Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate

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In *Drosophila*, the proneural genes of the *achaete-scute* complex encode transcriptional activators that can commit cells to a neural fate. We have isolated cDNAs for two *Xenopus achaete-scute* homologs, *ASH3a* and *ASH3b*, which are expressed in a subset of central nervous system (CNS) neuroblasts during early neurogenesis. After expressing either ASH3 protein in developing *Xenopus* embryos, we find enlargement of the CNS at the expense of adjacent non-neural ectoderm. Analysis of molecular markers for neural, epidermal, and neural crest cells indicates that CNS expansion occurs as early as neural plate formation. ASH3-dependent CNS enlargement appears to require neural induction, as it does not occur in animal cap explants. Inhibition of DNA synthesis shows that additional CNS tissue does not depend on cell division—rather it reflects conversion of prospective neural crest and epidermal cells to a neural fate. The differentiation of the early forming primary neurons also seems to be prevented by ASH3 expression. This may be secondary to the observed activation of *Xotch* transcription by ASH3.

In *Xenopus* embryos, the cells that form the central nervous system (CNS) arise from the ectoderm. During gastrulation, a contiguous region of dorsal ectoderm becomes committed to a neural fate in response to a vertical inductive signal from the underlying mesoderm and/or a planar inductive signal from the involuting mesoderm (Spemann 1938; Kintner and Melton 1987; Dixon and Kintner 1989; Doniach 1993; Ruiz i Altaba 1993). The protein Noggin may function as one such signal, because it has many of the properties required to be a neural inducer (Lamb et al. 1993). In the absence of induction, dorsal ectodermal cells become epidermis. However, recent experiments suggest that the default fate for ectoderm is neural, but this fate is negatively regulated in intact, uninduced ectoderm (Godsave and Slack 1989, 1991; Grunz and Tacke 1989; Hemmati-Brivanlou and Melton 1992; Ruiz i Altaba 1993). Induction would then become a process that removes inhibition.

In *Drosophila*, neural progenitors also arise from the ectoderm, and ectodermal cells select either an epidermal or neural fate. Analysis of mutations in *Drosophila* has led to the identification of a large number of genes that regulate the decision between epidermal and neural fates (for review, see Campuzano and Modolell 1992; Campos-Ortega 1993; Jan and Jan 1993). One group of these, the proneural genes, give ectodermal cells the potential to become neural progenitors. Mutations that inactivate one or more proneural genes prevent the forma-

tion of specific subsets of neural precursors (Jiménez and Campos-Ortega 1990; Campuzano and Modolell 1992), whereas ectopic expression of proneural genes causes additional cells to become neural precursors (Campuzano et al. 1986; Rodríguez et al. 1990; Domínguez and Campuzano 1993; Jarman et al. 1993a,b; Brand et al. 1993). The known proneural genes comprise four genes in the *achaete-scute* complex (*AS-C*), *atonal* (*ato*), and possibly *ventral nervous system condensation defective* (*vnd*). The four *AS-C* genes encode closely related members of the basic helix–loop–helix (bHLH) family of transcription factors (Villares and Cabrera 1987; Alonso and Cabrera 1988; González et al. 1989), whereas *ato* encodes a more distantly related bHLH protein (Jarman et al. 1993b). The AS-C and ato proteins bind to DNA as heterodimers with the bHLH protein daughterless, and activate transcription (Murre et al. 1989; Cabrera and Alonso 1991; Jarman et al. 1993a,b). Although the expression of the AS-C and ato bHLH proteins is restricted to the sites of neurogenesis, daughterless is ubiquitously expressed (Cronmiller and Cummings 1993). The molecular characterization of the *vnd* gene has not been described.

Two homologs of the *AS-C* genes have been isolated from the rat, *MASH1* and *MASH2* (Johnson et al. 1990). Subsequently, homologs of *MASH1* have been isolated from other vertebrates, including *Xenopus ASH1* (Ferreiro et al. 1992). These proteins are likely to function as

heterodimers with the ubiquitously expressed E proteins, a family of vertebrate bHLH proteins related to daughterless (Murre et al. 1989; Rashbass et al. 1992). MASH1 is expressed in neural progenitors in the developing nervous system (Lo et al. 1991), as is Xenopus ASH1 (Ferreiro et al. 1992), but only well after neural progenitors have formed in the ectoderm. Thus, MASH1 cannot regulate the initial decision between neural and epidermal fates. Recently, mice have been generated with a null mutation in the MASH1 gene (Guillemot et al. 1993). These mice lack neural progenitors for the olfactory epithelium and for the autonomic nervous system, apparently because of the death of these cells after they initially form. This suggests that MASH1 may regulate either the survival or differentiation of later progenitors within the nervous system. MASH2 is not expressed in the nervous system (Johnson et al. 1992).

As in Xenopus ectoderm, the default fate for cells in the ventral ectoderm of Drosophila embryos appears to be neural, attributable to the function of the proneural genes. Negative regulation restricts this function to a subset of cells, which will then form the CNS (for review, see Campos-Ortega 1993). Similarly, in Drosophila imaginal discs, proneural genes are activated in clusters of cells, but their function and expression is subsequently restricted to single cells within each cluster which will then form sense organs (for review, see Campuzano and Modolell 1992; Jan and Jan 1993). Thus, in Drosophila, the generation of neural progenitors reflects a balance between positive and negative regulation.

In both Drosophila embryos and larvae, restriction of proneural gene function is mediated by the products of the neurogenic genes, which include Notch and the Enhancer of split complex [E(spl)] (for review, see Campuzano and Modolell 1992; Campos-Ortega 1993). In addition to the neurogenic genes, the AS-C genes are negatively regulated by extramacrochaete and hairy in the imaginal discs (Moscoso del Prado and García-Bellido 1984). Vertebrate homologs of all of these genes have been identified and are expressed during neurogenesis, but their functions are not yet understood (Coffman et al. 1990; Benezra et al. 1990; Sasai et al. 1992; Feder et al. 1993; D. Turner and H. Weintraub, unpubl.).

We have isolated cDNAs for two achaete-scute homologs from Xenopus, designated ASH3a and ASH3b (achaete-scute homologs 3a and 3b), which are expressed in the prospective CNS, beginning during gastrulation. Expression of either ASH3 protein from RNA injected into Xenopus embryos leads to an enlargement of the CNS at the expense of other ectodermal derivatives. Our observations demonstrate that achaete-scute homologs can act as proneural genes in a vertebrate embryo.

Results

Isolation and characterization of Xenopus ASH3a and ASH3b

Fragments of achaete-scute-related genes, isolated by reverse transcriptase polymerase chain reaction (RT–PCR)

amplification of Xenopus embryo RNA, were used to identify corresponding cDNAs (see Materials and methods). Two cDNAs encode novel achaete-scute related proteins, ASH3a and ASH3b. Xenopus is pseudotetraploid, attributable to an ancestral genome duplication, and the degree of nucleotide identity between the ASH3 cDNAs (89% in the coding region) suggests that they represent a pair of genes arising from this duplication (Graf and Kobel 1991). During the course of this work, Zimmerman et al. (1993) reported the isolation of an ASH3a cDNA, and a partial ASH3b cDNA. The alignment of the complete ASH3a and ASH3b proteins to each other, and to several members of the achaete-scute bHLH family is shown in Figure 1. In addition to the conserved bHLH domain, we note that all of the proteins shown have a short conserved sequence located near the carboxyl terminus, which has been proposed to be a regulatory site for the Drosophila proteins (Villares and Cabrera 1987).

The expression of ASH3a/b mRNA in developing embryos was examined by whole-mount in situ hybridization (Harland 1991) and quantitative RT–PCR (Rupp and Weintraub 1991). Our in situ hybridization results are in agreement with those presented by Zimmerman et al. (1993), so we include only a summary here. Maternal mRNAs are not detected for either ASH3a or ASH3b by RT–PCR, but low levels of both mRNAs are present from midblastula transition (the start of zygotic transcription) until gastrulation, when levels of both mRNAs increase (R.A.W. Rupp, D.L. Turner, and H. Weintraub, unpubl.). At present, we do not know whether this early ASH3a/b mRNA is localized. ASH3a/b is first detected by in situ hybridization at midgastrulation (stage 11.5) in two patches within the presumptive neural plate. The initial ASH3a/b expression is in the posterior of the prospective CNS, but as neural tube forms, expression expands to include the future forebrain and retina (Fig. 2A,B). ASH3a/b expression is transient and appears to be restricted to zones within the CNS that contain proliferating precursors (Fig. 2C). Because the early expression of the ASH3a/b genes suggests a role in the initial formation of the CNS, we have focused our analysis on these genes.

Ectopic expression of ASH3a/b in Xenopus embryos leads to enlargement of the CNS

Ectopic expression of the Drosophila AS-C proteins in Drosophila larvae leads to the formation of additional neural structures (Campuzano et al. 1986; Rodríguez et al. 1990; Brand et al. 1993; Domínguez and Campuzano 1993), so we wanted to test whether ectopic expression of ASH3 could generate additional neural tissue in Xenopus embryos. We injected synthetic RNA that encoded either ASH3a or ASH3b into the animal hemisphere of one cell of Xenopus embryos at the two-cell stage. The first cleavage in Xenopus embryos usually defines the plane of bilateral symmetry, so such injections restrict the introduced RNA to one side of the developing embryo, allowing the uninjected side of an embryo to

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Xen. ASH3a                                                                            MEEQLSSKRQTKAPRKAASESGSPQRLRC
Xen. ASH3b                                                                            MEEQPSSDRRVKAPRKAATETESPQRLRC

Xen. ASH1a   MDNCVAAKIMDSNLSSQQQHFLQP-HCFF--------------------------PQNVQQLSPAEEQQAS------KAKPIKRQRSASPELMRC

rat MASH1    MESSGKMESGAGQQPQPPQPFLPPAACFFATAAAAAAAAAAAAAQSAQQQQQQQAPQQQAPQLSPVADGQPSGGGHKSAAKQVKRQRSSSPELMRC

Dros. l'sc                        MTSICSSKFQQQHYQLTNSNIFLLQHQHHHQTQQHQLIAPKIPLGTSQLQNMQQSQQSNVGPMLSSQ
Dros. sc                  MKNNNNTTKSTTMSSSVLSTNETFPTTINSATKIFRYQHIMPAPSPLIPGGNQNQPAGTMPIKTRKYTPRGMALTRCSES
Dros. ac                                                                                               MALGS
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                         basic                      helix-loop-helix
Xen. ASH3a   QRRSGSSSNTIGYSAP------SERRNERERNRVKLVNLGFAKLRQHVPQAQ--------------GPNKKMSKVETLRSAVEYIRALQSLLMER----
Xen. ASH3b   QKRSGSSANTIGLSAT------SERRNERERNRVKLVNMGFAKLRQHVPQAQ--------------GPNKKMSKVETLRSAVEYIRALQAVLIER----

