Development 120, 3649-3655 (1994)
Printed in Great Britain © The Company of Biologists Limited 1994

XASH genes promote neurogenesis in *Xenopus* embryos

Beatriz Ferreiro[1,*], Chris Kintner[2], Kathy Zimmerman[3,†], David Anderson[3] and William A. Harris[1,‡]

[1]Department of Biology and Center for Molecular Genetics, University of California San Diego, La Jolla, CA 92093-0357, USA
[2]Molecular Neurobiology Laboratories, The Salk Institute for Biological Science, P.O. Box 85800, La Jolla, CA 92038, USA
[3]Division of Biology, California Institute of Technology, Pasadena CA 91125, USA

*Present address: La Jolla Cancer Research Foundation, La Jolla, CA 92037, USA
†Present address: The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA
‡Author for correspondence

SUMMARY

Neural development in *Drosophila* is promoted by a family of basic helix-loop-helix (bHLH) transcription factors encoded within the *Achaete Scute-Complex (AS-C)*. *XASH-3*, a *Xenopus* homolog of the *Drosophila AS-C* genes, is expressed during neural induction within a portion of the dorsal ectoderm that gives rise to the neural plate and tube. Here, we show that *XASH-3*, when expressed with the promiscuous binding partner *XE12*, specifically activates the expression of neural genes in naive ectoderm, suggesting that *XASH-3* promotes neural development. Moreover, *XASH-3/XE12* RNA injections into embryos lead to hypertrophy of the neural tube. Interestingly, *XASH-3* misexpression does not lead to the formation of ectopic neural tissue in ventral regions, suggesting that the domain of *XASH* proneural function is restricted in the embryo. In contrast to the neural inducer *noggin*, which permanently activates the NCAM gene, the activation of neural genes by *XASH-3/XE12* is not stable in naive ectoderm, yet *XASH-3/XE12* powerfully and stably activates *NCAM*, *Neurofilament* and type III *β-tubulin* gene expression in *noggin*-treated ectoderm. These results show that the *XASH-3* promotes neural development, and suggest that its activity depends on additional factors which are induced in ectoderm by factors such as *noggin*.

Key words: *XASH*, basic helix-loop-helix transcription factor, neurogenesis, *Xenopus*

INTRODUCTION

The *AS-C* genes in flies comprise a set of four genes that are ascribed proneural functions, in that their activation augments neural deteremination (Alonso and Cabrera, 1988; Ghysen and Dambly, 1988; Campuzano and Modollel, 1992). They code for transcription factors of the basic-helix-loop-helix family (bHLH; Murre et al., 1989a), and are believed to promote neural differentiation in a manner similar to the proposed role of the myogenic bHLH proteins, MyoD, myf-5 and myogenin, in muscle development (Weintraub et al., 1991a). Flies that lack all of the *AS-C* genes have severe neural hypoplasia both in the central and peripheral nervous system, while ectopic expression of the *AS-C* genes can lead to additional neural differentiation (reviewed by Campos-Ortega and Jan, 1991; Campos-Ortega, 1993).

The *Xenopus* genes, *XASH-1* (Ferreiro et al., 1993) and *XASH-3* (Zimmerman et al., 1993) were cloned based on their sequence homology with the *Drosophila AS-C* genes. *MASH-1*, a mammalian *AS-C* homolog (Johnson et al., 1990), is required for the development of the peripheral autonomic and olfactory nervous system in mice (Guillemot et al., 1993). *XASH-1*, named because of its homology to *MASH-1*, is first expressed zygotically in the anterior neuropithelium of the embryo during folding of the neural tube in a pattern similar to but not identical with *MASH-1* (Ferreiro et al., 1993). *XASH-3*, however, can be detected much earlier, at stage 11.5, in an area fated to become part of the neural plate, including presumptive brain and spinal areas (Zimmerman et al., 1993). This makes *XASH-3* one of the earliest expressed neural-specific transcription factors in the *Xenopus* embryo. Since induction of the neural plate by the organizer begins at the onset of gastrulation (Kintner, 1992; Slack and Tannahill, 1992) just an hour and a half earlier, *XASH-3* could act early during neural induction to promote neural development.

To determine whether *XASH-3* promotes neural development in *Xenopus* embryos, we investigated whether *XASH-3* can activate neural genes in ectoderm by injecting *Xenopus* embryos with *XASH-3* RNA, along with RNA encoding the promiscuous bHLH heterodimer partner *XE12*. Ectodermal caps isolated from these embryos were found to express neural genes but not a mesoderm-specific gene. This activation of neural genes, however, appeared to be transient, and was not maintained in culture. The proneural activity of the *XASH* genes was reinforced by the finding that the expression of either *XASH-1/XE12* or *XASH-3/XE12* in embryos results in a stable enlargement of the neural tube. Ectopic *XASH* expression, however, did not cause the formation of neural tissue in ventral regions of the embryo. These results suggested that *XASH*-mediated activation of neural genes normally works in the context of a neural inducing signal and cannot substitute for this signal. In agreement with this idea, we found that

XASH-3/XE12 was a much more efficient activator of neuronal differentiation in *noggin* induced ectoderm. These results support the notion that the *XASH* genes are proneural in *Xenopus*, and that *XASH-3* proneural activity in the early neuroectoderm is enhanced by neural induction.

