Development 119, 221-232 (1993)
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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

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SUMMARY

We have isolated a novel *Xenopus* homolog of the *Drosophila achaete-scute* genes, called *XASH-3*. *XASH-3* expression is neural specific and is detected as early as stage $11\frac{1}{2}$, making it one of the earliest markers of neural induction so far described. Moreover, *XASH-3* expression within the neural plate is regionally restricted. Transverse bands of *XASH-3* mRNA mark discrete positions along the anteroposterior axis, while longitudinal bands mark a discrete position along the mediolateral axis. This latter site of *XASH-3* expression appears to demarcate the prospective sulcus limitans, a boundary zone that later separates the functionally distinct dorsal (alar) and ventral (basal) regions of the spinal cord. In sandwich explants lacking any underlying mesoderm, *XASH-3* is expressed in longitudinal stripes located lateral to the midline. This provides the first indication that planar or midline-derived inductive signals are sufficient to establish at least some aspects of positional identity along the mediolateral axis of the neural plate. By contrast, the transverse stripes of *XASH-3* expression are not detected, suggesting that this aspect of anteroposterior neural pattern is lost or delayed in the absence of vertically passed signals. The restricted mediolateral expression of *XASH-3* suggests that mediolateral patterning of the neural plate is an early event, and that this regionalization can be achieved in the absence of inducing signals derived from underlying mesoderm.

Key words: *Xenopus, achaete-scute*, basic helix-loop-helix, neural induction

INTRODUCTION

The identification of genes that determine cell type and pattern within the nervous system is a fundamental problem of developmental neurobiology. In *Drosophila*, the *achaete-scute* complex (AS-C) of four homologous genes (*achaete, scute, lethal of scute* and *asense*) is required for neuronal determination in both the central and peripheral nervous systems (Cabrera, 1992; Campuzano and Modolell, 1992). The AS-C genes are members of the basic helix-loop-helix (bHLH) family of transcription factors which includes myogenic determination factors such as *MyoD* (Weintraub et al., 1991). These tissue-specific proteins form heterodimers with more broadly expressed bHLH proteins such as mammalian *E12* and its *Drosophila* homolog, *daughter - less* (Murre et al., 1989a). These heterodimers mediate their transcriptional regulatory function through a six base pair core consensus binding sequence, termed the E box element (Murre et al., 1989b).

Two mammalian homologs of the *Drosophila achaete-scute* genes, *MASH1* and *MASH2*, have previously been isolated from a rat sympathoadrenal progenitor cell line (Johnson et al., 1990). These genes share approximately 80% identity to the *Drosophila achaete-scute* genes within their bHLH regions but diverge from the *Drosophila* genes, as well as from each other, outside of this region. Although the expression of *MASH2* in vivo is not neural specific (Johnson et al., 1992b; K. Zimmerman, unpublished data), *MASH1* expression is restricted to neural progenitor cells within discrete regions of the brain and spinal cord and to the developing sympathetic, parasympathetic and enteric ganglia in the peripheral nervous system (Guillemot and Joyner, 1993; Lo et al., 1991; L. Lo, unpublished observations). More recently, a *Xenopus achaete-scute* homolog has been isolated that shares 75% homology throughout its protein coding sequence and 100% identity within its bHLH domain with *MASH1* (Ferreiro et al., 1992). *XASH1* is not expressed at appreciable levels prior to neural tube closure (stage 20) but is then expressed in a regionally specific pattern within the developing brain and retina (Ferreiro et

al., 1992). Unlike *MASH1*, *XASH1* is not expressed in either the spinal cord or in any neural crest derivatives (Ferreiro et al., 1992).

The regionally and temporally restricted pattern of *MASH1* and *XASH1* expression suggested that additional *achaete-scute* homologs might be required at other places or times of neural development. Based on homology within the bHLH region, we have isolated a novel *achaete-scute* homolog from *Xenopus*, which we call *XASH3*. The expression of *XASH3* is neural specific and regionally restricted. Expression is detected as early as the mid-gastrula stage (stage 11-11$\frac{1}{2}$), making *XASH3* one of the earliest regional markers of neural induction thus far described.

We have used *XASH3* as a marker to examine the relative contributions of planar and vertical inductive signals to axial patterning of the neural plate. Previously, it had been shown that markers of anteroposterior position along the neuraxis exhibit appropriate regional localization in explants or exogastrulae lacking vertical inducing signals (Doniach, 1992; Papalopulu and Kintner, 1993; Ruiz i Altaba, 1990, 1992). Other studies have shown that some neuronal cell types characteristic of both dorsal and ventral regions of the spinal cord can differentiate in the absence of vertical inducing signals (Ruiz i Altaba, 1992). However, these latter studies leave open the issue of whether planar signals are sufficient to specify positional information correctly, independent of cell type, along the prospective dorsoventral axis. *XASH3* expression defines a unique position along this axis, demarcating the boundary between the presumptive dorsal and ventral halves of the neural tube. Analysis of *XASH3* expression in explants indicates that this boundary can form in the absence of vertical inducing signals. Taken together, these data support the idea (Yamada et al., 1993) that the inductive signals that determine at least some aspects of positional identity along the prospective dorsoventral axis operate very early, at the neural plate stage.

MATERIALS AND METHODS

Molecular cloning of *XASH3* cDNA

Random primed cDNA was prepared from embryonic stage 12-14 *Xenopus* total RNA. Fully degenerate oligonucleotide primers, which corresponded to the regions indicated in Fig. 1, were synthesized. Inosine residues were substituted at all positions of 4-fold degeneracy and a *Bam*HI or *Eco*RI restriction site was included on the 5′ or 3′ primer respectively. The sequence of the 5′ and 3′ primers were
ccggatccAA(C/T)(A/C)GIGTIAA(A/G)(C/T)TIGTIAA
and ccgaattcC(G/T)(A/G/T)AT(A/G)TA(C/T)TCIACIGC,
respectively. PCR amplification conditions were as recommended by the manufacturer (Perkin Elmer/Cetus) with an annealing temperature of 45˚C. PCR products were digested with *Bam*HI/*Eco*RI, subcloned into pGEM-3 and sequenced using standard methods (Sequenase/USB). The *XASH3* PCR amplified cDNA was used to probe a stage 17 neural plate library (Kintner and Melton, 1987). The two cDNA clones isolated, X3.A and X3.B, were subcloned in pBS-KS and the coding region of each was sequenced in its entirety on both strands.

Electrophoretic mobility shift and transcriptional activation assays

In vitro transcription, translation and electrophoretic mobility shift assays (EMSA) were according to previous experiments (Johnson et al., 1992a). The X3.A cDNA was linearized with *Hin*dIII or *Not*I and transcribed with T7 or T3 RNA polymerase to generate the sense and antisense transcripts, respectively. The sequence of the top strand of the oligonucleotide used in gel shift assays was as follows, GATCCCCCCAACACCTGCTGCCTGA. Transcriptional activation assays were as previously described (Johnson et al., 1992a). X3.A was subloned into the pRSVSV40 expression vector in both orientations (Johnson et al., 1992a). The pRSV*MASH1*, (2E)80MCKCAT and pRSVL constructs were as previously described (de Wet et al., 1987; Johnson et al., 1992a).