Xen. ASH1a   KRRLNFNG--FGYSLPQQQPAAVARRNERERNRVKLVNLGFATLREHVP---------NG------AANKKMSKVETLRSAVEYIRALQQLLDEH----

rat MASH1    KRRLNFSG--FGYSLPQQQPAAVARRNERERNRVKLVNLGFATLREHVP---------NG------AANKKMSKVETLRSAVEYIRALQQLLDEH----

Dros. l'sc   KKKFNYNNMPYGEQLP-----SVARRNARERNRVKQVNNGFVNLRQHLPQTVVNSLSNG----GRGSSKKLSKVDTLRIAVEYIRGLQDMLDDGTASS
Dros. sc     VSSLSPGSSPAPYNVDQSQ--SVQRRNARERNRVKQVNNSFARLRQHIPQSIITDLTKG--G-GRGPHKKISKVDTLRIAVEYIRSLQDLVDDLNGGS
Dros. ac     ENHSVFNDDEESSSAFNGP--SVIRRNARERNRVKQVNNGFSQLRQHIPAAVIADLSNGRRGIGPGANKKLSKVSTLKMAVEYIRRLQKVLHENDQQK
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Xen. ASH3a   -------------------------------------------------------------------------------------------------
Xen. ASH3b   -------------------------------------------------------------------------------------------------

Xen. ASH1a   -------------------------------------------------------------------------------------------------

rat MASH1    -------------------------------------------------------------------------------------------------

Dros. l'sc   TRHIYNSADESSNDGSSYNDYNDSLDSSQQFLTG---------------------------------------------------------------
Dros. sc     NIGANNAVTQLQLCLDESSSHSSSSSTCSSSGHNTYYQNRISVSPVQQQQQLQRQQFNHQPLTALSLNTNLVGTSVPGGDAGCVSTSKNQQTCHSPTS
Dros. ac     QKQLHLQQQHLHFQQQQQHQHLYAWHQELQLQSP---------------------------------------------------------------
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                              acidic region
Xen. ASH3a   --------SAGEGQERAGSDGLSPCGSSCSVD----SLSPGSY--CNSEDSSREDSSIPDADFFLTL-HGWG
Xen. ASH3b   --------SAGEGQ-RAGSDGLSPCGSSCSVDSGSMSLSPGSCSPCASEDSSREESSIPEADFFLTL-HGWGITRLTGHHIGTEDKEQTDYYKNISE

Xen. ASH1a   ------------DAVSAAFQSGVLSPTISPNYSHDMNSMAGSPVSSYSSDEGSYDPLSPEEQELLDF-TTWF

rat MASH1    ------------DAVSAAFQAGVLSPTISPNYSNDLNSMAGSPVSSYSSDEGSYDPLSPEEQELLDF-TNWF

Dros. l'sc   ---ATQSAQSRSYHSASPTPSYSGSEISGGGYIKQELQEQDLKFDSFDSFSDEQ----PDDEELLDYISSWQEQ
Dros. sc     SFNSSMSFDSGTYEGVPQQISTHLDRLDHLDNELHTHSQLQLKFEPYEHFQLDEEDCTPDDEELDYISLWQEQ
Dros. ac     ---------TGSTSSCNSISSYCKPATSTIPGATPPNNFHTKLEASFEDYRNNSCSSGTEDEDILDYISLWQDDL
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Figure 1. Alignment of ASH3a and ASH3b proteins to each other and to other members of the achaete-scute bHLH subfamily. Regions of sequence similarity between the vertebrate ASH1/ASH3 proteins and the *Drosophila* AS-C proteins are shaded. The bHLH domain of ASH3a/b has 65–71% amino acid identity with the bHLH domains of the *Drosophila* AS-C proteins (excluding the size difference in the loops), and 78% (ASH3b) or 82% (ASH3a) identity with the *Xenopus* ASH1a bHLH domain. In addition to the bHLH domain, all of these proteins have a related sequence at the carboxyl terminus, within a larger acidic region. ASH3b appears to have a second version of this sequence that is most similar to achaete (ac, underlined). The carboxyl terminus of the *Drosophila* AS-C proteins has been suggested to be a regulatory site (Villares and Cabrera 1987). Limited sequence similarity is also present amino-terminal to the basic region in the vertebrate proteins and lethal of scute (l'sc). (sc) Scute. Sources for sequences: ac and sc (Villares and Cabrera 1987); l'sc (Alonso and Cabrera 1988); MASH1 (Johnson et al. 1990); and ASH1a (Ferreiro et al. 1992).

be used as a control for the injected one. Embryos injected with *ASH3a* or *ASH3b* RNA show a dramatic increase in neural tissue on the injected side. This is especially clear in embryos that have been processed for whole-mount in situ hybridization with a probe for the neural specific *neural cell adhesion molecule* (*N-CAM*) mRNA (Fig. 2D,E; Kintner and Melton 1987). The extra tissue often delays or prevents neural tube closure. After injection of 500–700 pg of *ASH3a/b* RNA, ~80% of the injected embryos showed CNS expansion (n = 193). Enlargement of neural tissue was never observed in uninjected embryos nor after injection of RNAs that encode other proteins, including β-galactosidase, a multimerized Myc epitope tag (Roth et al. 1991), the bHLH protein E12 (Rashbass et al. 1992), and the bHLH protein hairy2a (see below; D. Turner and H. Weintraub, unpubl.). Most significantly, expression of a version of ASH3a in which the DNA-binding basic region is disrupted (Glu$_{51}$Arg$_{52}$ changed to Ala$_{51}$Gln$_{52}$) but the HLH dimerization do-

main remains intact, does not cause CNS expansion (data not shown). This last observation suggests that ASH3a requires DNA-binding for its activity and that it is not initiating neurogenesis by titrating out an inhibitor (such as Id; see Benezra et al. 1990) via dimerization through the HLH domain.

In addition to the enlarged neural tube, the closing blastopore was displaced toward the injected side of up to 50% of the embryos. At later stages, most of these embryos had a curved axis, disorganized somites, and/or reduced expression of the muscle-specific myosin heavy chain protein (Fig. 2M). About 10–15% of the injected embryos gastrulated abnormally. In an individual embryo, the degree of neural tube enlargement does not correlate with either the presence or absence of non-neural defects (e.g., see Fig. 2E).

To confirm that the affected side of the embryos was the injected side, we marked the injected side either by coinjection of RNA encoding β-galactosidase or by using
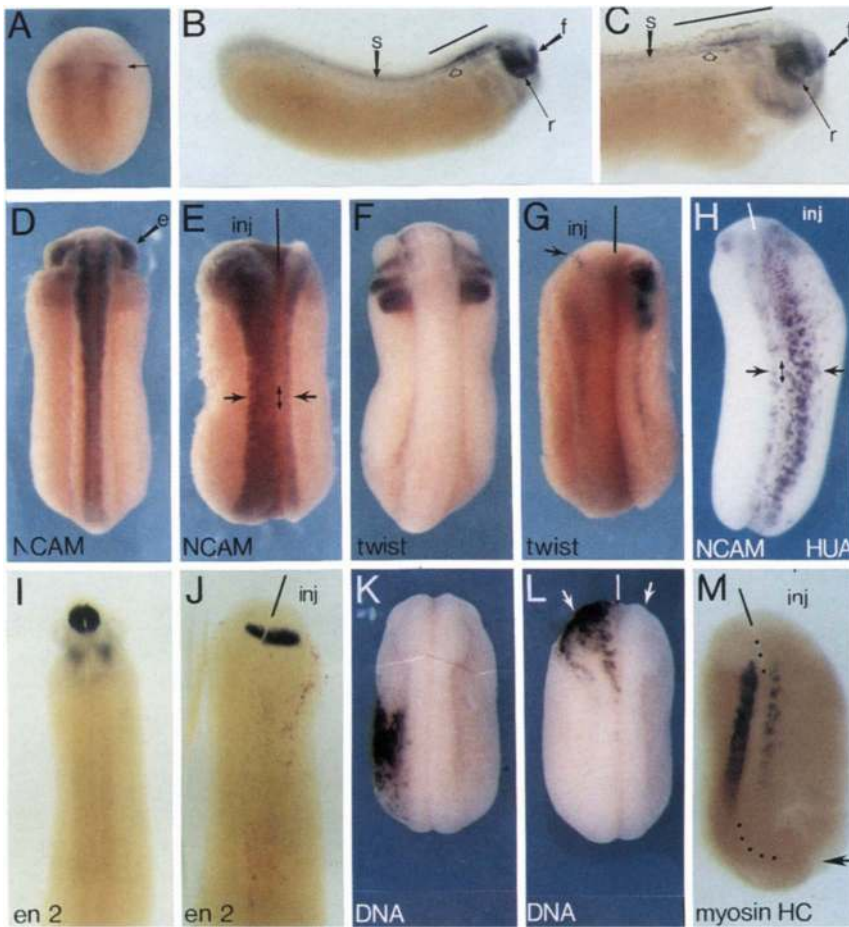
Figure 2. Expression of molecular markers in wild-type and *ASH3b* RNA-injected embryos. (*A–C*) Expression of *ASH3a* mRNA in wild-type embryos, detected by whole-mount in situ hybridization at three representitive stages. (*A*) An early neural plate embryo (stage 13.5) showing longitudinal stripes of *ASH3a* expression within prospective hindbrain and spinal cord. A transverse stripe is also present in the anterior of the future hindbrain (arrow). (*B*) Expression persists as a continuous longitudinal stripe (s) on each side of the spinal cord and hindbrain in an early tail bud embryo (stage 28), and *ASH3a* is also expressed in the retina (r) and forebrain (f). A second stripe of expression within the hindbrain is also visible (open arrow). The approximate extent of the hindbrain is shown by the bar above the embryos in both *B* and *C*. (*C*) The head of a later tail bud embryo (stage 34) showing the loss of ASH3a expression as neurons differentiate. ASH3a expression is shown by purple staining; brown dots are pigmented cells. The pigment epithelium of the eye is also brown. Note that expression in the retina is reduced to a band at the margin, the location of the retinal progenitors at this stage. The extent of expression in the forebrain and hindbrain is reduced, but the longitudinal stripes remain in the hindbrain and spinal cord (s). Anterior is toward the *right* in *B* and *C* and toward the *top* in *A* and *D–M*. Embryos in *B*, *C*, *I*, *J*, and *M* are cleared. *N-CAM* mRNA expression in a wild-type (*D*) and *ASH3b* RNA-injected embryo (*E*). A vertical bar shows the position of the midline and the injected side is labeled inj in *E*. The *N-CAM*-positive CNS is expanded laterally on the injected side along its entire length. Arrows indicate the boundaries of the prospective spinal cord; a double-headed arrow denotes the midline. The neural tube has failed to close because of the extra tissue. The eye vesicle (e) is labeled in *D* but is hidden in *E* because of the open neural tube. The embryo shown in *E* and other injected embryos shown in Figures 2–4 are examples of a strong but reproducible level of effect arising from *ASH3b* injection (see Materials and methods). (*F*) *twist* mRNA is expressed in the cranial neural crest but is almost absent after *ASH3b* injection (*G*). The arrow points out a small number of *twist*-positive cells that remain. N-CAM protein expression in an *ASH3b*-injected embryo treated with HUA (see text) also shows an expansion of the CNS (*H*). Arrows denote the borders of the prospective spinal cord; a double-headed arrow shows the midline. The injected sides of the embryos shown in *H* and *J* are marked by nuclear β-galactosidase expression (fine reddish dots). *en-2* mRNA is expressed at the midbrain–hindbrain boundary (*I*). Like *N-CAM*, *en-2* expression in expanded after *ASH3b* RNA injection (*J*). *K* and *L* show embryos injected with a DNA expression vector encoding an epitope-tagged ASH3b protein. The embryos have been stained with an antibody to the epitope tag (see Materials and methods). (*K*) ASH3b expression is outside of the dorsal ectoderm so the CNS does not show any enlargement. (*L*) ASH3b expression is present in the dorsal ectoderm and the future brain is expanded (arrows; vertical bar indicates midline). Expression in the embryo in (*L*) is restricted to the ectoderm (not shown). (*M*) An embryo injected with an *ASH3b* DNA expression vector showing the reduction of myosin heavy chain protein expression. Note that the blastopore is displaced toward the injected side (arrow). The curved axis is indicated. Similar effects were also seen after *ASH3b* RNA injection (not shown).