MATERIAL AND METHODS

Whole embryo analysis

cDNAs for *XASH-1* and *XASH-3* were subcloned into sp64T and transcribed into capped mRNA in vitro using SP6 polymerase (Kreig and Melton, 1987). One blastomere of 2-cell or 4-cell stage embryos were injected with 5 nl of mRNA solution. All injection solutions contained 80 pg β-gal mRNA (for doses of other RNAs see text and figure legends). In those cases where *XASH* RNA and *XE12* RNA (Rashbass et al., 1992) were injected together, they were mixed and added to the β-gal mRNA. Embryos were fixed in 4% paraformaldehyde, 15-36 hours after the injections, all were stained for β-gal activity with X-gal, and some were stained for *NCAM* or *Xtwist* message using a whole-mount protocol (Harland, 1991). The whole-mount embryos were cleared in benzyl-benzoate (Harland, 1991) causing the β-gal staining to fade eventually. Some of the embryos were embedded in paraffin for sectioning.

Animal cap studies

Both blastomeres of 2-cell stage embryos were injected with 5 nl of mRNA solution including the types and amounts described below. *Noggin* mRNA (Lamb et al., 1993) was generated from a PCR product corresponding to the *noggin* cDNA that was subcloned into sp64T. Six animal caps per condition were dissected out and cultured for 24 hours (or until undissected siblings reached N/F stage 24/25) on 1% agarose dishes in 0.5× MMR plus antibiotics. RNA was isolated from the cultured caps or from 3 control embryos and divided into a 90% portion, used for the RNase protection assay of the expression of *NF*, type III *β-tubulin*, *NCAM* and *EF-1A*, and a 10% portion, used for the RNase protection assay of the expression of muscle-specific actin. Probes are described in detail by Dixon and Kintner (1989) and Lamb et al. (1993). Approximately equivalent amounts of animal cap RNA were assayed in each case as monitored by the levels of *EF1α* transcripts or the cross-hybridization with cytoskeletal actin.

RESULTS

The effect of *XASH-3* on the expression of neural-specific genes in uninduced animal caps

Animal cap tissue isolated from the late blastula normally differentiates into epidermis but can be induced to become mesodermal or neural tissue if combined with a source of appropriate inducing signals (Kintner and Melton, 1987; Sharpe et al., 1987; Dixon and Kintner, 1989; Green and Smith, 1991). In order to determine whether *XASH-3* promotes neural development in ectoderm in the absence of neural inducing signals, animal caps were isolated from embryos injected at the one or two cell stage with *XASH-3*, cultured overnight, and then assayed for the expression of several neural-specific genes by an RNAase protection assay. Animal caps from embryos injected with just *XASH-3* alone (Fig. 1, lane d) expressed only very small amounts of the neural-specific transcript *NCAM*. Since *XASH-3*, like other bHLH transcription factors, is likely to act as a heterodimer with the promiscuous bHLH partner *E12*, the vertebrate homolog of *da* (which may be in limiting quantity in the embryo; Lassar et al., 1989; Murre et al., 1989b; Rashbass et al., 1992), *XASH-3* RNA was injected along with low amounts of *XE12* (Rashbass et al., 1992). Caps isolated from these embryos expressed *NCAM* transcripts as well as neuronal-specific transcripts for *Neurofilament (NF)* similar to those present in stage-matched embryos, even though the caps were cultured without any neural inducers (Fig. 1, *top* lanes e and i). Since ectoderm from embryos injected with just *XE12* or *XASH-3* alone failed to express significant levels of *NCAM* RNA (Fig. 1, *top* lanes c and d), *XASH-3* and *XE12* appear to act synergistically to induce neural gene expression.

Ectoderm from *XASH-3/XE12*-injected embryos was also examined the expression of the dorsal mesodermal marker,