In situ hybridization analysis

The X3.A cDNA was linearized with *Not*I or *Hin*dIII and transcribed with T3 or T7 RNA polymerase to generate antisense and sense *XASH3* probes, respectively. The full-length X3.A antisense probe was used for all in situ hybridization analyses shown. For control experiments, the X3.B cDNA was linearized with *Xba*I or *Hin*dIII and transcribed with T3 or T7 RNA polymerase to generate antisense and sense probes, respectively. In addition, each cDNA was linearized with *Pvu*II and transcribed with T3 RNA polymerase to generate antisense probes, which lacked the bHLH domain. A 500 bp 3′ *Bam*HI/*Eco*RI fragment of X3.A and a 600 bp internal *Bam*HI fragment from X3.B, which lacked the bHLH domain, were also used as probes in control experiments. The N-CAM cDNA, pTN1, has been described previously (Krieg et al., 1989).

Albino *Xenopus laevis* embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Whole-mount in situ hybridization analysis was performed using previously described techniques (Harland, 1991; Hemmati-Brivanlou et al., 1990). Modification to the published procedure included preblocking the alkaline phosphatase-conjugated digoxigenin antibody. *Xenopus* embryos were rehydrated, minced and incubated in PTw (PBS + 0.1% Tween-20) containing 10% sheep serum at room temperature for 1 hour. The liquid was then removed from the embryos and a 1:200 dilution of the digoxigenin antibody in PTw containing 1% sheep serum was added. The antibody was preblocked for greater than 3 hours prior to use. The ratio of embryo to antibody volume during this preblock was approximately 1:5. In addition, the washing steps following antibody incubation were extended to seven 1 hour washes. Reactions were stopped by fixation in MEMFA. Fixation was carried out at 4˚C overnight. The embryos were then dehydrated in methanol and cleared in a 2:1 mixture of benzyl benzoate:benzyl alcohol. Selected embryos were mounted in paraplast and sectioned at 10 μm thickness.

Preparation of explants

‘Closed face’ sandwich explants were prepared exactly as described by Keller and colleagues (Keller and Danilchik, 1988; Keller et al., 1992). All explants were made to include a radial sector of no more than 100˚ centered on the dorsal midline of stage 10+ embryos. While the entire height of the neural plate is included in the explants, the animal cap is excluded. Although the explants are made by initially apposing two similar pieces of tissue, extensive cell mixing between these two sheets eliminates this initial mirror duplication (Keller et al., 1992), so that the explant behaves like a single, integrated piece of tissue. Explants were analyzed at stage 17, a stage at which the longitudinal and transverse domains of *XASH3* expression are clearly visible in normal embryos (Fig. 7A). In contrast, previous studies using Keller sandwich explants to examine aspects of anteroposterior patterning have typically analyzed the explants at stage 26 (Doniach et al., 1992; Ruiz i Altaba, 1992). In our experiments, sibling explants from the same batches allowed to develop to stage 26 exhibited the typical, narrow constriction of the neurectoderm indicative of suc-

cessful convergent extension and an absence of invading mesoderm, as has been extensively documented in earlier studies (Doniach, 1992; Keller and Danilchik, 1988).

RESULTS

Isolation of *achaete-scute* homologs from *Xenopus*

We were interested in isolating *achaete-scute* homologs involved in early stages of neural determination in *Xenopus laevis*. We therefore prepared RNA and synthesized cDNA from embryos at the late gastrula/early neurula stages (stage 12-14) of development. PCR reactions were carried out using fully degenerate primers which represented conserved regions of the 5′ and 3′ helical regions of the *Drosophila achaete-scute* and mammalian *MASH* genes. Characterization of the products of these PCR reactions revealed two groups of sequences with homology to the *achaete-scute* genes. The first group was identical to *MASH1* in its protein coding sequence and was identical to the previously characterized *XASH1* gene (Ferreiro et al., 1992) in nucleotide sequence.

A second group of clones shared approximately 70% nucleotide sequence and 75% protein sequence homology with *MASH1* and *MASH2* within the region amplified (Fig.1, sequence between primers). The equal divergence of this gene from both *MASH1* and *MASH2*, as well as the isolation of a *Xenopus MASH1* homolog with 100% protein homology, suggests that this is not a *Xenopus* homolog of a previously isolated mammalian gene but is instead a unique vertebrate *achaete-scute* homolog. We have named this gene *XASH3*. The same gene has been isolated independently by D. Turner and H. Weintraub (D. Turner, personal communication). Attempts to identify a mammalian *XASH3* homolog using PCR and low-stringency Southern blot analysis have been unsuccessful (K. Zimmerman, unpublished data).

In order to characterize more fully the *XASH3* gene, a stage 17 neurula cDNA library (generously provided by C. Kintner and B. Harris) was screened. Two independent cDNA clones were isolated. These clones, designated X3.A and X3.B, were approximately 2.4 and 1.4 kb in length, respectively. The protein coding regions of both cDNA clones were sequenced. The X3.A clone contains a 160 amino acid open reading frame downstream of an in-frame methionine (Fig. 2). We are currently unable to determine whether this is the actual start codon for the X3.A protein as the cDNA clone extends only 81 bp 5′ to this methionine and no stop codons are located upstream. However, the region upstream of this potential start codon shares 5/6 nucleotides with the Kozak translation initiation consensus sequence (consensus CC(A/G)CCAUG, *XASH3* CCGCGAUG) including the important −3 position suggesting that this may be the actual start site of the *XASH3* protein (Kozak, 1984). The second cDNA clone, X3.B, begins 156 nucleotides 3′ of the X3.A clone and therefore contains no start codon.

Comparison of the X3.A and X3.B protein coding regions indicates that these clones encode related but distinct proteins sharing 75% amino acid homology (Fig. 2). The most obvious point of divergence is the C terminus of the protein where the X3.B coding region extends 25 amino acids beyond the X3.A stop codon due to a single nucleotide insertion in the X3.B clone. If this region is not included in the homology calculation, the degree of amino acid conservation is approximately 90%. The conservation of sequences outside of the bHLH domain, a region not conserved between *XASH3* and *XASH1*, suggests that the *X3.A* and *X3.B* genes may result from the duplication of the *Xenopus* genome rather than representing two unique *achaete-scute* homologs. The homology between *XASH3* and the *MASH1* and *MASH2* proteins within the bHLH region is approximately 90% (Fig. 1). *XASH3* shares only 60% homology with the *Drosophila scute* protein, the most related member

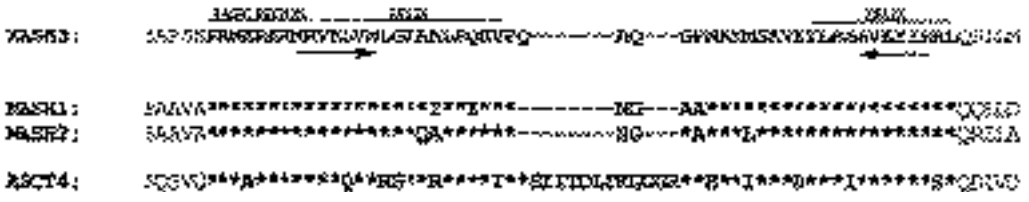


Fig. 1. Comparison of *XASH3* bHLH domain to previously characterized genes. Primers used in PCR amplifications are indicated by arrows. Stars represent conserved residues within the bHLH domain. Dashes indicate a gap in the protein alignment.