an epitope-tagged version of ASH3a/b (see Materials and methods). By either method, the affected side of the embryo was always the marked side (n>120). Injection of *ASH3a/b* RNA into both cells of a two-cell embryo resulted in similar effects on both sides of the embryo (not shown). Most of the analysis we present is based on embryos injected with *ASH3b* RNA. However, embryos injected with *ASH3a* RNA showed identical effects when scored by morphology or neural markers (not shown).

Extra CNS tissue does not require cell division after gastrulation

Harris and Hartenstein (1991) have shown that *Xenopus* embryos can develop almost normally until tailbud stages after cell division is arrested at gastrulation by hydroxyurea/aphidicoline (HUA) treatment. To test whether the enlarged CNS was attributable to additional proliferation of neural precursors, we injected embryos

with *ASH3b* RNA and then blocked cell division at mid-gastrulation. The injected HUA-treated embryos had increased numbers of N-CAM-expressing cells on the injected side, despite the lack of cell proliferation (Fig. 2H), indicating that ectopic *ASH3b* converts additional cells to a neural fate. Although cell proliferation is not necessary for the expansion of the CNS in *ASH3b*-injected embryos, proliferation may also contribute to this expansion (in the absence of HUA).

Ectopic ASH3b acts in ectoderm, at or near the site of expression

To restrict the area of ectopic ASH3b expression, we injected one cell of two- or four-cell embryos with a DNA vector that expresses epitope-tagged ASH3b (see Materials and methods). Because of the mosaic distribution of template DNA, these embryos expressed the ectopic ASH3b protein within smaller regions than RNA-injected embryos (Fig. 2K,L; data not shown). Embryos that expressed ASH3b protein in ventral epidermis, outside of the neural tube, did not show neural tube expansion (Fig. 2K), whereas embryos that expressed ASH3b protein in dorsal ectoderm, crossing the border of the neural tube, had an expanded CNS (Fig. 2L). As in *ASH3b* RNA-injected embryos, expansion of N-CAM protein expression was restricted to dorsal ectoderm, contiguous with the CNS (not shown). We also observed CNS enlargment in embryos in which ASH3b expression was present only in the ectoderm, and not the mesoderm or endoderm. These results suggest that the effects of ASH3b on the CNS are attributable to local effects on the dorsal ectoderm.

Because ASH3b functions within the ectoderm, we tested the effect of ASH3a/b expression in uninduced ectoderm by isolating animal caps from blastula-stage embryos that had been bilaterally injected with *ASH3a* or *ASH3b* RNA. We did not detect the activation of either N-CAM protein or mRNA in such explants (not shown). Thus, ASH3a/b does not initiate neurogenesis in uninduced animal cap ectoderm. Given the current thinking about induction removing inhibition (see introductory section), these results suggest that the function of ASH3a/b may be subject to negative regulation, which is relieved by neural induction.

Cranial neural crest is reduced in ASH3b-injected embryos

The *Xenopus* cranial neural crest derives mostly from cells at the edge of the neural plate, adjacent to the prospective midbrain and hindbrain. These cells migrate away from the neural tube as it closes and give rise to cartilage and mesenchyme in the branchial arches and facial structures, as well as some peripheral neurons (Sadaghiani and Thiebaud 1987). The *Xenopus twist* gene is expressed in most cranial neural crest cells, both before and during migration (Fig. 2F; Hopwood et al. 1989). After migration, *twist* expression is restricted to structures comprised of cartilage and mesenchyme (Hopwood

et al. 1989; D. Turner and H. Weintraub, unpubl.), suggesting that *twist* expression marks a population of neural crest cells with the potential to generate these non-neural cell types. In *ASH3b*-injected embryos, *twist* mRNA expression is reduced or absent on the injected side (Fig. 2G). Instead, many of the presumptive neural crest cells express high levels of *N-CAM*, suggesting that they have adopted a neural fate (Fig. 2E).

The *engrailed-2* (*en-2*) mRNA is expressed at the border between the midbrain and the hindbrain (Fig. 2I; Hemmati-Brivanlou et al. 1991). Like *N-CAM*, *en-2* expression is extended laterally, into the additional cells at the edge of the neural tube in *ASH3b*-injected embryos (Fig. 2J). This suggests that these cells have become part of the CNS, as *en-2* is normally expressed only in the CNS and not in the adjacent neural crest. The presence of the *en-2* at the midbrain–hindbrain border in the extra neural tissue also suggests that the laterally expanded CNS has retained its normal anterior–posterior patterning.

Enlargement of the CNS is attributable to changes in cell fate during gastrula or early neural plate stages

To determine whether the effects of ASH3b expression reflect an early alteration in cell fate, we examined the expression of the *N-CAM* mRNA, *twist* mRNA, and EpA, an epidermal antigen (Jones and Woodland 1986), shortly after the formation of the neural plate (stage 13.5–14; see Fig. 3A). *N-CAM* mRNA is present in most of the prospective CNS, except for the notoplate (the prospective floorplate). *N-CAM* is not found in the premigratory cranial neural crest at the edge of the neural plate (Fig. 3A,B; also see Kintner and Melton 1987). *twist* mRNA is found in two patches of the prospective cranial neural crest, adjacent to the region of *N-CAM* expression (Fig. 3A,C) (Hopwood et al. 1989). Ectoderm outside the neural plate forms primarily epidermis, which expresses the EpA antigen (Fig. 3A,D,E; Jones and Woodland 1986). In embryos injected with *ASH3b* RNA, the region of *N-CAM* expression was expanded laterally on the injected side, into the regions that would normally express *twist* or EpA (Fig. 3B,F). The extra *N-CAM* expression was always contiguous with the normal region of *N-CAM* expression and never included the notoplate. The expression of *twist* was reduced or absent on the injected side of most embryos (Fig. 3C), and the dorsal limit of EpA expression was farther from the midline (Fig. 3D,E). We also examined the expression of the mRNA for hairy2a, a *Xenopus* homolog of the *Drosophila* hairy bHLH protein (D.L. Turner, R.A.W. Rupp, and H. Weintraub, in prep.) in injected embryos. At neural plate stages, *hairy2a* mRNA is expressed in a band of ectodermal cells at the border of the neural plate and in the notoplate, so that *hairy2a*-expressing cells surround the region of *N-CAM* expression (Fig. 3A,G). In ASH3b-injected embryos, *hairy2a* expression in the notoplate was unaffected, but expression at the neural plate border was farther from the midline on the injected side (Fig. 3G). This creates a larger region of ectoderm surrounded
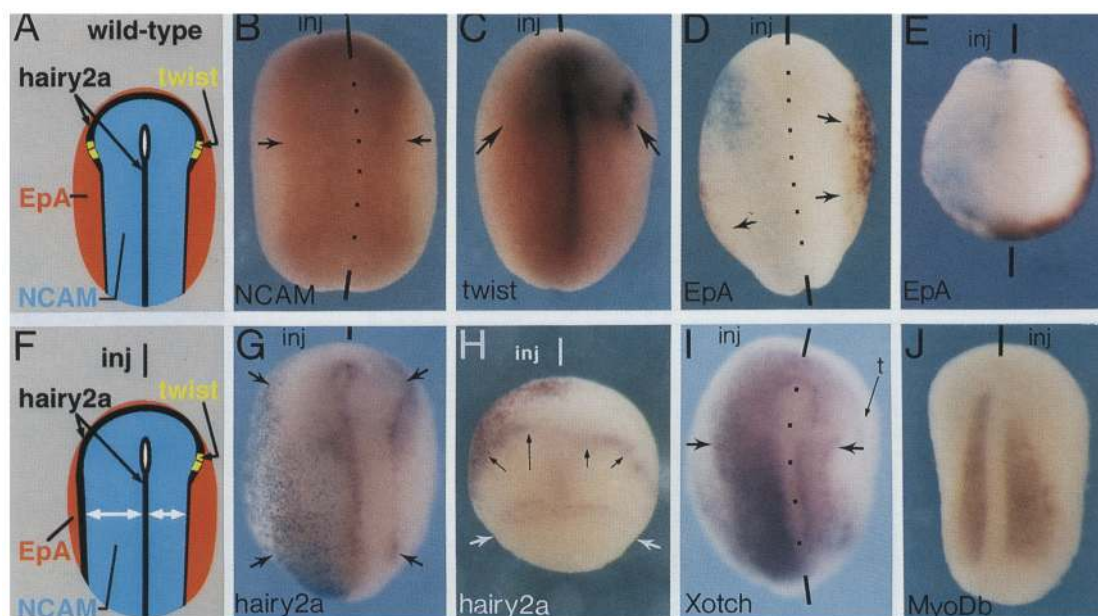
Figure 3. Expression of molecular markers at neural plate and gastrula stages in *ASH3b* RNA-injected embryos. (*A–D, F, G, I, J*) The dorsal side of stage 13.5–14 embryos. Anterior is at the *top*. (*A,F*) Schematic diagrams showing the expression patterns of *N-CAM*, *twist*, *hairy2a*, and EpA molecular markers in a wild-type embryo (*A*) and an injected embryo (*F*) (see text for references). (*B*) CNS tissue, identified by *N-CAM* mRNA expression, is expanded on the injected side (inj). The arrows show the lateral limits of *N-CAM* expression; the position of the midline is marked. (*C*) *twist* mRNA is present in the prospective neural crest on the control side but not on the injected side (arrows). Midline staining in *C* is *twist* expression in the notochord and head mesoderm (unaffected by ASH3b). The dorsal limit of expression of the epidermal antigen EpA (brown staining) is moved away from the midline after *ASH3b* RNA injection (*D*). (*E*) The anterior end of a different EpA-stained embryo. In *D, E*, and *G* the injected side is marked by nuclear β-galactosidase staining (blue dots). (*G*) *hairy2a* mRNA expression at the border of the neural plate is broader, at a lower level, and farther laterally from the midline on the injected side (compare location of arrows), but midline expression appears unaffected. (*H*) Dorsal view of a gastrula embryo (stage 11.5) showing the expansion of the area surrounded by *hairy2a* (purple staining) on the injected side (arrows). The injected side is marked by red staining for nuclear β-galactosidase. The position of the blastopore lip is shown (white arrows). (*I*) Expression of *Xotch* mRNA is elevated on the *ASH3b*-injected side and wider (compare distance of arrows from the unstained midline). The trigeminal placode (t) is separate from the neural plate on the uninjected control side but contained within the expanded *Xotch* staining on the injected side. (*J*) *myoDb* mRNA is expressed at normal levels on both sides of this injected embryo, but the expressing cells do not extend as far anteriorly and spread farther laterally on the injected side.