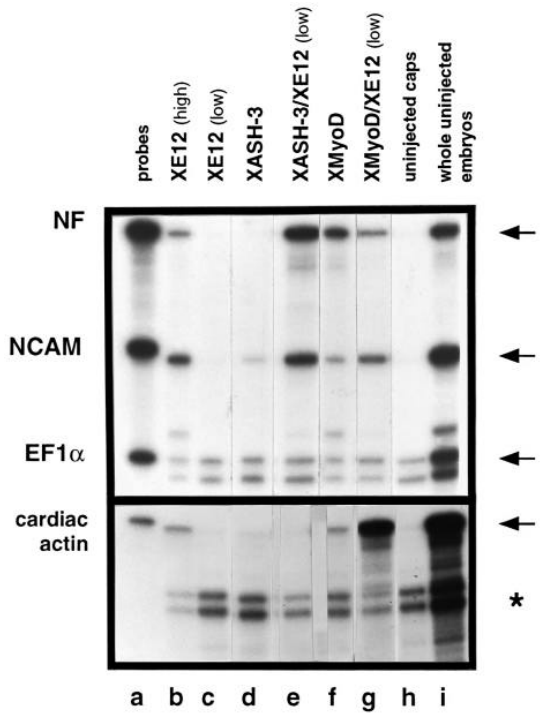

Fig. 1. The specificity of expression of neural and muscle transcripts in animal caps from embryos injected with different bHLH encoding mRNAs. Top: 125 pg (high) but not 25 pg (low) of *XE12* mRNA induces NCAM and neurofilament expression (lanes b and c). 833 pg of *XASH-3* mRNA alone induces only slight *NCAM* expression, but when it is combined with 25 pg of *XE12* mRNA, there is a dramatic increase in *NCAM* and *NF* expression (lanes d and e). Note that the level of neural transcripts in the *XASH-3*/*XE12*-injected condition are close to the levels in whole uninjected embryos (lane i). 833 pg of *XMyoD* mRNA induces both NF and NCAM expression, but when this is combined with 25 pg of *XE12*, the levels of NCAM and NF expression are not increased (lanes f and g). Bottom: 125 pg of *XE12* mRNA induces some expression of muscle-specific actin (lane b), but neither *XE12* at 25 pg or*XASH-3* at 833 pg alone, or in combination, induce muscle actin expression (lanes c-e). However, *XMyoD* at 833 pg does activate muscle actin expression and this is dramatically enhanced by combination with 25 pg of *XE12* mRNA (lanes f and g). These results were replicated twice in independent experiments. The asterisk shows cytoskeletal actin mRNA, which is recognized by the cardiac actin probe and acts as a loading control. These lanes were cut and rearranged from a single exposure of one experiment.

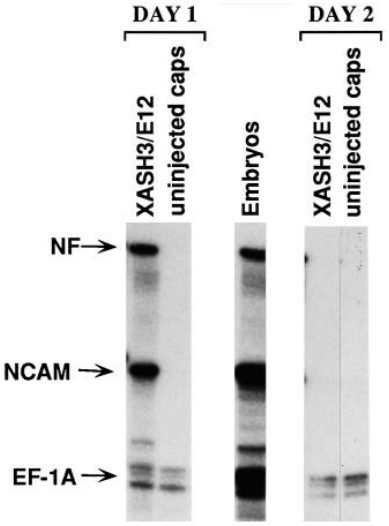

Fig. 2. *XASH3/XE12* transiently activates neural genes. Neurofilament and *NCAM* are induced strongly in naive ectoderm by the injection of *XASH-3/XE12* when the caps are assayed 1 day after the injection (lane a). If the caps are cultured for a second or third day, the level of these neural genes is dramatically reduced (lane d). Control caps (lanes b and e) show no neural gene expression on day 1 or 2. This experiment was repeated at least three times, and although there was some variation in the extent of the decrease in *NCAM* and *NF* expression *XASH-3/XE12* induced caps, the decrease was always dramatic by 3 days.

Cardiac Actin or *Muscle Specific Actin* (*MSA*; Hopwood and Gurdon, 1990). In contrast to the clear induction of neural genes, there was no induced expression of *MSA* (Fig. 1, lane e), suggesting that the effect of *XASH-3* on neural development is direct in the sense that the neural induction it is not mediated through the primary induction of dorsal mesoderm in these caps.

Specificity of *MyoD/XE12* for myogenesis and *XASH-3/XE12* for neurogenesis

In order to assess further the specificity of *XASH-3*, we also examined the effects of *XMyoD* (Hopwood et al., 1989), a bHLH transcription factor that was previously shown to induce the expression of muscle-specific genes (Weintraub et al., 1991b), and *MSA* expression in animal cap assays (Hopwood and Gurdon, 1990). Surprisingly, injection of *XMyoD* RNA alone induced low levels of *NCAM* and *NF* as well as *MSA* RNA expression (Fig. 1, lane f). Reducing the amounts *XMyoD* RNA injected into embryos, simultaneously reduced the levels of *MSA* and of *NF* and *NCAM* RNA. Thus, we could not find a dose of *XMyoD* RNA that induced *MSA* but not neural gene expression. Previous work with *XMyoD* had shown that coexpression with *XE12* results in formation of heterodimers, and an enhancement in *XMyoD*'s ability to activate muscle-specific genes (Rashbass et al., 1992). Thus, we injected embryos with *XMyoD* plus *XE12*, at a concentration of *XE12* that alone did not induce any of the marker genes. We saw a specific enhancement of *MSA* expression, and no enhancement of either *NCAM* or *NF* expression (Fig. 3, lane g). This result is similar to the above result with *XASH-3*, which when combined with *XE12*, also greatly enhanced the expression of the the neural-specific markers, but still did not cause any induction of the muscle-specific marker (Fig. 3, lanes d and e). This indicates that, in the presence of *XE12*, *XASH-3* is a specific activator of neural gene expression while *XMyoD* is primarily an activator of muscle gene expression.