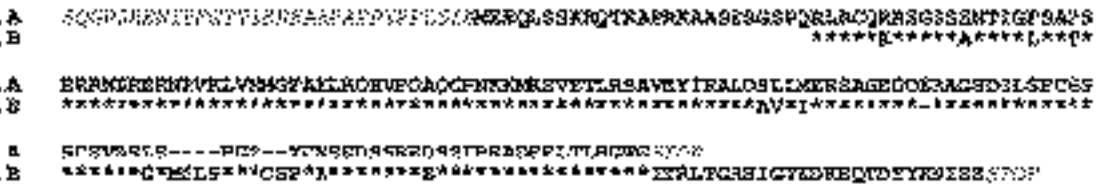


Fig. 2. Deduced protein coding sequence of X3.A and X3.B cDNA clones. Residues upstream of the first methionine in the X3.A protein are indicated in italics as we are currently unable to determine if these residues are contained within the *XASH3*.A protein (see text). Stars indicate conserved residues. Dashes indicate a gap in the protein alignment. The nucleotide sequences are available from the GenBank data base, accession numbers L20214 (X3.A) and L20215 (X3.B).

of the *achaete-scute* family, but the divergence between these two proteins is largely accounted for by the additional ten amino acids within the *scute* loop region (Fig. 1). Both *XASH3* proteins diverge from the *Drosophila achaete-scute* genes and their previously characterized vertebrate homologs outside of the bHLH region.

XASH3 functions as a transcriptional regulator

In vitro characterization of the binding sites of a number of bHLH-containing proteins has established a consensus binding sequence of 6 nucleotides (CANNTG) termed the E box element (Murre et al., 1989b). To assess the function of *XASH3*, we first tested its ability to bind an E box sequence in a gel shift assay, using protein synthesized by in vitro transcription and translation from a cloned cDNA template. Previous studies with the *achaete-scute* proteins as well as with *MASH1* and *MASH2* have demonstrated that they bind E box sequences only as heterodimers with another member of the bHLH family, the *Drosophila daughterless* protein and its vertebrate homolog, *E12*, respectively (Johnson et al., 1992a; Murre et al., 1989b). The combination of the in vitro-synthesized *XASH3* and *E12* proteins also binds an E box-containing oligonucleotide, and the resulting DNA-protein complex is equal in intensity to that observed for the combination of the *MASH1* and *E12* proteins (Fig. 3A, lanes 3 and 4). This result not only confirms that *XASH3* is a DNA-binding protein, but also establishes that the protein encoded within the X3.A cDNA clone, even if not full length, is sufficient to carry out this function.

We next tested the ability of *XASH3* to act as a transcriptional regulator. *MASH1* and *MASH2* have been shown to activate specifically transcription in transfected 10T½ cells via an E box sequence located within the muscle creatine kinase (MCK) promoter (Johnson et al., 1992a). We employed the same assay to test the transcriptional regulatory capacity of the *XASH3* protein. 10T½ cells were cotransfected with a *XASH3* expression construct and an MCK/CAT reporter construct, which contained a minimal MCK promoter with two upstream E box-containing oligonucleotide sequences. In *XASH3*-transfected cells, an approximately 10-fold increase in CAT activity was detected (Fig. 3B, lane 4). A similar enhancement was seen in *MASH1*-transfected cells (Fig. 3B, lane 2). These results indicate that *XASH3*, like other members of the bHLH family, is a DNA-binding, transcriptional regulatory protein.

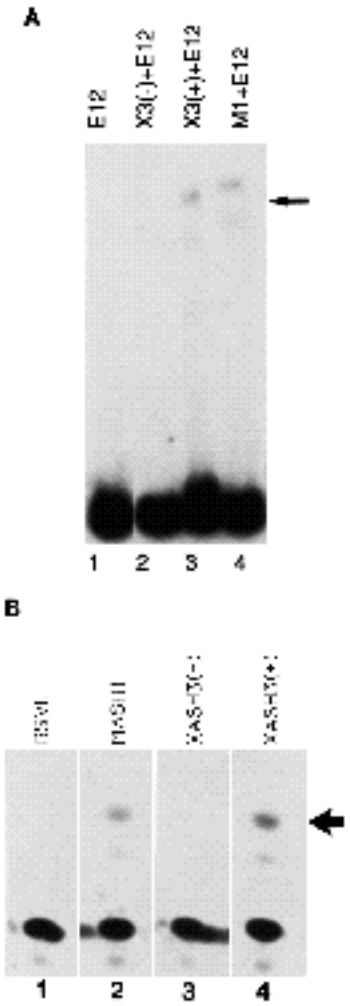


Fig. 3. *XASH3* binds DNA and activates transcription of an MCK/CAT reporter construct in 10T½ cells. (A) Electrophoretic mobility shift assays were performed with in vitro translation extracts prepared with in vitro transcribed *XASH3* (sense and antisense), *MASH1* and *E12* RNA transcripts. Extracts were combined as follows E12 (1), XASH3 (antisense) + E12 (2), XASH3 (sense) +12 (3), MASH1(sense) +E12 (4), mixed with a ^{32}P-labelled, E-box containing oligonucleotide and analyzed by electrophoresis as previously described (Johnson et al., 1992a). Arrow indicates position of gel shifted band. (B) Cells were co-transfected with an MCK/CAT reporter construct, a CMV β-gal construct, and either an RSV luciferase construct (1), RSVMASH1 (2), RSVXASH3 in the antisense orientation (3), or RSVXASH3 in the sense orientation (4) construct. Samples were standardized for transfection efficiency by quantitation of β-gal activity prior to CAT enzymatic assay. Arrow indicates acetylated chloramphenicol reaction product.

The pattern of *XASH3* expression during development

We have characterized the expression of *XASH3* during *Xenopus* embryonic development using the technique of whole-mount in situ hybridization (Hemmati-Brivanlou et al., 1990). For most studies, the entire X3.A cDNA clone was used as a probe. To confirm that the expression pattern that we observed with this probe was not due to cross-hybridizing genes, we also used two non-bHLH-containing probes from both the *X3.A* and *X3.B* genes. All probes tested yielded a similar expression pattern indicating that the hybridization pattern that we observe is specifically that of the *XASH3* gene rather than related bHLH-containing genes. We were not able to distinguish whether both the *X3.A* and *X3.B* genes are expressed in a similar pattern or if the signal that we observe is due to cross-hybridization, as all regions of the two genes cross-hybridize at high stringency in plasmid blotting assays. For this reason, the expression pattern described will be referred to as that of *XASH3* without reference to the specific gene. Comparison of the signal intensity of *XASH3* to that of control probes such as *Krox-20*, *actin* and *Xtwist* indicated that *XASH3* mRNA is a very low abundance transcript, and may explain our failure to detect it by northern analysis (data not shown).