by *hairy2a*-expressing cells, consistent with the expansion of *N-CAM* expression. The *hairy2a* border expression was often broader, and at a lower level, on the injected side (Fig. 3G). *Xotch*, the *Xenopus* homolog of *Notch* (Coffman et al. 1990, 1993), is expressed at various levels throughout the neural plate, except for the notoplate. *Xotch* mRNA is also expressed in the trigeminal placode, and around the closing blastopore (Fig. 3I). Later, *Xotch* is expressed in neuroblasts, but not in differentiated neurons (Coffman et al. 1990; data not shown). In *ASH3b*-injected embryos, *Xotch* mRNA expression is expanded laterally on the affected side at neural plate stages, and the level of mRNA is elevated, relative to the control side (Fig. 3I). Like *N-CAM*, *Xotch* is not expressed in the notoplate of injected embryos. At tailbud stages, much of the additional neural tissue in *ASH3b*-injected embryos continues to express *Xotch* (not shown). This suggests that many of the additional neural cells in ASH3b-injected embryos may remain as undifferentiated neuroblasts until relatively late in development.

Taken together, the observed alterations of *N-CAM*, *hairy2a*, *twist*, EpA, and *Xotch* expression show that the prospective CNS expands at the expense of the adjacent cranial neural crest and epidermis in ASH3b-injected embryos (Fig. 3F). Hoechst labeling of nuclei in injected embryos confirms that more cells are present on the injected side within the ectoderm bordered by *hairy2a* (not shown). Because HUA treatment shows that extra neural cells form in injected embryos without proliferation, it appears that ASH3b converts prospective neural crest and epidermal cells to a neural fate.

The effects of ASH3b described thus far were assayed several hours after neural induction is thought to have been completed (about stage 11; for review, see Kintner 1992). To determine whether the effects of *ASH3b* RNA injection could be detected during gastrulation, we examined the expression of *hairy2a* at stage 11.5. At this time, *hairy2a* mRNA expression outlines the prospective neural plate. It is also present in cells of the superficial layer of the organizer region and the adjacent non-involuting marginal zone: cells that are forming the no-

toplate. Although *hairy2a* expression in the organizer/ notoplate was unaffected, the area of the ectoderm surrounded by *hairy2a* was increased on the injected side of embryos (Fig. 3H). Thus, the effects of *ASH3b* RNA injection begin during gastrulation, probably during the normal time of neural induction.

Excess CNS tissue persists

ASH3b-injected embryos were allowed to develop into swimming tadpoles (stage 45–46; Fig. 4A) to assess the fate of the expanded neural tissue. Most embryos showed enlargement of brain and/or anterior spinal cord. Both the normal and expanded CNS regions were positive for *N-CAM* mRNA or protein (Fig. 4B–D; data not shown), indicating that the extra tissue remained neural. The monoclonal antibody 2G9, which recognizes differentiated neural cells (Jones and Woodland 1989), also labeled both the normal and enlarged regions of the CNS (Fig. 4E). These observations indicate that the extra neural tissue present in injected embryos is maintained as part of the CNS and can eventually differentiate into neurons. Because injected RNA is usually absent by this

time in development (4 days after fertilization), it is likely that transient expression of ASH3b leads to an irreversible change in cell fate.

Reduction or loss of the cranial neural crest-derived cartilage and mesenchyme was permanent, as the gills and eye socket were reduced or absent on the affected side of swimming tadpoles (Fig. 4D). Tadpoles arising from injected embryos often showed other alterations on the injected side, including enlargement of the olfactory organ (Fig. 4B,C), and poorly organized or missing lateral line organs (Fig. 4E). We did not observe defects in the epidermis of later stage *ASH3b*-injected embryos. Many epidermal cells are unaffected by *ASH3b* injection, so they may be able to compensate for missing epidermal cells by proliferation.

Neuronal differentiation is altered in ASH3b-*injected embryos*

In *Xenopus* and other lower vertebrates, a small number of primary neurons differentiate early to form a simplified nervous system that mediates larval escape responses (see Hartenstein 1993 and references therein).

Figure 4. Persistence and differentiation of the enlarged CNS after *ASH3b* RNA injection. (A–D) N-CAM mRNA detected in swimming tadpoles (stage 45) by whole-mount in situ hybridization; staining is purple. Some brown pigmentation is present in the pigment epithelium of the eye, but these animals are otherwise albino. (A) A wild-type animal. (B–D) A tadpole arising from an injected embryo (inj: injected side). On the uninjected control side (B), the eye (e) is normally positioned within the mostly neural crest-derived eye socket, and the gill arches (g) and vagus complex (v) are normal sized. However, on the injected side (D), the eye is adjacent to the brain because of the reduced eye socket, the vagus complex is smaller, and the gill arches are absent (large open arrow). A dorsal view (C) shows the massive enlargement of the brain and anterior spinal cord on the injected side. Small open arrows in B and D indicate the olfactory organs. The olfactory organ on the injected side is enlarged. (E) Dorsal view of a tadpole (stage 45) stained for the 2G9 neural epitope (purple). The 2G9 epitope stains differentiated neurons. The brain and anterior spinal cord are expanded laterally on the injected side (black line shows midline), but the anterior–posterior patterning of the brain, revealed by staining intensity variations, appears normal. Some lateral line organs (o) are disorganized or missing on the affected side, whereas the gill arches are smaller (open arrow). The eye is also positioned closer to the brain on the injected side, reflecting a slightly reduced eye socket. (F) A dorsal view of tailbud embryos (stage 31) stained with an antibody to NF-M. The lower embryo is an uninjected control; the upper embryo was injected with *ASH3b* RNA on the marked side. At this age, NF-M is present in early differentiated neurons (primary neurons) and their processes. On the affected side of the upper embryo, the more anterior NF-M-expressing cells in the hindbrain are disorganized (arrow), whereas NF-M-expressing cells are almost absent from the spinal cord (posterior to arrow). The embryos in F are cleared. Anterior is to the *left* in A–C, E, and F and to the *right* in D.
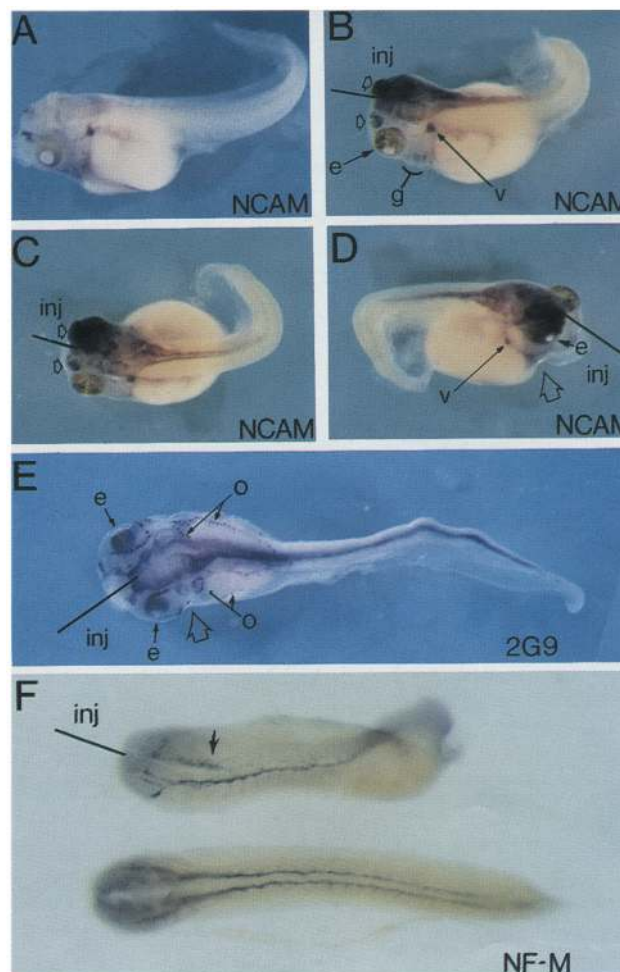
The primary neurons become postmitotic at early neural plate stages and begin to differentiate as the neural tube closes (Hartenstein 1989, 1993). Many primary neurons can be identified shortly after differentiation by expression of neurofilament-M (NF-M) protein (Fig. 4F; Szaro and Gainer 1988; Szaro et al. 1989). In *ASH3b*-injected embryos, the number of differentiated primary neurons is drastically reduced at tailbud stages, with the few remaining primary neurons appearing poorly organized (Fig. 4F). Consistent with the absence or disruption of the primary neurons, tadpoles derived from injected embryos often fail to respond to touch on the injected side (data not shown).