It is not clear why *XMyoD* RNA injected alone activates low levels of *NCAM* and *NF* RNA expression. We noted, however, that animal caps isolated from embryos injected with five times the dose of *XE12* RNA (125 pg instead of 25 pg) also expressed RNAs for neural-specific genes as well as *MSA* (Fig. 3, lane b). Misexpression of bHLH proteins may have indirect effects because of their propensity to heterodimerize indiscriminately, and to dilute out potential inhibitors such as *id* (Benezra et al., 1990) or activators such as *E12*. (Parkhurst et al., 1990; Jarman et al., 1993). Since *XASH-3* binds the mammalian muscle creatine kinase (MCK) E-box in vitro and transactivates the MCK/CAT reporter gene in transfected 10T1/2 cells (Zimmerman et al., 1993), it is perhaps surprising that *XASH-3* activates neural gene expression but not muscle gene expression in vivo. This suggests that *XASH-3* is *not* a promiscuous in vivo, and that it may show this particular specificity in the context of unidentified factors that exist in cells of the developing embryo.

Activation of neural gene expression in animal caps by *XASH-3* is unstable

Activation of neural gene expression by *XASH-3* suggests that *XASH-3* promotes neural differentiation. Similar studies using injection of *XmyoD* RNA, however, have shown that *XMyoD* can activate muscle gene expression in animal cap assays without causing the differentiation of ectoderm cells into muscle (Hopwood and Gurdon, 1990). In the case of *XMyoD*, muscle gene expression can be detected in ectoderm after 1 day in culture, but the expression is then lost, presumably following the loss of the misexpressed *XMyoD* transcripts and protein. Similarly, ectoderm from embryos injected with *XASH-3/XE12*, after 1 day in culture, expressed large amounts of *NCAM* and *NF* RNA, but after 2-3 days in culture, the levels of these RNAs dropped, and in some experiments disappeared altogether (Fig. 2). These results indicate that while *XASH-3* can activate neural gene expression in naive ectoderm, *XASH-3* is probably *not* acting as a switch, causing cells that express it transiently to become neural. In this sense, the injection of *XASH-3/XE12* RNA does not mimic the normal events of neural induction. This result also agrees with our whole embryo misexpression studies (see below), and indicates that *XASH-3* probably does not activate neural genes by inducing a neural inducer, as all neural inducers so far studied stably activate neural development.

The effect of *XASH-3* in animals caps is very sensitive to concentration. Relatively large amounts of *XASH-3/XE12* RNA are needed to turn on neural gene expression, yet when the *XASH-3* RNA has been diluted out even 2- to 3-fold, there is little or no activation (data not shown). The sharp dose response curve is consistent with the idea that *XASH-3* acts by activating its own expression as has been proposed for the myogenic bHLH transcription factors (Weintraub et al., 1991a). *XASH-3* injection, however, does not appear to turn on *XASH-1* expression in isolated ectoderm (data not shown) suggesting that cross-regulation between the *XASH* genes may not be a factor in this dosage sensitivity.

XASH genes in embryos lead to neural hypertrophy in dorsal regions of the embryo

To explore the role of *XASH* genes in neural development in vivo, we examined the phenotypes of embryos that had been exposed to ectopic *XASH-1* and *XASH-3* by RNA injection. When embryos were injected with the same levels of *XASH-*