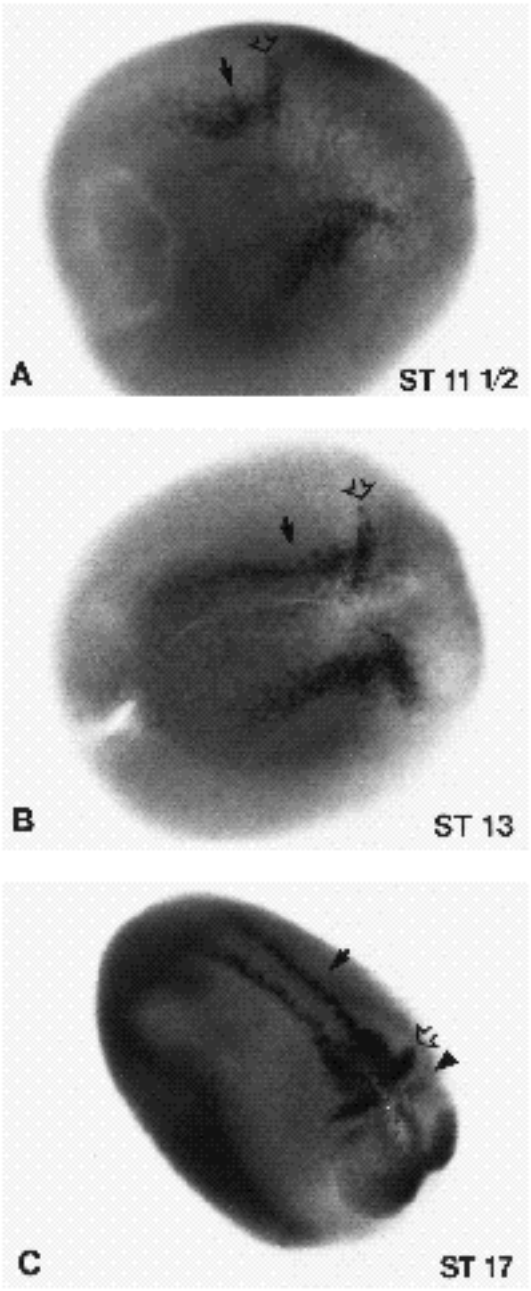


Fig. 4. *XASH3* expression during mid-gastrula and neurula stages of *Xenopus* development. Whole-mount in situ analysis was performed at early stages of *Xenopus* development. Arrows mark specific aspects of *XASH3* expression pattern: closed arrow (A-C) indicates stripes appearing at the midline of the mediolateral axis of the neural plate; in B and C these stripes parallel the anteroposterior axis of the embryo. Open arrow (A-C) indicates expression in transverse 'eyebrow' stripes within prospective hindbrain region. Arrowhead (C) marks anterior stripe of expression near hindbrain/midbrain junction. In all panels, anterior is to the right although the exact orientations are different.

XASH3 expression is first detected at the mid-gastrula stage (stage 11-11½) of development in two symmetric patches of expression lateral to the midline (Fig. 4A, closed arrow). In neurula stage embryos (stage 14-20), these patches become condensed and extended, appearing as two bilaterally symmetric stripes (Fig. 4B and C, closed arrow). These stripes run the length of the presumptive spinal cord and terminate anteriorly within the hindbrain region. A transverse band of *XASH3* expression is observed at the anterior end of each stripe, first detectable as a tangential 'eyebrow' like zone of expression at stage 11-11½ (Fig. 4A-C, open arrow). Cohybridization of late neurula embryos (stage 18) with a mixture of *Krox-20* and *XASH3* probes indicates that the 'eyebrow' stripes of *XASH3* expression are located immediately anterior to the third rhombomere (data not shown). A fainter horizontal stripe of *XASH3* expression is observed in late neurula stage embryos (stage 17+) just anterior to the 'eyebrow' stripes, near the presumptive hindbrain/midbrain junction region (Fig. 4C, arrowhead). Diffuse staining of *XASH3* is observed in more anterior regions of the developing nervous system with more intense signal being detected at the anterior most end of the prosencephalon (Figs 4C, 5A). In addition, faint staining is detected in scattered cells located immediately lateral to the cephalic neural plate (Fig. 5A, open arrow). These cells may represent a subset of prospective cranial neural crest cells.

Comparison of the *XASH3* expression pattern to that of the neural cell adhesion molecule, N-CAM, at stage 13 indicates that the *XASH3* expression domain is restricted within the region of the neural plate defined by N-CAM expression (Fig. 5A,D). To define more clearly the mediolateral boundaries of *XASH3* expression within the neural plate, we examined cross-sections of early neurula stage embryos (stage 13/14) that had been hybridized with the *XASH3* probe. Within the region of the embryo where *XASH3* appears as bilaterally symmetric longitudinal stripes, expression is restricted to a group of cells located near the midpoint of the medial and lateral extremes of the neural plate, above the center of the somites (Fig. 5B). By contrast, N-CAM mRNA is more broadly distributed along the mediolateral axis and is less abundant at this stage (Fig. 5E). In the region containing the 'eyebrow' stripes, the *XASH3* expression domain is expanded along the mediolateral axis although expression is absent from the ventral midline (Fig. 5C).

Following neural tube closure (stage 20+), *XASH3* expression persists in the neural tube as two bilaterally symmetric longitudinal stripes. Cross-sections of embryos following neural tube closure reveal that *XASH3* expression is restricted to a group of cells located at the midline of the dorsoventral axis, the sulcus limitans (Fig. 6B,C, closed arrows). Expression continues within this region at least until stage 38, the latest stage which we have examined. A second, more ventral, stripe of *XASH3* expression is also observed in the hindbrain region at later stages (stage 20+) of development (Fig. 6A, closed arrow). At stage 25+, this ventral stripe of *XASH3* expression can be clearly resolved in whole-mount specimens into three longitudinally oriented dashes. These dashes are longer than the length of a single rhombomere by comparison to *Krox-20* expression in whole mounts (data not shown). Cross-sections within the hindbrain region reveal that this second stripe of *XASH3* expression is located in a region of the neural tube immediately adjacent to the floor plate (Fig. 6C, open arrow).

XASH3 continues to be expressed in a diffuse pattern throughout midbrain and forebrain regions in all embryonic