Ectopic ASH3b affects the somitic mesoderm

Because myosin heavy chain expression was reduced in some *ASH3a/b*-injected embryos, we examined the expression of *Xenopus myoDb* mRNA, which can be detected in the somitic mesoderm beginning at gastrulation. Athough the level of *myoDb* expression appeared normal, *myoDb*-positive cells on the injected sides of some embryos did not extend as far anteriorly and, instead, spread farther laterally than on the uninjected sides (Fig. 3J). This suggests that ASH3b expression does not block the formation of the somitic mesoderm but may alter the behavior of cells in the somitic mesoderm during gastrulation (see Keller 1991 and references therein). In extreme cases this may lead to the disruption of gastrulation. Ectopic expression of other bHLH proteins also can disrupt gastrulation (R.A.W. Rupp, J.E. Lee, D.L. Turner, and H. Weintraub, unpubl.), so this phenotype does not appear specific to ASH3a/b. The subsequent loss of myosin heavy chain expression after *ASH3b* injection may reflect either an indirect effect on differentiation, or a competition for bHLH dimerization partners in the mesoderm (Murre et al. 1989). We do not observe alterations of either the level or pattern of *twist* expression in the head mesoderm, notochord, or sclerotome (Hopwood et al. 1989), suggesting that these structures are not affected by ASH3b (Fig. 3C, and data not shown).

Discussion

We have isolated two *Xenopus* genes *ASH3a* and *ASH3b*, which encode bHLH proteins related to the *Drosophila* AS-C proteins and the vertebrate ASH1 proteins (see also Zimmerman et al. 1993). *ASH3a/b* are among the earliest genes expressed in the prospective CNS after neural induction, making them candidates for regulating neuroblast formation. Ectopic expression of ASH3a/b in *Xenopus* embryos leads to a striking enlargement of the CNS, at the expense of other ectodermal cell types (Figs. 2E and 4B-E). At early stages, the neural plate is extended laterally in injected embryos, but this extension ends abruptly (Fig. 2E), perhaps because of inhibition within the ventral ectoderm. CNS enlargement seems to require neural induction, because ASH3 does not convert cells to a neural fate in animal cap explants. In many

cases, the additional neural tissue shows proper anterior–posterior patterning (Figs. 2J and 4E). In addition, ASH3a/b expression appears to inhibit early neuronal differentiation, perhaps by activating *Xotch* transcription.

ASH3b converts ectodermal cells to a neural fate

Enlargement of the CNS after *ASH3b* injection does not require the additional proliferation of neural progenitors, because additional neural cells are found in embryos in which cell division is blocked from midgastrula onward by HUA treatment (Fig. 2H). This indicates that ASH3b expression leads cells that are normally outside the prospective CNS to adopt a neural fate. Analysis of embryos with mosaic ASH3b expression from a DNA vector suggests that ASH3b directly affects the ectoderm, near the site of expression, although it is impossible to rule out effects from earlier transient expression of ASH3b at other locations in these embryos. ASH3b can affect the mesoderm (perhaps by altering mesoderm movements during gastrulation), but it is unlikely that neural expansion arises because of changes in the mesoderm, as the degree of neural expansion does not correlate with the presence or absence of mesoderm defects.

Analysis of molecular markers for neural, epidermal, and neural crest cells shows that the prospective CNS expands laterally, into ectoderm that would normally form the cranial neural crest and epidermis (Fig. 3A–I). During subsequent development, cartilage and mesenchyme arising from the cranial neural crest are reduced or absent. From these observations we conclude that ASH3b converts prospective neural crest and epidermis to a neural fate. We have not observed the activation of neural markers by ectopic ASH3b in either the mesoderm or the endoderm, suggesting that ASH3b cannot convert cells in these germ layers to a neural fate. Ferreiro et al. also have observed enlargement of the CNS in *Xenopus* embryos after coinjection of *ASH3a* and *E12* RNA (B. Ferreiro, K. Zimmerman, D. Anderson, C. Kintner, and W. Harris, pers. comm.).

CNS expansion in *ASH3b*-injected embryos is detected as early as gastrula stages (Fig. 3H). Thus, the effects of ASH3b begin during or immediately after the time when cells in a wild-type embryo adopt a CNS fate. This is also when *ASH3a/b* expression begins during normal development. These observations suggest that ectopic ASH3b is altering the initial choice of cell fate within the ectoderm, rather than directing non-neural cells to a neural fate at some later time.

achaete-scute homologs as vertebrate proneural genes

The early expression of *ASH3a/b* in the prospective CNS, prior to other neural-specific genes such as *N-CAM*, is consistent with a proneural function for these genes during embryogenesis. Alternately, ectopic *ASH3a/b* expression may mimic another proneural gene. This would be analogous to the effect of ectopic *asense* in *Drosophila* imaginal discs. The *asense* gene is dispensable for the formation of most sense organ pre-

cursor cells but is required for subsequent sense organ differentiation (Domínguez and Campuzano 1993; Jarman et al. 1993a). However, ectopic *asense* acts as a proneural gene to generate additional sense organ precursors (Brand et al. 1993; Domínguez and Campuzano 1993). A similar situation exists in mice with the myogenin protein. During normal development, *myogenin* is required for muscle differentiation but not for myoblast formation (Hasty et al. 1993; Nabeshima et al. 1993). However, expression of myogenin can convert cultured cells into myoblasts (Wright et al. 1989). Both ectopic *asense* and *myogenin* seem to mimic the normal functions of genes that encode closely related bHLH proteins. In imaginal discs, *asense* appears to mimic the normal functions of *achaete* and *scute* (Brand et al. 1993; Domínguez and Campuzano 1993; Jan and Jan 1993), whereas in mammalian cells, *myogenin* appears to mimic the function of *myoD* and *myf-5* (Rudnicki et al. 1993; Weintraub 1993). Thus, without a loss-of-function phenotype, our experiments cannot prove that *ASH3a/b* function as proneural genes during normal development. Nonetheless, the proneural capability of ASH3a/b in vertebrate embryos demonstrates that a developmental mechanism for generating neural cells has been conserved from *Drosophila* to vertebrates.

Recently, the loss-of-function phenotype has been determined for the mouse *MASH1* gene (Guillemot et al. 1993). In *MASH1* mutant mice, neural progenitors in the olfactory epithelium and the autonomic nervous system form, but fail to differentiate, and apparently die. Although this phenotype demonstrates an essential role for *MASH1* in neural development, it remains unclear whether *MASH1* normally regulates cell fate decisions.

ASH3b may keep cells as undifferentiated neuroblasts by elevating Xotch expression

ASH3b expression inhibits the formation or differentiation of the primary neurons in injected embryos. *ASH3b* injection also leads to elevated *Xotch* expression throughout the expanded CNS (Fig. 3G). We have also observed elevated expression of two *Xenopus E(spl)* homologs, *ESR1* and *ESR3*, after *ASH3b* RNA injection (D. Turner and H. Weintraub, unpubl.). As far as we are aware, there is no evidence that proneural genes in *Drosophila* activate *Notch* transcription, although it has been shown recently that lethal of scute can activate *E(spl)* and *Delta* transcription (Hinz et al. 1994). *Drosophila Notch* regulates neuronal differentiation in both the developing retina and the external sense organs. In the retina, *Notch* appears to regulate the number of progenitors that differentiate as defined cell types. Although the loss of *Notch* function leads to an excess of specific retinal cell types (Cagan and Ready 1989), an activated version of the Notch protein delays or prevents retinal cell differentiation (Fortini et al. 1993). Similarly, an activated version of the Xotch protein appears to prevent neural differentiation in *Xenopus* embryos (Coffman et al. 1993). We speculate that elevated levels of Xotch (and perhaps also a Xotch ligand) activates or enhances a

Xotch-mediated signaling pathway that blocks the formation of primary neurons in *ASH3b*-injected embryos. The regulation of *Xotch* transcription may reflect a normal function for ASH3b, as the expression of *Xotch* and *ASH3a/b* overlaps during CNS formation (cf. Fig. 2A and Fig. 3I; D. Turner and H. Weintraub, unpubl.). Perhaps the later expression of *ASH3a/b*, and also *ASH1a/b* (Ferreiro et al. 1992; D. Turner and H. Weintraub, unpubl.) is attributable to a requirement for proneural gene function to maintain cells as undifferentiated neuroblasts. This would be consistent with the loss of neuroblasts in mice lacking *MASH1* (Guillemot et al. 1993), as well as with the later effects of *asense* mutations (Domínguez and Campuzano 1993; Jarman et al. 1993a).