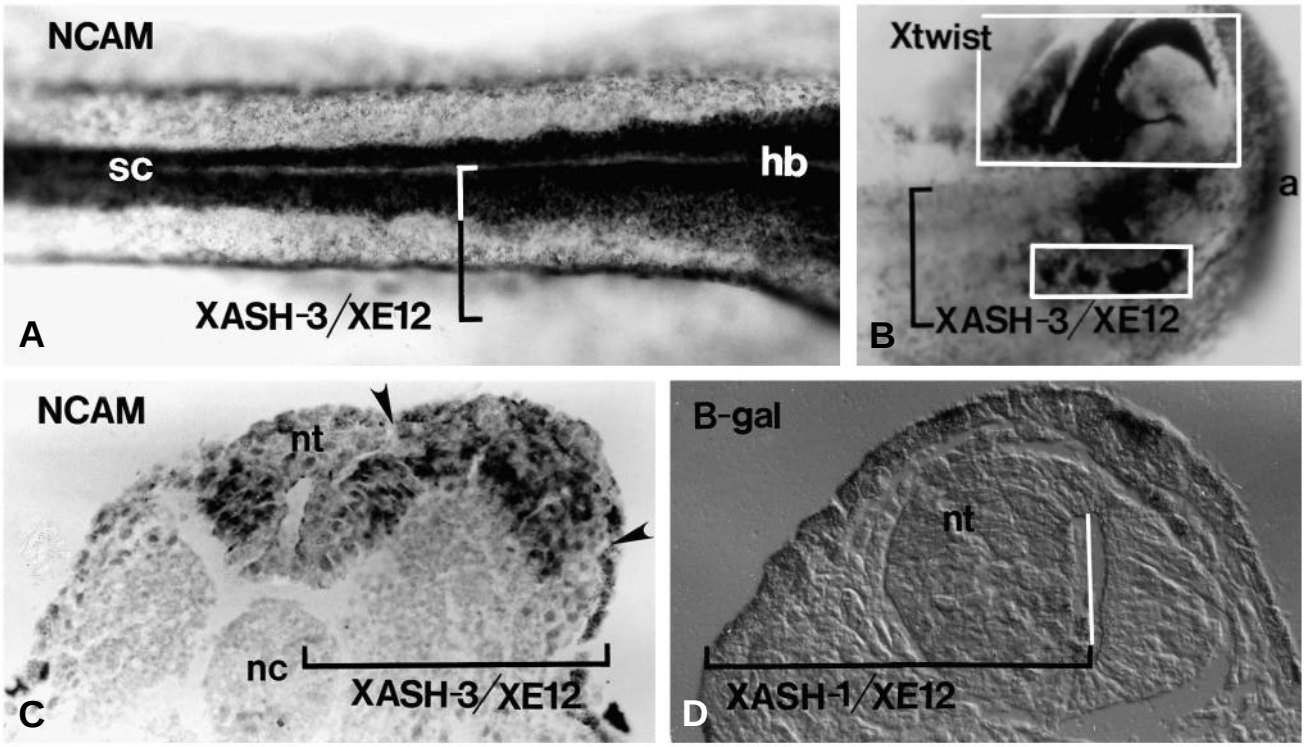

Fig. 3. Phenotypes of *XASH/XE12*-injected embryos. (A). Direct dorsal view of a whole-mount embryo injected with 90 pg of*XASH-3* mixed with *XE12* in the right side (anterior to right), and stained for the expression of *NCAM* mRNA by in situ hybridization (Harland, 1991). The neural tissue on the injected side (brackets) is obviously much wider along its entire length including hindbrain (hb) and spinal cord (sc) than that on the uninjected side. (B) Direct dorsal view of a late neural plate embryo shows a striking reduction in ectodermal *Xtwist* expression (in white boxes) on the *XASH-3/XE12*-injected right side (brackets). Anterior (a) is to the right. (C) A section through the spinal region of an embryo like that in A. In this section, also labeled for *NCAM* expression, we note the lateral expansion (between arrowheads) of the neural tissue on the injected right side (brackets). The normally sized notochord (nc), marking the midline, is visible directly below the neural tube (nt). (D) Expansion of the neural tube (nt) in a *XASH-1/XE12*-injected embryo. Here the tube was photographed using Nomarski optics. The white vertical bar marks the midline of the neural tube.

3/XE12 RNA that led to the activation of neural genes in the animal cap assay, they failed to gastrulate normally and thus could not be analyzed. Therefore, we examined embryos that had been injected with a ten-fold dilution of *XASH-3* RNA. At this dose, *XASH-3/XE12* did not have any effect on neural gene expression in the animal cap assay (see below). However, when these embryos were allowed to develop until neural plate or neural tube stages, they showed extra neural tissue when they were examined both histologically and for the expression of the neural-specific gene *NCAM*. (Fig. 3A,C). Histological analysis reveals that the neural plate of younger embryos and the neural tube of older ones expands dramatically on the injected side, spreading out laterally (Figs 3C, 4). No expansion of the notochord was observed (Fig. 3C), nor was there enhanced expression of the notochord-specific gene *Xlim-1* (Taira et al., 1992) (data not shown). Similar, though less dramatic, results were obtained with *XASH-1/XE12* injections (Figs 3D, 4). This, combined with the evidence from animal caps above, confirms that the expansion of the neural tube is not a secondary consequence of the *XASH* genes leading to extra inducing tissue on the injected side. In addition, the expansion of the neural tube is not simply the result of injecting the *XE12* molecule. We also looked at embryos in which *XE12* was injected alone at twice the dose of the *XASH-3/XE12* experiments and in no case did we see an expansion of the neural tube (Fig. 4). Thus, *XASH* RNA can perturb the amount of neural tissue that forms in embryos at doses where it has no effects on isolated animal cap tissue.

Importantly, the enlargement of neural tissue following the injection of low amounts of *XASH-3/E12* RNA was autonomous and restricted. Neural hypertrophy was only in the side of the embryo where the ectopic *XASH-3/XE12* was expressed, and only in the region of the neural plate/tube. If, for instance, *XASH-3/XE12* was expressed in the posterior part of the embryo, then the neural plate would expand only in the region of ectopic expression. When *XASH-1/XE12* or *XASH-3/XE12* was misexpressed in ventral regions of the embryo, this did not lead to an extra neural tissue, suggesting that the proneural function of *XASH* is restricted to the dorsal ectoderm, near its normal zone of expression.

Since the lateral expansion of the neural tube could be at the expense of tissue around the neural plate, we looked at the ectodermal expression of *Xtwist*, a cephalic neural crest marker, expressed at the lateral borders of the anterior neural plate (Hopwood et al., 1989). Consistent with this idea, embryos misexpressing *XASH-3/XE12* showed reduced *Xtwist* expression on the injected side (Figs 3B, 4). Similar results on the expansion of the neural tube at the expense of lateral ectoderm in *XASH-3* injected embryos have been found by Turner and Weintraub (1994).