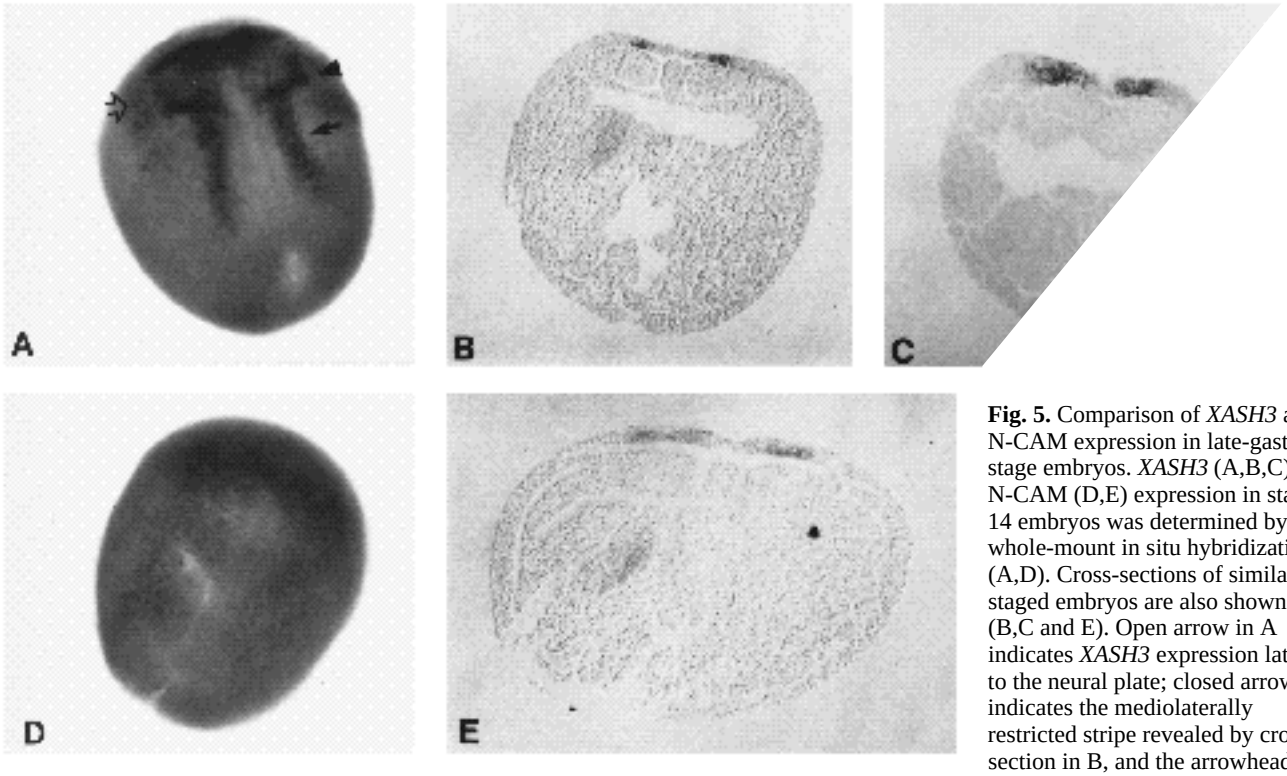


Fig. 5. Comparison of *XASH3* and N-CAM expression in late-gastrula stage embryos. *XASH3* (A,B,C) and N-CAM (D,E) expression in stage 14 embryos was determined by whole-mount in situ hybridization (A,D). Cross-sections of similarly staged embryos are also shown (B,C and E). Open arrow in A indicates *XASH3* expression lateral to the neural plate; closed arrow indicates the mediolaterally restricted stripe revealed by cross-section in B, and the arrowhead indicates the transverse 'eyebrow' stripe revealed by cross-section in C. In A, anterior is towards 11 o'clock and, in D, it is towards 1 o'clock. Note the restricted expression of *XASH3* along the mediolateral axis of the neural plate (B) in comparison to that of N-CAM (E).

stages examined. *XASH3* expression is also detected in the developing retina (Fig. 6A). In the eye vesicle, *XASH3* appears as early as stage 21 and continues to be expressed within the retinal marginal zone as late as stage 38 (data not shown). Later stages of development have not yet been examined.

XASH3 expression in explant cultures

The expression of *XASH3* in the neural plate appears to demarcate the presumptive sulcus limitans, a major landmark of dorsoventral pattern in the spinal cord. As other aspects of dorsoventral pattern have been shown to be dependent on vertical inducing signals, we examined whether the pattern of *XASH3* expression was similarly dependent on such signals (Ruiz i Altaba, 1992; Ruiz i Altaba and Jessell, 1992). We chose to address this question using sandwich explants (Keller and Danilchik, 1988). The absence of vertical mesoderm-ectoderm contact in properly made explants is well documented and therefore the specific effects of planar inducing signals can be examined in the absence of vertical signalling (Keller and Danilchik, 1988; Keller et al., 1992). We examined two components of *XASH3* expression: the longitudinal stripes in the spinal cord/hindbrain region (Fig. 7A, dark arrow) and the 'eyebrow' stripes in the anterior hindbrain (Fig. 7A, open arrow). These aspects of *XASH3* pattern are readily visible in stage 17 embryos, the stage at which the explants were examined (Fig. 7A). In 12/13 explants, *XASH3* expression was observed in the neuroectodermal region of the explant. Moreover, *XASH3* expression was restricted with respect to the mediolateral axis of the neural plate. In some cases (4/13), two parallel longitudinal stripes similar to those seen in normal embryos were observed (Fig. 7B and C, dark arrows).

Several aspects of the *XASH3* expression pattern in explant cultures differed from those observed during normal development. In a number of explants (8/13), particularly those in which convergence and extension of the neuroectoderm was asymmetric, *XASH3* expression was not bilaterally symmetric (Fig. 7D, dark arrow). The mediolateral position of *XASH3* expression also differed from that seen in normal embryos. *XASH3* expression no longer defined the midpoint of the mediolateral axis of the neuroectoderm but was instead located more laterally, particularly within the posterior 'neck' of the explant. Another striking difference in the pattern of *XASH3* expression in explant cultures was the consistent absence of the 'eyebrow' stripes of *XASH3* expression within the hindbrain. *XASH3* expression was not detected at this position in any explant examined (0/13). Thus in sandwich explants lacking an underlying source of mesoderm-derived vertical inducing signals (see Materials and Methods), the longitudinal and transverse components of the *XASH3* expression pattern can be dissociated from one another. The longitudinal component is retained while the transverse component is apparently lost.

DISCUSSION

XASH3 expression

The *Drosophila achaete-scute* proteins function as neuronal determination factors in both the central and peripheral

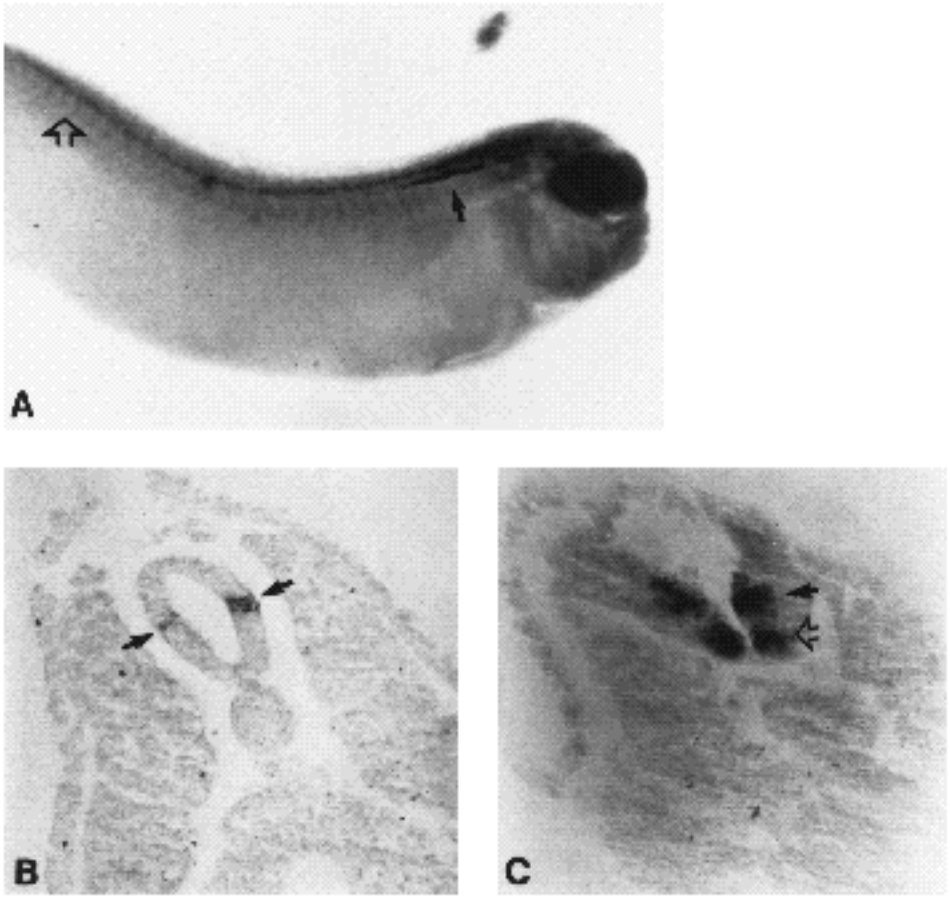


Fig. 6. Expression of *XASH3* in embryos following neural tube closure. *XASH3* expression as determined by whole-mount in situ analysis at stage 28 is shown in A; anterior is to the right. Cross sections of the spinal cord region from similarly staged embryo are shown in B and C; dorsal is up. Open and closed arrows in A indicate approximate planes of section in B and C, respectively. Closed arrows in B and C indicate the sulcus limitans; open arrow in C indicates expression adjacent to the floorplate.

