*). Multimerized LexA binding sites were cloned upstream of two reporter genes, the *HIS3* gene and the β-*galactosidase* gene. The *Saccharomyces cerevisiae* L40 strain (*22*) was transformed with a plasmid encoding LexA-Da protein and maintained in medium lacking leucine. Yeast clones ($2.2 \times 10^7$) were transformed with a cDNA library plasmid preparation. Before being subjected to histidine selection, these transformants were maintained for 16 hours in medium lacking leucine and tryptophan. The cells were then selected on plates lacking histidine, leucine, tryptophan, uracil, and lysine. Of $2.2 \times 10^7$ transformants, 2640 clones (approximately one in $1.2 \times 10^4$) were $His^+$, and of these approximately 90 percent were also $LacZ^+$. The plasmids were rescued from $His^+/LacZ^+$ clones, and the LexA-Da plasmids were removed by transforming them into HB101 strain ($leu^-$) and growing them in the absence of leucine. The plasmids encoding VP16-cDNA fusion proteins were harvested and transformed into yeast L40 strain harboring LexA-lamin. Of these transformants, 20 percent were $LacZ^-$. We classified 410 clones that were $His^+/LacZ^+$ with LexA-Da and $His^+/LacZ^-$ with LexA-lamin. Among these clones, 120 clones represented previously identified bHLH genes, including *Id-1,2,3*, *tal-1,2*, and a previously cloned bHLH gene called *Th-1* (*22*). *neuroD* represents one of the previously unidentified bHLH genes among the remaining clones. We also obtained other previously reported bHLH genes in the pools that contained strong $LacZ^+$ with LexA-Da, and weak $LacZ^+$ with LexA-lamin. These pools contained *Id-1,2,3*, *tal-1,2*, *NSCL*, and *myogenin*.
26. The mouse cDNA library was made from MF1 strain and the genomic library from 129/sv strain.
27. R. L. Davis and H. Weintraub, *Science* **256**, 1027 (1992).
28. Inbred albino mouse strain, CD1, was used for in situ hybridization. The day of the vaginal plug was considered as embryonic day 0.5 (e0.5). The techniques used before hybridization including collection, dehydration, paraffin embedding, and prior treatment of the sections with proteinase K and acetic anhydride have been described in (*29*). A murine digoxigenin-labeled *neuroD* RNA probe (1.7 kb) was used for the hybridization. The hybridization solutions and all steps after hybridization were identical to the *Xenopus* whole mount in situ hybridization procedures described in (*20*).
29. D. G. Wilkinson and J. Green, *Postimplantation Mammalian Embryos: A Practical Approach*, A. J. Copp and D. L. Cockroft, Eds. (Oxford Univ. Press, New York, 1990), pp. 155–171.
30. J. Lee and H. Weintraub, unpublished data.
31. Although most of the embryonic *neuroD* expression seems transient, we also detected *neuroD* expression in the adult brain, in the granule layers of cerebellum and hippocampus. Some expression in the cortex and midbrain was also observed.
32. J. E. Johnson, S. J. Birren, D. Anderson, *Nature* **346**, 858 (1990); L.-C. Lo, J. E. Johnson, C. W. Wuenschell, T. Saito, D. J. Anderson, *Genes Dev.* **5**, 1524 (1991); F. Guillemot and A. Joyner, *Mech. Dev.* **42**, 171 (1993); the in situ hybridization using a digoxigenin-labeled *MASH* RNA probe was done as described (*28*).
33. R. Harland and H. Weintraub, *J. Cell Biol.* **101**, 1094 (1985); J.-D. Graf and H. R. Kobel, *Methods Cell Biol.* **36**, 19 (1991).
34. The *Xenopus* cDNA clone was obtained by screening a stage 17 *Xenopus* head library made by C. Kintner and D. Melton (*45*), with the mouse cDNA clone insert as a probe with low stringency hybridization, which was performed with 50 percent formamide and 4× SSC at 33°C and washing with 2× SSC and 0.1 percent SDS at 40°C.