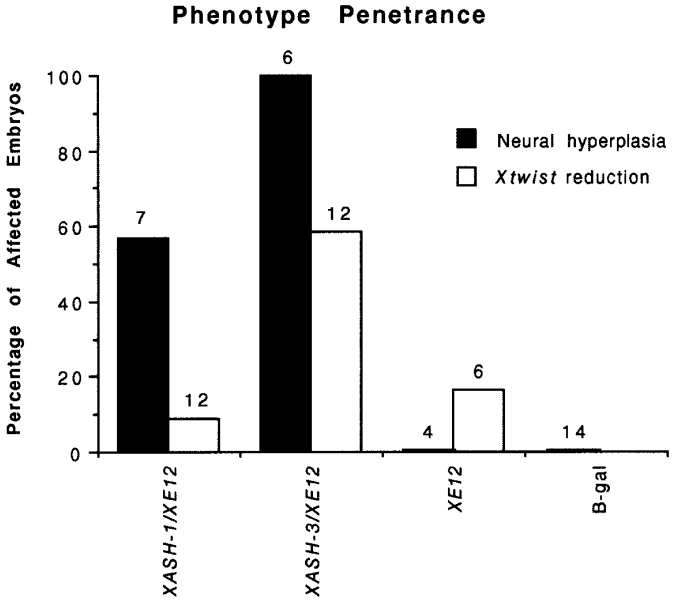

Fig. 4. Phenotypic penetrance of injected embryos. Percentages of embryos injected unilaterally that express the neural hypertrophy and the *Xtwist* reduction phenotypes in one experiment. *XASH-3*/*XE12* strongly produced both phenotypes in a high fraction of the embryos studied. *XASH-1*/*XE12* injections were less effective. Small numbers on top of the bars indicate numbers of embryos studied in each condition. For these experiments, embryos at the two- or four-cell stage were injected with the mRNAs listed, in combination with β-gal mRNA. They were fixed in paraformaldehyde when they reached neural plate through neural tube stages, stained with X-gal, and then paraffin sectioned. If the embryo showed clear unilateral blue staining in the region of the neural plate or tube, it was then determined whether there was an obvious expansion of this tissue on the injected side. For the *Xtwist* phenotype, similarly injected embryos were simultaneously stained for β-gal activity and *Xtwist* expression by in situ hybridization (Harland, 1991). A reduction of *Xtwist* staining on the β-gal expression side was counted as a positive. The experiment looking at enlargement of the neural tube with *XASH-3/E12* was carried out at least four times with five or more embryos, and in each case all the misexpressing embryos showed neural hypertrophies.

XASH-3 promotes neuronal differentiation in *noggin*-treated animal caps

The result described above indicates that *XASH-3* can promote neural development but only in regions of the embryo where neural induction normally takes place. To test this further, we examined the effects of *XASH-3* expression in ectoderm that had been induced to form neural tissue by the neural inducer *noggin* (Lamb et al., 1993). *Noggin*-treated ectoderm expresses large amounts of *NCAM* RNA as well as the anterior CNS homeodomain transcript *OtxA* (Lamb et al., 1993). In contrast to *XASH-3*-expressing ectoderm, however, *noggin*-induced ectoderm does not appear to undergo neuronal differentiation as marked by the expression of neuronal genes such as type III neural-specific tubulin (Lamb et al., 1993). Thus, we injected *XASH-3/E12* RNA along with *noggin* RNA into animal caps and assayed for type III tubulin expression. The results of this analysis shows that *noggin*-injected ectoderm does not express type III tubulin (Fig. 5 lane 2), as shown previously (Lamb et al., 1993). However, when these caps are co-injected with *XASH-3/E12*, they express type III tubulin at levels near those of control embryos (Fig. 5 lanes 4 and 5). Importantly, the levels of *XASH-3* required for the induction of neural tubulin in *noggin* induced ectoderm are at least 10-fold less than the levels required to induce tubulin in naive ectoderm, and similar to the levels required to cause neural expansion in whole embryos. When this amount of *XASH-3/XE12* RNA is injected without *noggin*, there is no induction of either *NCAM* or tubulin (Fig. 5 lane 3). In addition, the effects of *XASH-3/XE12* expression on tubulin expression in *noggin* induced caps appears to be stable for up to 2 days in culture (data not shown). Thus, these results support the idea that *XASH-3* is more effective in promoting neural development in the context of *noggin*-induced ectoderm than it is in uninduced ectoderm.