nervous systems (Cabrera, 1992; Campuzano and Modolell, 1992). Two mammalian homologs of these genes, *MASH1* and *MASH2*, have previously been characterized and a *Xenopus* homolog of *MASH1*, called *XASH1*, has also been identified (Ferreiro et al., 1992; Johnson et al., 1990). We have isolated a novel *Xenopus* homolog of the *Drosophila achaete-scute* genes, which we call *XASH3*. Expression of the *XASH3* gene is neural specific and appears in a regionally restricted population of central nervous system precursor cells at the time of neural induction. The early and restricted expression of *XASH3* superficially parallels the spatially restricted and progenitor-specific expression patterns observed for the *AS-C* genes during *Drosophila* neural development (Campuzano and Modolell, 1992).

Possible role of *XASH3* in patterning the neural plate

The expression of *XASH3* in mid-gastrula stage embryos makes it one of the earliest markers of neural regionalization thus far described. Moreover, *XASH3* is expressed in a regionally restricted manner with respect to both the anteroposterior and mediolateral axes of the neural plate. The mediolateral restriction is particularly striking because it appears to correspond to the presumptive sulcus limitans, a boundary that separates the functionally distinct alar and basal plates of the spinal cord. Emerging data from several organisms suggest that diffusible signals produced at both the midline and prospective dorsal margins of the neural plate may control patterning of the dorsoventral axis of the developing spinal cord (Basler et al., 1993; Placzek et al., 1993; Yamada et al., 1991, 1993). Later in development, regulatory genes such as *Pax-3* and *Pax-6* are expressed in a non-graded manner within the alar or basal plates, respectively, and terminate abruptly at the emerging sulcus limitans (Goulding et al., 1991; Gruss and Walther, 1992; Walther and Gruss, 1991) (Fig. 8C). This suggests that graded, continuous information produced at the prospective dorsal and ventral extremes of the neural tube may first be converted into several discontinuous mediolateral zones of regulatory gene expression, prior to the specification of cell type (Fig. 8C,D). Such a mechanism would be analagous to the early stages of anteroposterior axis patterning in *Drosophila* (Nüsslein-Volhard, 1991).

The expression of *XASH3* in the presumptive sulcus limitans may play an important role in this conversion, by establishing or maintaining an early zone of discontinuity along the mediolateral axis of the neural plate (Figs 5B, 8B). One possibility is that this zone may form a boundary that sharpens the transition point from dorsal to ventral character of the neural tube, for example by perturbing gradients of diffusible signals (see Fig. 8C). Alternatively, *XASH3* could be a consequence rather than a cause of boundary formation, and might determine cell fates appropriate to that region. Whatever the case, the zone of *XASH3* expression could identify one of the first responses of neural plate cells to dorsal or ventral midline-derived gradient signals, and may define an important early event in converting this continu-

ous information to discontinuous domains of cellular differentiation. While we have thus far failed to detect *XASH3* homologs in higher vertebrates, a homeobox gene specifically expressed at the sulcus limitans has been identified in both chick (Rangini et al., 1991) and mouse (Lu et al., 1992), supporting the idea that molecular specification of this

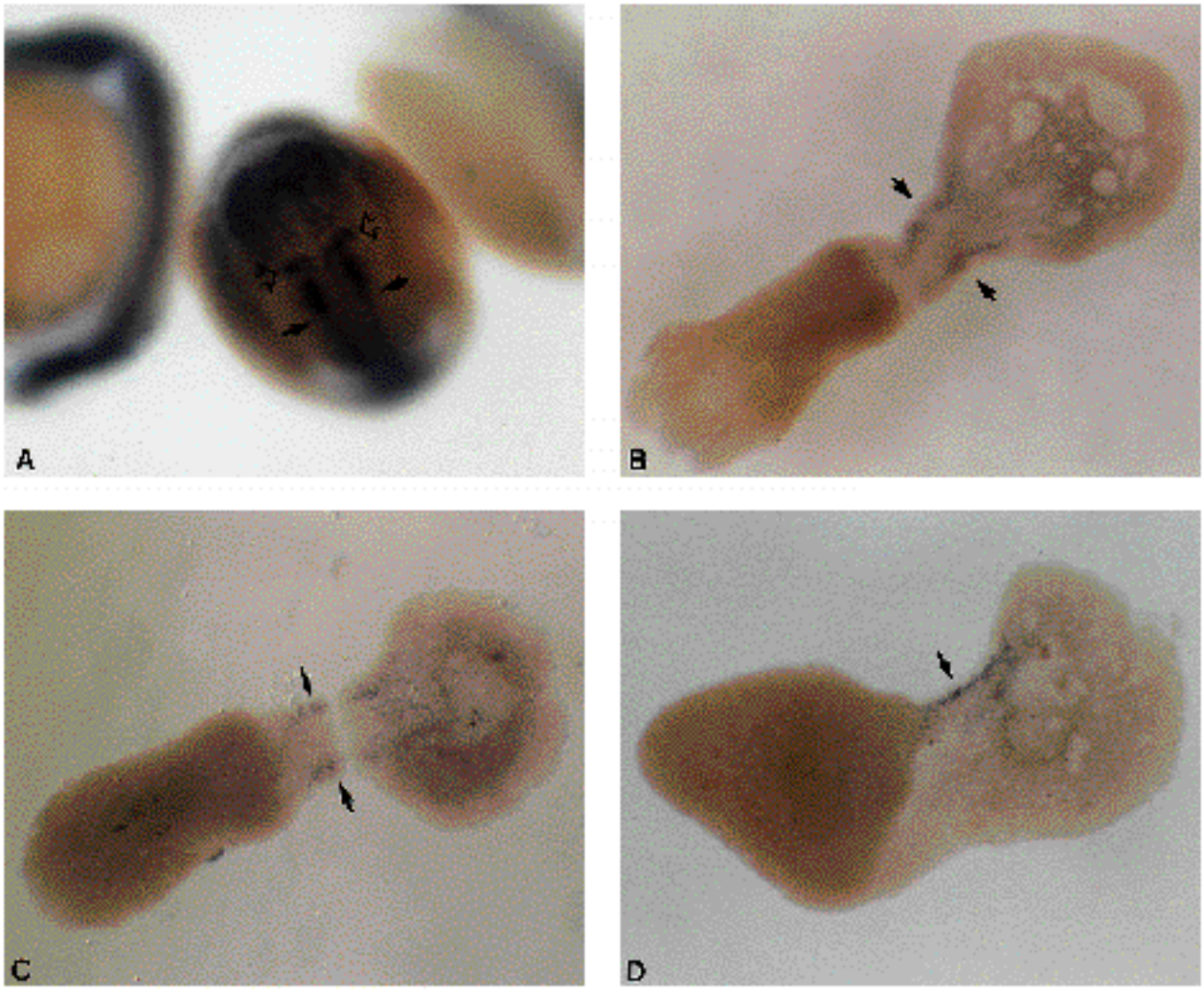
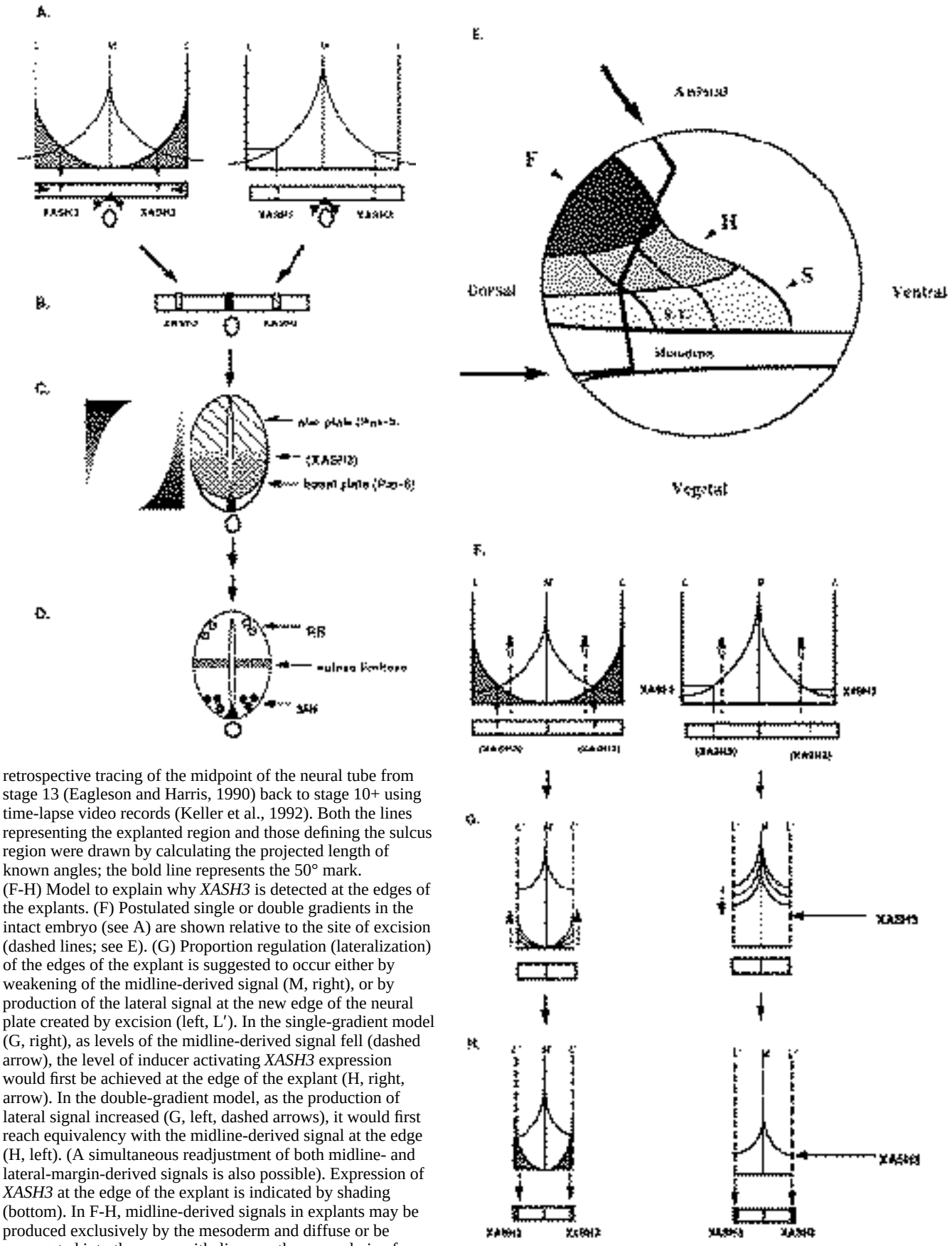