The expansion of the CNS in *ASH3a/b* RNA-injected embryos is superficially similar to the enlargement of the CNS observed after injection of RNA encoding an activated Xotch protein (Coffman et al. 1993). However, expansion of the neural plate appears to be an early, and perhaps direct consequence of ASH3a/b expression, whereas neural expansion appears to be a slightly later, and more complex outcome of activated Xotch expression. At early neural plate stages, activated Xotch inhibits the expression of markers in many cell types, including *ASH3a/b* in the posterior CNS (Coffman et al. 1993; D.L. Turner and C. Kintner, unpubl.), but during late neurula, the neural plate expands in *Xotch*-injected embryos. These apparently opposite effects of activated Xotch might be reconciled by supposing that activated Xotch initially delays cell fate commitment, and after injected *Xotch* RNA decays, additional uncommitted ectodermal cells are present that can adopt a neural fate (Coffman et al. 1993). In contrast, in *ASH3a/b*-injected embryos, expansion of the neural plate begins earlier, during late gastrula/early neurula. This suggests that additional cells adopt a neural fate at an earlier time after *ASH3a/b* injection than after the injection of RNA encoding activated Xotch, probably reflecting a more direct effect of ASH3a/b.

Negative regulation of ectopic ASH3 and neuroblast formation

Explants of animal cap ectoderm, taken from *Xenopus* embryos prior to neural induction, form epidermis but not neural tissue. However, dissociation of animal caps leads to the formation of neural cells without induction (Godsave and Slack 1989, 1991; Grunz and Tacke 1989). This suggests that neurogenesis is the ground state or default pathway for ectoderm, and local signaling between ectodermal cells inhibits the formation of neuroblasts. A dominant-negative activin receptor activates neural markers without induction (Hemmati-Brivanlou and Melton 1992), again suggesting that an external signal, perhaps dependent on activin, represses neurogenesis. In the embryo, mesoderm appears to be the primary inducer of neural tissue. Possibly an inductive signal from the mesoderm removes negative regulation present in intact animal caps. Although microinjected *ASH3a/b* RNA can convert ectodermal cells to a neural fate in

intact embryos, this does not occur in uninduced animal cap explants. The combined data suggest that proneural gene function in the intact ectoderm is repressed via an activin-like signaling pathway; neural induction might then relieve this repression. This explanation may also address how injected *ASH3a/b* causes such a remarkably uniform lateral expansion of the CNS, at the expense of adjacent ectodermal cell types (Fig. 2E). Perhaps an inducing signal originates at the midline and spreads laterally as a graded signal. The lateral extent of the CNS might then be determined by the balance between repression and the level of inducer. Expression of excess ASH3a/b would make cells more sensitive to the same level of inducer.

One candidate to mediate repression of ASH3a/b in uninduced ectoderm is the *Xenopus hairy2a* gene, because maternal *hairy2a* mRNA is present in animal cap ectoderm (D.L. Turner, R.A.W. Rupp, and H. Weintraub, in prep.). In *Drosophila*, both normal and ectopic AS-C expression is subject to repression by *hairy* (Moscoso del Prado and Garcia-Bellido 1984). We have also isolated additional *hairy* homologs, as well as homologs of the E(spl) bHLH proteins from *Xenopus* (D. Turner, R.A.W. Rupp, and H. Weintraub, unpubl.). Future experiments will determine whether these proteins regulate the function of ASH3a/b and whether they are the targets for regulation by the neural induction mechanism.

Materials and methods

PCR, cDNA isolation, and RACE

Completely degenerate oligonucleotide primers were designed based on two conserved amino acid sequences within the bHLH domain of the *Drosophila* AS-C proteins: ERNRVK (CGA<u>CTC-GAGA</u>[GA][AC]GNAA[TC][AC]GNGTNA; added *Xho*I site underlined) and AVEYI (GG<u>GAATTCG</u>[GAT]AT[GA]TA[TC]T-CACNGC; added *Eco*RI site underlined). These primers (10 μg each) were used for a 50-μl PCR. The first three cycles used an annealing temperature of 48°C for 1 min, followed by a 1-min ramp from 48°C to 60°C, and then a 72°C extension for 1 min. Subsequent cycles (42) used a 60°C annealing temperature for 1 min and a 72°C extension for 1 min. The PCR template was cDNA reverse transcribed from total RNA from stage 14 or 17 *Xenopus* embryos (1 μg; primed with random hexamers). PCR products of the appropriate size were purified by gel electrophoresis, subcloned, and sequenced. Cloned PCR fragments were used as a mixed probe to screen a stage 17 embryo cDNA library in λgt10 (kindly provided by Chris Kintner, Salk Institute, La Jolla, CA). cDNAs that encode four AS-C-related proteins were identified. One 3.4-kb cDNA (originally designated *ASX28*) encodes ASH3a, whereas a 3.0-kb cDNA (originally designated *ASX31*) encodes ASH3b. Other cDNAs encode either of two proteins with extensive homology to MASH1. One is identical to the *Xenopus* ASH1 protein (referred to here as ASH1a; Ferreiro et al. 1993); we have designated the other ASH1b. A partial nucleotide sequence of the ASH1b-coding region is 91% identical to the ASH1a nucleotide sequence (not shown), suggesting that *ASH1a* and *ASH1b* also represent a pair of genes arising from the *Xenopus* genome duplication (Graf and Kobel 1991). The expression of the *ASH1b* mRNA is similar to that described for *ASH1a* (Ferreiro et al. 1993), although we detect it in a few cells in the prospective midbrain as early as stage 16 (not shown).

A 1.6-kb *Eco*RI fragment that contained the 5' end of the *ASH3a* cDNA was subcloned into pBluescript II (Stratagene) and designated pASX28R1. The coding region and 5'-untranslated region were sequenced. The *ASH3b* cDNA was consistently rearranged after subcloning, so that the *ASH3b* cDNA insert was PCR amplified from the phage using primers flanking the λgt10 cloning site (gt10LX: CAGATCTCGAGCAAGT-TCAGCCTGGTTA and gt10RX: CGTCTCGAGAGGTGGCT-TATGAGTATT). The coding region was directly sequenced from the PCR products. A primer to the 3'-untranslated region (ASX31R, CACC<u>CTCGAG</u>GTCATGTATAAATAAC; added *Xho*I site underlined) and the gt10LX primer were then used to PCR amplify the coding region for subcloning (using Vent polymerase from New England Biolabs to minimize the possibility of errors).

Because the *ASH3b* cDNA did not contain the entire coding sequence, the RACE (rapid amplification of cDNA ends) procedure (Frohman 1990) was employed to obtain the 5' end of the *ASH3b*-coding region. Total RNA from stage 20–21 embryos was used as a template for reverse transcription. The primers and conditions were exactly as described in Vojtek and Cooper (1993), except that the gene-specific primers were (1) AS171A, TTGTTCCGGTCCCCCTGAAGCCTTTGA, and (2) RR57, GTTTCACCCTGTTCCTCTC. RACE products were subcloned into pT7Blue (Novagen) and sequenced. The sequence from the RACE products was confirmed by directly sequencing the corresponding genomic region amplified by PCR (with a 5'-end primer based on the RACE sequence ASX31F, GAC-<u>GAATTC</u>ATTCACCACTAGGAGACAA, added *Eco*RI site underlined, and RR57). The GenBank accession numbers for the *ASH3* sequences are U09768 (*ASH3a*) and U09769 (*ASH3b*).

We find a single in-frame ATG upstream of the bHLH domain in both ASH3a and ASH3b, located at the same position in each open reading frame. This is likely to be the initiation codon for each protein, as the amino acid sequence 5' to it is not conserved between ASH3a and ASH3b (because of multiple deletions/insertions, as well as nucleotide substitutions), whereas the amino acid sequence after it is 89% identical. Our sequence for ASH3b is in agreement with the partial sequence of Zimmerman et al. (1993), whereas our sequence for ASH3a shows two differences: Phe $_{42}$ is Tyr in our sequence, and Glu $_{150}$ is Asp in our sequence. These conservative amino acid substitutions result from single-base changes and may reflect allelic variation.

For insertion into an expression vector, the *ASH3a*-coding sequence was PCR amplified from the original phage cDNA with the 5' primer ASX28M (TC<u>AGATCTTCGAATTC</u>CGC-GATGGAGGAGCAGCTG; *Bgl*II and *Eco*RI sites underlined) and the 3' primer ASX28R2 (CG<u>TCTAGA</u>CTCCTACGTGGT-GTCCTGG; *Xba*I site underlined), using Vent polymerase. The full length coding sequence of the *ASH3b* gene was constructed by ligating the 5' genomic PCR fragment, cut at a unique *Mlu*I site, to the original partial cDNA, also cut with *Mlu*I. This ligation then served as a template for PCR with *Vent* polymerase. The primers used were ASX31M (GCC<u>AGATCTTCGAAT-TC</u>CGCTATGAGGAACAGCCC; added *Bgl*II and *Eco*RI sites underlined) and the ASX31R primer.

All direct sequencing of PCR products and some plasmid sequencing was performed on an Applied Biosystems sequencing system, using *Taq* dye terminator cycle sequencing. Other sequencing was performed with Sequenase (U.S. Biochemical).

CS2 vectors

The coding regions of *ASH3a* and *ASH3b*, without 5'- or 3'-untranslated regions [obtained as described above (PCR, cDNA

isolation, and RACE)], were subcloned into the multipurpose expression vectors CS− and CS2+ MT (constructed by D.L. Turner and R.A.W. Rupp). The CS/CS2 vectors will be described elsewhere (a detailed description is available upon request). These vectors contain the simian cytomegalovirus IE94 enhancer/promoter upstream of a polylinker. The simian IE94 promoter functions in most mammalian tissue culture cells, as well as in Xenopus and zebrafish embryos. An SP6 promoter is present in the 5'-untranslated region of the IE94-driven transcript to allow in vitro RNA synthesis. An SV40 late polyadenylation site is present after the polylinker, followed by restriction sites for linearizing the vector for RNA synthesis. CS2+ and CS− also contain an f1 origin for single-stranded DNA preparation (+ / − refers to the orientation of the f1 origin) and an ampicillin resistance gene. The CS2+ MT vector is identical to CS2+, except that it contains six copies of the Myc epitope tag at the 5' end of the polylinker (see Roth et al. 1991). The epitope-tagged versions of ASH3a and ASH3b generate the same phenotype as the wild-type proteins, when expressed from injected RNA or from DNA expression vectors in Xenopus embryos.

Capped SP6 RNAs synthesized from CS or CS2 vectors linearized 3' to the SV40 polyadenylation site appear to generate at least 10-fold more protein than other SP6 expression vectors (such as SP64) after microinjection into Xenopus embryos. This appears to be attributable to polyadenylation of the injected RNA (Rupp et al. 1994).