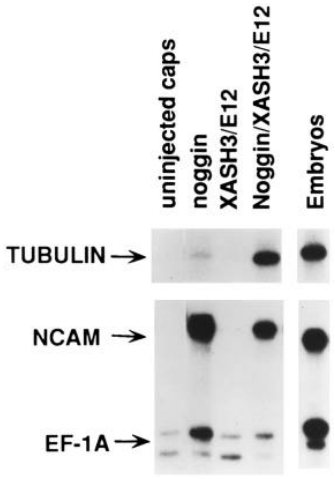

Fig. 5. *XASH-3/XE12* efficiently induces neural genes in *noggin* caps. Animal caps from embryos injected with *noggin* RNA are induced to express *NCAM*, but not the more differentiated neural marker type III *β-tubulin* (lane b). Low doses (90 pg) of *XASH-3* mixed with *XE12* induce neither (lane c). When *noggin* RNA is combined with the low dose of *XASH-3/XE12*, the result is the induction of *NCAM* and *β-tubulin* that mimic the levels in normal embryos (lanes d and e).

DISCUSSION

The principle finding of this study is that *XASH-3* acts as a proneural gene. In embryos, *XASH-3/XE12* results in an increase in the size of the neural tube, perhaps by increasing the lateral extent of the neural plate. In the animal cap assay, *XASH-3* when combined with *XE12* induces neural but not muscle gene expression. Thus, these assays provide strong support for the conclusion that *XASH-3* is a proneural gene. Our results with misexpression corroborate the proneural role of the vertebrate *AS-C* homologs that has been suggested by the knock out of *MASH-1* in the mouse (Guillemot et al., 1993).

In overexpression studies with bHLH proteins, the question of specificity must arise. Because bHLH genes heterodimerize, they may have non-specific effects when overexpressed. The classic example of this is the effect of *hairy* on sex determination in flies (Parkhurst et al., 1990). Thus, in the context of the whole organism, where a myriad of bHLH proteins may be operating in a dosage sensitive way, the overexpression of a single member of this class can create a phenotype which may be less informative about the overexpressed gene than it is about its partner. We found that either *XmyoD* and *XE12*, when expressed alone in animal caps at high enough levels, lead to the expression of both neural and muscle-specific markers. Why either turns on neural genes in the animal cap assay is unclear. Perhaps they have less target specificity as homodimers, perhaps they are diluted out or compete with an endoge-

nous inhibitory protein (Benezra et al., 1990; Ellis et al., 1990; Garrell and Modolell, 1990; Ruezinsky et al., 1991), or perhaps they form activating heterodimers with the low levels of maternal *XASH-1* (data not shown). The problem of specificity is overcome to some extent in the animal cap studies where a direct comparison of several different bHLH members can be made on exactly the same tissue. Thus, when *XASH-3* or *XMyoD* were combined with low doses *XE12* (at doses where *XE12* did not by itself activate neural or muscle genes), we found that *XASH-3/XE12* heterodimers strongly induce neural genes without inducing muscle genes, while *XMyoD/XE12* heterodimers preferentially activate myogenic genes over neural genes. The heterodimers thus show exquisite tissue specificity that presumably reflects their normal site of action.

Another major finding to emerge from these studies is that the ability of *XASH-3* to act in naive ectoderm appears to be compromised relative to its ability to act in the embryo or in *noggin*-induced ectoderm. Thus, while *XASH-3* will turn on neural genes in naive ectoderm, this occurs only when relatively large amounts of *XASH-3* RNA are injected and in most experiments, the effects on neural gene expression is only transient. In contrast, 10-fold less *XASH-3* RNA is required to expand the neural tube in embryos or to activate neuronal gene expression in *noggin*-induced ectoderm, and in both cases, the effects appear to be irreversible. These observations strongly suggest that the normal expression of *XASH-3* is not sufficient to allow ectoderm to take on the neural fate. Neural induction, therefore, cannot be viewed simply as the activation of *XASH-3* expression.

One of the important unresolved questions raised by these studies, concerns where *XASH-3* normally acts during the early stages of neural development. In this respect, it is interesting to compare the effects obtained by the expression of *XASH-3* in ectoderm with those obtained by treating ectoderm with the neural inducer, *noggin*. While ectoderm treated with *noggin* appears to differentiate into neural tissue as measured by the expression of large amounts *NCAM* RNA, it does not appear to undergo neuronal differentiation as measured by the expression of genes such as *NF* or tubulin (Lamb et al., 1993). In contrast, ectoderm injected with large amounts of *XASH-3/XE12* RNA goes on to express large amounts of both *NF* and tubulin RNA relative to the amounts of the neural marker *NCAM*. Moreover, tubulin expression can be activated in *noggin*-treated caps even by relatively low levels of *XASH-3/XE12* RNA. Thus, one model based on these observations is that *XASH-3* acts downstream of neural induction to promote neuronal differentiation. In this model, the primary role of the proneural *XASH* genes is not to establish the neuroepithelium of the neural plate and tube, but rather to act within this region of the embryo to promote the neuronal fate.