Fig. 7. *XASH3* expression in explants. Closed-face Keller sandwiches were prepared as described in Materials and Methods, and analyzed at the equivalent of stage 17 for *XASH3* expression by whole-mount in situ hybridization. For comparison, a normal stage 17 embryo hybridized with *XASH3* is shown in A; closed arrows mark the mediolaterally restricted longitudinal stripes of *XASH3* expression, open arrows mark the transverse 'eyebrow' stripes in the hindbrain (anterior is toward 11 o'clock). Explants in B-D are oriented with anterior to the right. Specimens in B and C have undergone symmetric convergent extension and show bilateral symmetric stripes of *XASH3* expression displaced towards the lateral edges of the explants (arrows). In D, convergent extension was asymmetric and a single stripe of *XASH3* is observed.

Fig. 8. Models to explain the control of *XASH3* expression in embryos (A-D) and in Keller sandwich explants (E-H). (A) The site of *XASH3* expression along the mediolateral axis of the neural plate may be determined by a particular value of a single, midline-derived gradient (right), or by the point of intersection of two opposing gradients, one (shaded) derived from the lateral margins of the neural plate (left). These models apply specifically to the control of *XASH3* expression and not necessarily to the determination of other spinal cord cell types. (B) Schematic of stage $11\frac{1}{2}$ neural plate showing sites of *XASH3* expression relative to midline (black bar) and notochord (circle). (C) Following neural tube closure, the boundary zone defined by *XASH3* expression may be important in converting graded, continuous signals deriving from the dorsal and ventral margins of the tube (stippled drawing on left) to discontinuous zones of gene expression within the forming alar and basal plates, perhaps by sharpening the transition from dorsal to ventral character of the spinal cord. (D) After subdivision of the neural tube into alar and basal zones, further steps lead to the differentiation of specific dorsal and ventral cell types such as Rohon Beard neurons (RB) and motorneurons (MN). (E) The position of the explanted dorsal marginal zone used in sandwich explants (arrows, bold line) is superimposed on the fate map of a stage 10+ gastrula (see Materials and Methods). Prospective forebrain (F), hindbrain (H), spinal cord (S) and sulcus limitans (S.L.) are indicated. Explanted tissue excludes the prospective sulcus limitans as determined by



retrospective tracing of the midpoint of the neural tube from stage 13 (Eagleson and Harris, 1990) back to stage 10+ using time-lapse video records (Keller et al., 1992). Both the lines representing the explanted region and those defining the sulcus region were drawn by calculating the projected length of known angles; the bold line represents the 50° mark.
(F-H) Model to explain why *XASH3* is detected at the edges of the explants. (F) Postulated single or double gradients in the intact embryo (see A) are shown relative to the site of excision (dashed lines; see E). (G) Proportion regulation (lateralization) of the edges of the explant is suggested to occur either by weakening of the midline-derived signal (M, right), or by production of the lateral signal at the new edge of the neural plate created by excision (left, L′). In the single-gradient model (G, right), as levels of the midline-derived signal fell (dashed arrow), the level of inducer activating *XASH3* expression would first be achieved at the edge of the explant (H, right, arrow). In the double-gradient model, as the production of lateral signal increased (G, left, dashed arrows), it would first reach equivalency with the midline-derived signal at the edge (H, left). (A simultaneous readjustment of both midline- and lateral-margin-derived signals is also possible). Expression of *XASH3* at the edge of the explant is indicated by shading (bottom). In F-H, midline-derived signals in explants may be produced exclusively by the mesoderm and diffuse or be propagated into the neuroepithelium, or they may derive from the notoplate (Ruiz i Altaba, 1992).

boundary region is a common feature of vertebrate neural development. It is not known whether this homeobox gene is expressed as early as *XASH3*, however.