In vitro RNA synthesis

ASH3a and ASH3b RNAs were synthesized from 2.5 μg of CS/CS2-derived vectors, linearized with Asp718. Reactions were as described by Harland and Weintraub (1985), with nucleotide concentrations of 0.5 mM each for GTP, ATP, CTP, and UTP, and 2.5 mM for the GTP cap analog (New England Biolabs 1407). SP6 polymerase (Promega) was used (50 U/50 μl reaction), with an additional 25–50 units added after 2 hr. Total synthesis time was 4–5 hr. After DNase treatment, RNAs were extracted with phenol–chloroform, purified using Sephadex G50 spin columns, and ethanol precipitated. RNAs were resuspended in water for injection. Nuclear localized β-galactosidase mRNA was synthesized by the same procedure, but the template was 5 μg of CS− nuclear β-galactosidase (constructed by J.E. Lee and D.L. Turner), linearized with NotI.

Microinjection of mRNA and DNA

Embryos from albino females were fertilized in vitro with sperm from either pigmented or albino males. Animals were obtained from Xenopus One. We note that the offspring from a few apparently albino males were partially pigmented at late stages (see Fig. 4). Embryos were dejellied with 2% cysteine 1 hr after fertilization and then kept at 15°C in 0.1× modified Barth's saline (MBS) until injection. Embryos were injected at the two-cell stage, with two injections (4–5 nl each; usually 70 pg/nl in water) into the animal hemisphere of one cell. Injections were made near the upper surface of the egg, so that the yolk was often visibly displaced. In some experiments, ~50 pg of RNA encoding nuclear β-galactosidase was coinjected with the ASH3 RNA. For DNA injections, a single injection of ~60–75 pg of CS2+ MT ASH3b DNA (15 pg/nl in water) was made into each embryo. After injection, embryos were allowed to develop at either 15°C or 23°C in 0.1× MBS to the desired stage. Embryos were staged according Nieukwoop and Faber (1967). For HUA treatment, injected and control embryos were transferred to

HUA at stage 10–10.25, as described in Harris and Hartenstein (1991).

Analysis of injected embryos

More than 350 ASH3a/b-injected embryos were scored for alterations in the expression of a variety of markers, using either in situ hybridization or immunohistochemistry. All markers shown were scored on embryos from at least two different sets of injected embryos (at least 14 embryos per probe; >30 embryos in most cases). All markers shown (as well as additional neural markers not shown) gave consistent results on injected embryos. The degree of neural expansion depends on the dose of RNA: At 25 pg/embryo of ASH3b RNA, a weak phenotype was observed (not shown). At >1 ng/embryo of ASH3b RNA, the number of embryos with severe gastrulation defects increased, making accurate assessment of neural expansion difficult. At a given concentration of injected RNA or DNA, a range of phenotypes was observed (see Results), probably reflecting slight variations in the location and/or volume of each injection. Most embryos shown exhibit strong phenotypes; this level of effect was reproducibly present among affected embryos. Injection of DNA vectors consistently yielded a weaker phenotype than RNA injections.

We have primarily analyzed embryos injected on a single side, as described above and in the text. The uninjected side serves as an internal control in such embryos, because the expression of molecular markers on the uninjected side is generally the same as in uninjected control embryos (not shown). Some morphological distortions of the uninjected side of embryos do occur, because of failure of the neural tube to close and/or axial defects caused by the injected RNA.

In situ hybridization and immunohistochemistry

The whole-mount in situ procedure of Harland (1991) was used with some modifications. A detailed protocol is available on request. Briefly, embryos were fixed as described and then stored in 100% ethanol at −20°C. For in situ hybridization, embryos were placed in sieves constructed of 1.6-cm high polypropylene cylinders (cut from Falcon 2063 or Wheaton 225402 tubes) with stainless steel or nylon mesh fused to the bottoms. Sieves were transferred through the standard solutions (1–2 ml each) in the wells of 24-well tissue culture dishes (Costar). All steps were performed in the sieves/24-well dishes, including hybridization and alkaline phosphatase staining, to facilitate the processing of 12–18 probes simultaneously. Tris (100 mM at pH 7.4) buffer with 150 mM NaCl was substituted for phosphate-buffered saline (PBS) during incubation with the alkaline-phosphatase-conjugated anti-digoxigenin antibody and subsequent washes.

Whole-mount in situ hybridization for ASH3a was done with an antisense probe from pASX28R1. Whole-mount in situ hybridization with an ASH3b probe, or with an ASH3a probe processed without RNase (which should detect both ASH3a and ASH3b), yields an expression pattern that is apparently identical to ASH3a (not shown).

Immunohistochemistry was done essentially as described by Hemmati-Brivanlou and Harland (1989), except that embryos were processed in sieves/24-well dishes as described above, and embryos stained for 2G9 or NF-M were fixed in Dent's fixative [20% dimethylsulfoxide/80% methanol (Dent et al. 1989)]. EpA and 2G9 supernatants (Jones and Woodland 1986, 1989) were used undiluted, whereas MF20 (anti-myosin heavy chain; Developmental Studies Hybridoma Bank) and 9e10 (anti-Myc epitope tag; Evan et al. 1985) were diluted 1:5. XC10C6 ascites

(anti-*Xenopus* neurofilament-M; Szaro and Gainer 1988) was diluted 1:5000. Anti-N-CAM (Balak et al. 1987) was diluted 1:500. Primary antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse secondary antibody (diluted 1:4000; Boehringer-Mannheim 605 31), or, for N-CAM, with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (diluted 1:2000; Boehringer-Mannheim 605 230). After either in situ hybridization or immunohistochemistry, some embryos were cleared in 2:1 benzyl benzoate/benzyl alcohol (Dent et al. 1989).

β-Galactosidase staining

Embryos coinjected with RNA for nuclear β-galactosidase were stained for β-galactosidase activity after fixation, but prior to ethanol/methanol storage. Embryos were rinsed several times in PBS after fixation. β-Galactosidase staining conditions were 10 mM potassium ferrocyanide, 10 mM potassium ferricyanide, 1 mM MgCl$_2$, and 1 mg/ml of X-gal (blue staining) or Salmon-gal (red staining; Biosynth-AG) in PBS at 37°C. After staining, embryos were rinsed in PBS three times and transferred to methanol or ethanol for storage at -20°C. We note that the nuclear β-galactosidase usually stained fewer cells per embryo than were identified by immunohistochemistry for epitope-tagged ASH3a/b. Therefore, whereas β-galactosidase activity does mark the injected side of the embryo, it does not necessarily identify all cells that express the coinjected ASH3a/b protein. This may reflect a difference in the sensitivity of the two detection systems, or a difference in RNA or protein stability.

Acknowledgments

References

Alonso, M.C. and C.V. Cabrera. 1988. The *achaete-scute* gene complex of Drosophila melanogaster comprises four homologous genes. *EMBO J.* 7: 2585–2591.

Balak, K., M. Jacobson, J. Sunshine, and U. Rutishauser. 1987. Neural cell adhesion molecule expression in Xenopus embryos. *Dev. Biol.* 119: 540–550.

Benezra, R., R.L. Davis, D. Lockshon, D.L. Turner, and H. Weintraub. 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* 61: 49–59.

Brand, M., A.P. Jarman, L.Y. Jan, and Y.N. Jan. 1993. *asense* is a Drosphophila neural precursor gene and is capable of initi-

ating sense organ formation. *Development* 119: 1–17.

Cabrera, C.V. and M.C. Alonso. 1991. Transcriptional activation by heterodimers of the *achaete-scute* and *daughterless* gene products of Drosophila. *EMBO J.* 10: 2965–2973.

Cagan, R.L. and D.F. Ready. 1989. *Notch* is required for successive cell decisions in the developing Drosophila retina. *Genes & Dev.* 3: 1099–1112.

Campos-Ortega, J.A. 1993. Mechanisms of early neurogenesis in Drosophila melanogaster. *J. Neurobiol.* 24: 1305–1327.

Campuzano, S. and J. Modolell. 1992. Patterning of the Drosophila nervous system: The *achaete-scute* gene complex. *Trends Genet.* 8: 202–208.

Campuzano, S., L. Balcells, R. Villares, L. Carramolino, L. Garcia-Alonso, and J. Modolell. 1986. Excess function *hairy-wing* mutations caused by *gypsy* and *copia* insertions within structural genes of the *achaete-scute* locus of Drosophila. *Cell* 44: 303–312.

Coffman, C., W. Harris, and C. Kintner. 1990. *Xotch*, the Xenopus homolog of Drosophila *Notch*. *Science* 249: 1438–1441.

Coffman, C.R., P. Skolglund, W.A. Harris, and C.R. Kintner. 1993. Expression of an extracellular deletion of *Xotch* diverts cell fate in Xenopus embryos. *Cell* 73: 659–671.

Cronmiller, C. and C.A. Cummings. 1993. The daughterless gene product in Drosophila is a nuclear protein that is broadly expressed throughout the organism during development. *Mech. Dev.* 42: 159–169.

Dent, J.A., A.G. Polson, and M.W. Klymkowsky. 1989. A whole-mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in Xenopus. *Development* 105: 61–74.

Dixon, J.E. and C.R. Kintner. 1989. Cellular contacts required for neural induction in Xenopus embryos: Evidence for two signals. *Development* 106: 749–757.

Dominguez, M. and S. Campuzano. 1993. *asense*, a member of the Drosophila *achaete-scute* complex, is a proneural and neural differentiation gene. *EMBO J.* 12: 2049–2060.

Doniach, T. 1993. Planar and vertical induction of anteroposterior pattern during the development of the amphibian central nervous system. *J. Neurobiol.* 24: 1256–1275.

Evan, G.I., G.K. Lewis, G. Ramsay, and J.M. Bishop. 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* 5: 3610–3616.

Feder, J.N., L.Y. Jan, and Y.N. Jan. 1993. A rat gene with sequence homology to the Drosophila gene hairy is rapidly induced by growth factors known to influence neuronal differentiation. *Mol. Cell Biol.* 13: 105–113.

Ferreiro, B., P. Skoglund, A. Bailey, R. Dorsky, and W.A. Harris. 1992. *XASH1*, a Xenopus homolog of *achaete-scute*: A proneural gene in anterior regions of the vertebrate CNS. *Mech. Dev.* 40: 25–36.