35. The mouse and frog proteins show 77 percent sequence similarity in sequence overall [274 of 358 amino acid residues (aa)], 95 percent sequence identity in the bHLH domain (50 of 52 aa), and 80 percent in the region carboxyl terminal to the bHLH domain (162 of 203 aa). The amino-terminal regions were less conserved (60 percent identity, 61 of 102 aa), but both contain highly acidic domains (Fig. 1). Such acidic domains often function as activation domains in other transcription factors [K. Struhl, *Cell* **49**, 295 (1987); S. Hahn, *Cell* **72**, 481 (1993)]. The region that is carboxyl-terminal to the bHLH domain is proline-rich, suggesting a potential transcriptional activation domain [N. Mermod, E. A. O'Neill, T. J. Kelly, R. Tijan, *ibid.* **58**, 741 (1989)].
36. R. Wilson *et al.*, *Nature*, in press; J. Kirsten, unpublished data; the name of the plasmid encoding a *C. elegans* homolog of NeuroD is CELC34E10 and its Gene Bank accession number is U10402.
37. Embryonic stages were designated in accordance with P. D. Nieuwkoop and J. Faber [*Normal Table of* Xenopus laevis (Daudin) (North-Holland, Amsterdam, 1967).
38. The whole-mount in situ hybridization was performed as described by R. M. Harland, [*Methods Cell Biol.* **36**, 675 (1991)], with modifications described in (*20*).
39. J. Lamborghini, *J. Comp. Neurol.* **189**, 323 (1980);

H. J. ten Donkelaar and R. de Boer-van Huizen, *Anat. Embryol.* **183**, 589 (1991); V. Hartenstein, *J. Comp. Neurol.* **328**, 213 (1993); V. Korzh, T. Edlund, S. Thor, *Development* **118**, 417 (1993).
40. B. Sadaghiani and C. H. Thiebaud, *Dev. Biol.* **124**, 91 (1987); A. Baroffio, E. Dupin, M. M. Le Douarin, *Development* **112**, 301 (1991); H.-H. Epperlein and J. Lofberg, *Ann. Anat.* **175**, 483 (1993).
41. M. Jacobson, *Dev. Biol.* **17**, 219 (1968); C. E. Holt, T. W. Bertsch, H. M. Ellis, W. A. Harris, *Neuron* **1**, 15 (1988); R. Wetts, J. H. Kook, S. E. Fraser, *Dev. Dynamics* **198**, 54 (1993).
42. P. A. Krieg and D. A. Melton, *Methods Enzymol.* **155**, 397 (1987).
43. The *Xenopus* cDNA clone was restricted at the position corresponding to the 11th amino acid and fused in-frame to the Myc epitope of the pCS2$^+$MT vector described in (*20*). In vitro RNA synthesis was as described (*20, 42*).
44. Immunohistochemistry was as described in (*20*).
45. C. R. Kintner and D. A. Melton, *Development* **99**, 311 (1987); K. Balak, M. Jacobson, J. Sunshine, U. Rutishauser, *Dev. Biol.* **119**, 540 (1987); an antibody to N-CAM was used at 1:500 dilution.
46. L. Dale and J. M. W. Slack, *Development* **99**, 527 (1987).
47. Neural specific *class II* β-*tubulin* [K. Richter, H. Grunz, I. G. Dawid, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8086 (1988)]; acetylated α-tubulin [G. Piperno and M. T. Fuller, *J. Cell Biol.* **101**, 2085 (1985)]; *tanabin* [A. Hemmati-Brinvanlou, R. W. Mann, R. M. Harland, *Neuron* **9**, 417 (1992)]; NF-M [B. G. Szaro and H. Gainer, *J. Comp. Neurol.* **273**, 344 (1988)]; Xen-1 and Xen-2 [A. Ruiz i Altaba, *Development* **115**, 67 (1992)]. The antibodies were used at the following dilutions: acetylated α-tubulin (1:2000, unpublished data), Xen-1 (1:1), Xen-2 (1:1, unpublished data), and NF-M (1:2000). Primary antibodies were detected with alkaline phosphatase-conjugated goat antibody to mouse or rabbit antibodies diluted at 1:2000 (Boehringer Mannheim). Embryos stained for NF-M were fixed in Dent's solution (20 percent dimethyl sulfoxide and 80 percent methanol) and cleared in a 2:1 mixture of benzyl benzoate and benzyl alcohol [J. A. Dent, A. G. Polson, M. W. Klymkowsky, *Development* **105**, 61 (1989)]. In situ hybridization was performed with *tanabin* and β-*tubulin*. In situ hybridization with β-*tubulin* without ribonuclease treatment can also detect *tubulin* expression in the ciliated epidermal cells (Fig. 4, E and F).