Planar and vertical inductive interactions

Classical experiments suggest that both planar and vertical inducing signals derived from the mesoderm may control the patterning of the amphibian neural plate (Spemann, 1938). In exogastrulae and explant cultures, two experimental systems in which no vertical contact between mesoderm and ectoderm is established, planar interactions are sufficient for inducing both general neural properties such as N-CAM expression (Dixon and Kintner, 1989; Kintner and Melton, 1987) and a number of markers of anteroposterior position (Doniach et al., 1992; Papalopulu and Kintner, 1993; Ruiz i Altaba, 1990, 1992). Vertical interactions are important for the patterning of the ventral spinal cord in *Xenopus*. In both exogastrula and UV-irradiated embryos (which lack a notochord), the floor plate, a ventral midline structure, does not develop and the number of ventral motor neurons is greatly reduced (Clarke et al., 1991; Ruiz i Altaba, 1992; Ruiz i Altaba and Jessell, 1992).

The appearance of *XASH3* in bilaterally symmetric stripes in our explants indicates that vertical signals are not essential for inducing at least some aspects of mediolateral patterning. In these explants, *XASH3* may be induced exclusively by planar signals derived from adjacent mesoderm; alternatively planar inductive signals may derive from midline sources within the neurectoderm such as the notoplate, which has been suggested to account for axial patterning in *Xenopus* exogastrulae (Ruiz i Altaba, 1992). In a number of explants, *XASH3* was not expressed at equivalent levels on both sides. This lack of bilateral symmetry was particularly apparent in cases where convergence and extension movements were asymmetric (Fig. 7D). A similar asymmetry is observed in N-CAM staining in such explants (J. Shih, unpublished data) suggesting a general failure to achieve symmetric neural induction. This variability may reflect an insufficient concentration and/or transmission of mesoderm-derived signals or an asymmetric orientation of the notoplate in these explants, and is consistent with the idea that in vivo supplementary vertical signals ensure consistent and symmetric induction (Spemann, 1938).

The apparent absence of the transverse 'eyebrow' stripes of *XASH3* expression was surprising, given that other markers of anteroposterior position, such as *Krox-20*, are expressed in explants assayed at later stages (Doniach et al., 1992; Ruiz i Altaba, 1992). While we cannot rule out that the transverse stripes were simply distorted or reoriented longitudinally, such an explanation is not supported by maps of the distortions that occur in anterior regions of Keller sandwiches (Keller et al., 1992). Whether this result indicates an absolute requirement of *XASH3* for vertical inducing signals, or rather a delay in the onset of its expression in explants, remains to be determined. Preliminary data suggest that *Krox-20* induction in explants may in fact be delayed relative to its normal time of onset in vivo (K. Zimmerman and J. Shih, unpublished data). Such a delay could reflect the time required for the transmission of planar inducing signals to the anterior region of the explant. This delay would not occur in vivo because the underlying axial mesoderm would provide a local source of vertical inducing signals.

Previous studies have indicated that specific neuronal cell types characteristic of dorsal and ventral spinal cord can form in the absence of vertical inducing signals (Ruiz i Altaba, 1992). The restricted expression in explants of *XASH3*, a marker of proliferating neuroepithelial precursor cells, suggests that planar or notoplate-derived signals are also sufficient to determine aspects of positional identity, as well as cell type, along the mediolateral axis of the neural plate. The apparent requirement for underlying mesoderm to induce the most medial markers of the neural plate, such as *pintallavis* (Ruiz i Altaba and Jessell, 1992), suggests that different components of the mediolateral pattern may require different amounts and/or types of inducing signals. Furthermore, the mediolateral restriction of *XASH3* expression in explants which presumably lack *Pintallavis* expression (Ruiz i Altaba and Jessell, 1992), indicates that *Pintallavis* itself is not normally responsible for exclusion of *XASH3* expression from the midline region.

Control of *XASH3* expression in embryos and explants

How is the *XASH3* expression domain positioned precisely at the midpoint of the medial and lateral limits of the neural plate? Recent studies in the chick and rat spinal cord have identified diffusible signals deriving from both the medial and lateral margins of the neural plate as important determinants of pattern (Basler et al., 1993; Placzek et al., 1993; Yamada et al., 1993; Yamada et al., 1991). The site of *XASH3* expression could be determined by a particular scalar value of a single gradient (Fig. 8A, right) (Yamada et al., 1991), or by the intersection of two opposing signal gradients (Fig. 8A, left). Such models could also account for the lateral displacement of *XASH3* expression observed in sandwich explants. The tissue excised to generate the explant (see Materials and Methods) excludes the prospective sulcus limitans (Fig. 8E, see legend). Thus this explant should also exclude the prospective domain of *XASH3* expression (Fig. 8F). The recovery of *XASH3* expression suggests that the explant undergoes some degree of proportion regulation along the mediolateral axis. This is consistent with previous studies that have documented proportion regulation in explants (Holtfreter, 1965). As the edge of the explant progressively acquired more lateral character (Fig. 8G), it would be expected to achieve positional values that induce *XASH3* expression before achieving values characteristic of an extreme lateral margin. *XASH3* would therefore first appear at the edge of the explant. Alternatively, the edge of the explant might undergo incomplete lateralization to a value permitting *XASH3* expression, and then stop. Distinguishing these possibilities by examining *XASH3* expression in later stage explants is complicated by the extreme narrowing of the posterior neural plate. Nevertheless, these considerations illustrate how models that invoke gradient signals to explain the positioning of *XASH3* in normal embryos (Fig. 8A) predict that *XASH3* should be detected at the edge of explants that undergo lateralization (Fig. 8F-H), consistent with our experimental observations.

In summary, we have identified a novel vertebrate

homolog of *achaete-scute* that, like the *Drosophila* genes, is specifically expressed in the developing nervous system. The expression pattern of this gene suggests that neural induction at its earliest stages is accompanied by subdivision of the neural plate along its mediolateral axis, in particular by the establishment of a boundary between the prospective alar and basal plates of the spinal cord. Experiments in explants suggest that this aspect of presumptive dorsoventral pattern, like some components of anteroposterior pattern, can be induced in the absence of vertical signals deriving from underlying mesoderm. Such observations suggest that regionalization along the prospective dorsoventral axis of the neural tube occurs at a much earlier stage of development than previously anticipated.

The authors are particularly grateful to Drs Beatriz Ferreiro, William Harris and Chris Kintner for providing cDNA libraries, technical advice and scientific discussion. The authors would also like to thank Drs Akiko Kumagai, Bill Dunphy, Paul Skoglund and Ariel Ruiz i Altaba for sharing their technical expertise at various stages of this project. We especially thank Dr Scott Fraser for support (grant HD 29304) and the use of his laboratory facilities. We also thank Drs David Wilkinson and Chris Kintner for providing the *Xenopus Krox-20* and N-CAM probes respectively, David Turner for sharing his results prior to publication, and Adam Strassberg for help with computer graphics. The authors are also grateful to Drs Ariel Ruiz i Altaba and Tom Jessell for many helpful discussions, and to Bill Harris, Tom Jessell, Barbara Wold and Scott Fraser for their critical comments on the manuscript. K. Z. was supported by an NIH post-doctoral fellowship and is currently an Associate of the Howard Hughes Medical Institute. D. J. A. is an Associate Investigator of the Howard Hughes Medical Institute.

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(Accepted 22 June 1993)