Fortini, M.E., I. Rebay, L.A. Caron, and S. Artavania-Tsakonas. 1993. An activated Notch receptor blocks cell-fate commitment in the developing Drosophila eye. *Nature* 365: 555–557.

Frohman, M. 1990. Rapid amplification of cDNA ends (RACE): User friendly cDNA cloning. *Amplifications* 5: 11–15.

Godsave, S.F. and J.M.W. Slack. 1989. Clonal analysis of mesoderm induction in Xenopus laevis. *Dev. Biol.* 134: 486–490.

———. 1991. Single cell analysis of mesoderm formation in the Xenopus embryo. *Development* 111: 523–530.

Gonzalez, F., S. Romani, P. Cubas, J. Modolell, and S. Campuzano. 1989. Molecular analysis of the *asense* gene, a member of the *achaete-scute* complex of Drosophila melanogaster, and its novel role in optic lobe development. *EMBO J.* 8: 3553–3562.

Graf, J.-D. and H.R. Kobel. 1991. Genetics of *Xenopus laevis*.

Turner and Weintraub

Methods Cell Biol. 36: 19–34.

Grunz, H. and L. Tacke. 1989. Neural differentiation of Xenopus laevis ectoderm takes place after disaggregation and delayed reaggregation without inducer. Cell Differ. and Dev. 28: 211–218.

Guillemot, F., L.-C. Lo, J.E. Johnson, A. Auerbach, D.J. Anderson, and A.L. Joyner. 1993. Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell 75: 1–20.

Harland, R. 1991. In situ hybridization: An improved whole-mount method for Xenopus embryos. Methods Cell Biol. 39: 685–695.

Harland, R. and H. Weintraub. 1985. Translation of mRNA injected into Xenopus oocytes is specifically inhibited by antisense RNA. J. Cell Biol. 101: 1094–1099.

Harris, W.A. and V. Hartenstein. 1991. Neural determination without cell division in Xenopus embryos. Neuron 6: 499–515.

Hartenstein, V. 1989. Early neurogenesis in Xenopus: The spatio-temporal pattern of proliferation and cell lineages in the embryonic spinal cord. Neuron 3: 399–411.

———. 1993. Early pattern of neuronal differentiation in the Xenopus embryonic brainstem and spinal cord. J. Comp. Neurol. 328: 213–231.

Hasty, P., A. Bradley, J.H. Morris, D.G. Edmondson, J.M. Venuti, E. Olsen, and W.H. Klein. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature 364: 501–506.

Hemmati-Brivanlou, A. and R.M. Harland. 1989. Expression of an engrailed-related protein is induced in the anterior neural ectoderm of early Xenopus embryos. Development 106: 611–617.

Hemmati-Brivanlou, A. and D.A. Melton. 1992. A truncated activin receptor inhibits mesoderm induction and formation of axial structures in Xenopus embryos. Nature 359: 609–614.

Hemmati-Brivanlou, A., J.R. de la Torre, C. Holt, and R.M. Harland. 1991. Cephalic expression and molecular characterization of Xenopus En-2. Development 111: 715–724.

Hinz, U., B. Giebel, and J.A. Campos-Ortega. 1994. The basic helix-loop-helix domain of Drosophila lethal of scute protein is sufficient for proneural function and activates neurogenic genes. Cell 76: 77–87.

Hopwood, N.D., A. Pluck, and J.B. Gurdon. 1989. A Xenopus mRNA related to Drosophila twist is expressed in response to induction in the mesoderm and the neural crest. Cell 59: 893–903.

Jan, Y.N. and L.Y. Jan. 1993. HLH proteins, fly neurogenesis, and vertebrate myogenesis. Cell 75: 827–830.

Jarman, A.P., M. Brand, L.Y. Jan, and Y.N. Jan. 1993a. The regulation and function of the helix-loop-helix gene, asense, in Drosophila neural precursors. Development 119: 19–29.

Jarman, A.P., Y. Grau, L.Y. Jan, and Y.N. Jan. 1993b. atonal is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system. Cell 73: 1307–1321.

Jimenez, F. and J.A. Campos-Ortega. 1990. Defective neuroblast commitment in mutants of the achaete-scute complex and adjacent genes of D. melanogaster. Neuron 5: 81–89.

Johnson, J.E., S.J. Birren, and D.J. Anderson. 1990. Two rat homologues of Drosophila achaete-scute specifically expressed in neuronal precursors. Nature 346: 858–861.

Johnson, J.E., K. Zimmerman, T. Saito, and D.J. Anderson. 1992. Induction and repression of mammalian achaete-scute homologue (MASH) gene expression during neuronal differentiation of P19 embryonal carcinoma cells. Development 114: 75–87.

Jones, E.A. and H.R. Woodland. 1986. Development of the ectoderm in Xenopus: Tissue specification and the role of cell association and division. Cell 44: 345–355.

———. 1989. Spatial aspects of neural induction in Xenopus laevis. Development 107: 785–791.

Keller, R. 1991. Early embryonic development of Xenopus laevis. Methods Cell Biol. 36: 61–113.

Kintner, C. 1992. Molecular bases of early neural development in Xenopus embryos. Annu. Rev. Neurosci. 15: 251-0284.

Kintner, C.R. and J. Dodd. 1991. Hensen's node induces neural tissue in Xenopus ectoderm. Implications for the action of the organizer in neural induction. Development 113: 1495–1505.

Kintner, C.R. and D.A. Melton. 1987. Expression of Xenopus N-CAM RNA in ectoderm is an early response to neural induction. Development 99: 311–325.

Lamb, T.M., A.K. Knecht, W.C. Smith, S.E. Stachel, A.N. Economides, N. Stahl, G.D. Yancopolous, and R. Harland. 1993. Neural induction by the secreted peptide Noggin. Science 262: 713–717.

Lo, L.-C., J.E. Johnson, C.W. Wuenschell, T. Saito, and D.J. Anderson. 1991. Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. Genes & Dev. 5: 1524–1537.

Moscoso del Prado, J. and A. Garcia-Bellido. 1984. Genetic regulation of the achaete-scute complex of Drosophila melanogaster. Wilhelm Roux's Arch. Dev. Biol. 193: 242–245.

Murre, C., P.S. McCaw, H. Vaessin, M. Caudy, L.Y. Jan, Y.N. Jan, C.V. Cabrera, J.N. Buskin, S.D. Hauschka, A.B. Lassar, H. Weintraub, and D. Baltimore. 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58: 537–544.

Nabeshima, Y., K. Hanaoka, M. Hayasaka, E. Esumi, S. Li, I. Nonaka, and Y. Nabeshima. 1993. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature 364: 532–535.

Nieuwkoop, P.D. and J. Faber. 1967. Normal table of Xenopus laevis (Daudin), 2nd ed. North-Holland Publishing Co., Amsterdam, The Netherlands.

Rashbass, J., M.V. Taylor, and J.B. Gurdon. 1992. The DNA-binding protein E12 co-operates with XMyoD in the activation of muscle-specific gene expression in Xenopus embryos. EMBO J. 11: 2981–2990.

Rodriguez, I., R. Hernandez, J. Modolell, and M. Ruiz-Gomez. 1990. Competence to develop sensory organs is temporally and spatially regulated in Drosophila epidermal primordia. EMBO J. 9: 3583–3592.

Roth, M.B., A.M. Zahler, and J.A. Stolk. 1991. A conserved family of nuclear phosphoproteins localized to sites of polymerase II transcription. J. Cell Biol. 115: 587–596.

Rudnicki, M.A., P.N.J. Schnegelsberg, T. Braun, H.-H. Arnold, and R. Jaenisch. 1993. MyoD or Myf-5 is required in a functionally redundant manner for the determination of skeletal myoblasts. Cell 75: 1351–1360.

Ruiz i Altaba, A. 1993. Induction and axial patterning of the neural plate: Planar and vertical signals. J. Neurobiol. 24: 1276–1304.

Rupp, R.A.W. and H. Weintraub. 1991. Ubiquitous MyoD transcription at the midblastula transition precedes induction-dependent MyoD expression in presumptive mesoderm of X. laevis. Cell 65: 927–937.

Rupp, R.A.W., L. Snider, and H. Weintraub. 1994. Xenopus embryos regulate the nuclear localization of XMyoD. Genes &

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Dev. (in press).

Sadaghiani, B. and T. Thiebaud. 1987. Neural crest development in the *Xenopus laevis* embryo, studied by interspecific transplantation and scanning electron microscopy. *Dev. Biol.* **124:** 91–110.

Sasai, Y., R. Kageyama, Y. Tagawa, R. Shigemoto, and S. Nakanishi. 1992. Two mammalian helix-loop-helix factors structurally related to *Drosophila hairy* and *Enhancer of split*. *Genes & Dev.* **6:** 2620–2634.

Spemann, H. 1938. *Embryonic development and induction.* Yale University Press, New Haven, CT; reprinted by Garland (1988), New York.

Szaro, B.G. and H. Gainer. 1988. Identities, antigenic determinants, and topographic distributions of neurofilament proteins in the nervous systems of adult frogs and tadpoles of Xenopus laevis. *J. Comp. Neurol.* **273:** 344–358.

Szaro, B.G., V.M.-Y. Lee, and H. Gainer. 1989. Spatial and temporal expression of phosphorylated and non-phosphorylated forms of neurofilament proteins in the developing nervous system of Xenopus laevis. *Dev. Brain Res.* **48:** 87–103.

Villares, R. and C.V. Cabrera. 1987. The *achaete-scute* gene complex of D. melanogaster: Conserved domains in a subset of genes required for neurogenesis and their homology to *myc. Cell* **50:** 415–424.

Vojtek, A.B. and J. Cooper. 1993. Identification and characterization of a cDNA encoding mouse CAP: A homolog of the yeast adenylyl cyclase associated protein. *J. Cell Sci.* **105:** 777–785.

Weintraub, H. 1993. The MyoD family and myogenesis: Redundancy, networks, and thresholds. *Cell* **75:** 1241–1244.

Wright, W.E., D.A. Sassoon, and V.K. Lin. 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell* **56:** 607–617.

Zimmerman, K., J. Shih, J. Bars, A. Collazo, and D.J. Anderson. 1993. *XASH-3*, a novel Xenopus *achaete-scute* homolog, provides an early marker of planar neural induction and position along the mediolateral axis of the neural plate. *Development* **119:** 221–232.