48. HNK-1 [R. H. Nordlander, *Dev. Brain Res.* **50**, 147 (1989)]; *shaker-1* [A. B. Ribera and D.-A. Nguyen, *J. Neurosci.* **13**, 4988 (1993)]; *shaker-1*, unpublished data; Islet-1 [J. Ericson, S. Thor, T. Edlund, T. M. Jessell, T. Yamada, *Science* **256**, 1555 (1992); V. Korzh, T. Edlund, S. Thor, *Development* **118**, 417 (1993)]. The antibodies were used at the following dilutions: Islet-1 (1:500), and HNK-1 (1:1).
49. AC explants were dissected at around stage 9 and maintained in 0.5 × MBS [H. B. Peng, *Methods Cell Biol.* **36**, 659 (1991). AC explants showed ectopic N-CAM staining after 36 hours post fertilization.
50. N. D. Hopwood, A. Pluck, J. B. Gurdon, *Cell* **59**, 893 (1989).
51. J. Lee, L. Snider, H. Weintraub, unpublished data.
52. *Xtwi* coding sequence was obtained with primers based on the published sequence (*53*). The *Xtwi* insert was subcloned into pCS2$^+$ vector (*20, 53*). RNA transcription and injection was performed as described for *neuroD*.
53. R. Rupp, L. Snider, H. Weintraub, *Genes Dev.* **8**, 1311 (1994).
54. E. A. Jones and H. R. Woodland, *Cell* **44**, 345 (1986); *Development* **107**, 785 (1989), J. Lee and H. Weintraub, unpublished data.
55. We performed immunostaining on *neuroD* injected embryos with an antibody to GFAP (G-A-5, Boehringer Mannheim, No. 814369). Staining (in both wild-type and injected embryos) was only observed in embryos fixed in Dent's solution (*47*). The staining pattern indicated that no ectopic GFAP-expressing cells were observed on the *neuroD* injected side and that the staining in the CNS may even be reduced (unpublished data). Since GFAP stains only astrocytes, we cannot rule out the possibility that ectopically expressed *neuroD* may generate other glial cell types.
56. T. Tsuchida *et al.*, *Cell* **79**, 957 (1994); B. Lewin, *Cell* **79**, 935 (1994).
57. Although we have seldom noticed ectopic neuron formation in tissues derived from other germ layers, we frequently observed that the somites of embryos injected with *neuroD* were poorly formed and deficient in immunostaining against the muscle marker myosin heavy chain protein. It is difficult to interpret these results because *neuroD* may be titrating endogenous E proteins or Id proteins, leading to a non-specific effect. We also performed injections into the vegetal pole of two-cell stage embryos and into the bottom tier of 32-cell stage embryos. Although we observed reproducible morphological effects (that is, a bulging mass protruding from the abdomen), we did not see any ectopic N-CAM staining in these embryos except for one case. One embryo showed severely disturbed internal organ development and dispersed but strong N-CAM staining in the abdominal cavity.
58. T. W. Chu and M. W. Klymkowsky, *Dev. Biol.* **136**, 104 (1989).
59. B. Kramatschek and J. A. Campos-Ortega, *Development* **120**, 815 (1994); A. Singson, M. W. Leviten, A. G. Bang, X. H. Hua, J. W. Posakony, *Genes Dev.* **8**, 2058 (1994).
60. P. M. Rong, M.-A. Teillet, N. M. Le Douarin, *Development* **115**, 657 (1992); B. Christ, B. Brand-Saberi, M. Grim, J. Wilting, *Anat. Embryol.* **186**, 505 (1992); N. Buffinger and F. F. Stockdale, *Development* **120**, 1443 (1994).
61. M. Brand, A. P. Jarman, L. Y. Jan, Y. N. Jan, *Development* **119**, 1 (1993); M. Dominguez and S. Campuzano, *EMBO J.* **12** (5), 2049 (1993).
62. C. J. Marshall, *Cell* **80**, 179 (1995); C. S. Hill and R. Treisman, *ibid.*, p. 199.
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