In *Drosophila*, *Notch* mutant embryos make neural tissue at the expense of ventral epidermis, suggesting that the cells of the ventral neurogenic region may flip between these two states (Artavanis-Tsakonas and Simpson, 1991). Yet in mutants for *AS-C*, although there is a severe neural deficiency, there is no noticeable extra epidermal tissue (Wieschaus et al., 1984; Jimenez and Campos-Ortega, 1990). In *AS-C/Notch* double mutants, the neural hypertrophic phenotype is repressed, yet these embryos still cannot make ventral epidermis (Martinez Arias, 1993). These results suggest that *AS-C* function is not a simply a switch between the epidermal and neural state, and show that the lack of *AS-C* function does not lead cells to take on an epidermal fate. While we have no data on the loss of *XASH* function, we note that loss of *MASH-1* in the mouse leads to a loss of the olfactory neurons, but not to the neuroepithelium from which these neurons arise. From these data, we suggest that the loss of *XASH-3* may not cause cells in the neural plate to take on an epidermal fate, but rather to differentiate into an alternative fate within the neural tube such as, for example, into ependymal cells.

Our studies are directly comparable to overexpression studies in flies, using a heat shock promoter to induce the temporary expression of *scute* throughout the developing fly deficient for the endogenous *achaete* and *scute* gene function (Rodriguez et al., 1990). One finding from such studies concerns the redundancy of the *AS-C* genes, such that heat shock expression of one of them can substitute for the expression of another. Thus, sensory bristles that are lost in *achaete* mutants are regained in these flies where *scute* or *asense* are ubiquitously expressed through heat shock (Rodriguez et al., 1990; Dominguez and Campuzano, 1993). Like the *AS-C* genes, the two *XASH* genes are expressed in distinct but overlapping spatiotemporal domains, yet either one has the potential, when overexpressed, to cause neural hypertrophy, suggesting that they also may have functional redundancy.

A critical comparison between our results and and those in the fly concerns the finding that the *AS-C* genes can only function in restricted regions of neurogenic potential, so that even when there is ubiquitous heat-shock induced expression of one of these genes, sensory organs still tend to appear only at their appropriate sites on the fly (Rodriguez et al., 1990; Dominguez and Campuzano, 1993). The spacing between sensory bristles is regulated in part by the inhibitor of *AS-C* function, *emc* (Ellis et al., 1990; Garrell and Modolell, 1990). As a HLH protein lacking a basic domain, *emc* has an anti-neural function: it inactivates *AS-C* proteins by forming 'dead' heterodimers with them that are incapable of binding DNA (Ellis et al., 1990; Garrell and Modolell, 1990). In *emc* mutants, heat shock induced *AS-C* activity leads to ectopic bristles, suggesting that the spatial restriction of *AS-C* proneural function in these flies is in part controlled by the endogenous pattern of *emc* expression (Cubas and Modolell, 1992). We have previously shown that *emc* similarly inhibits the *XASH-1* DNA binding in vitro (Ferreiro et al., 1993). Similarly, in the blastoderm there appears to negative regulation of the *AS-C* genes by *twist* and *snail* in ventral region during establishment of the mesoderm (Kosman et al., 1991), and in dorsal regions through the action of *dpp* during the establishment of the dorsal epidermis (Ferguson and Anderson, 1992). Thus, the dorsal restriction we see in the proneural function of overexpressed *XASH*, in *Xenopus* embryos could in part be due to the expression of inhibitory factors in ventral regions of the embryo. Neural induction may therefore not only turn on *XASH-3* expression, but also eliminate negative regulators thereby providing a domain in which the proneural properties of *XASH-3* are effective. We cannot, of course, rule out the possibility that there may be spatially restricted proneural factors co-expressed with *XASH-3* in the neural plate and tube that help to turn on neural genes.

In sum, these studies suggest that *XASH-3* plays a role in amphibian embryos in promoting neural development during the progression of ectoderm into neural tissue. It will be of

interest to determine what factors are normally responsible for activating *XASH* gene expression within the neural plate, and whether other genes, such as the vertebrate homologs for the *Drosophila* neurogenic genes or the antineural genes, regulate the activity of the *XASH* genes in the neural plate and tube.

We thank Drs John Gurdon and David Turner for the gift of *XMyoD*, *XE12* and *Xtwist* cDNAs respectively. We thank Drs E. Bier, M. Levine, C. Murre, J. Posakony, D. Tanahill and S. Bray for their critical reading of the manuscript and R. Hartong for his input into this work. The technical contribution of J. Voss is gratefully acknowledged. Financial support was provided by the NIH, the HHMI, and McKnight Foundation.

REFERENCES

Alonso, M. C. and Cabrera, C. V. (1988). The *achaete-scute* gene complex of Drosophila melanogaster comprises four homologous genes. *EMBO J.* **7,** 2585-91

Artavanis-Tsakonas, S. and Simpson, P. (1991). Choosing a cell fate: a view from the *Notch* locus. *Trends Genet.* **7,** 403-8.

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

Campos-Ortega, J. A. (1993). Early neurogenesis in *Drosophila melanogaster*. In: *The Development of Drosophila melanogaster*. (ed. Bate, M. and Martinez Arias, A.), pp. 1091-1130.Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Campos-Ortega, J. and Jan, Y. (1991). Genetic and molecular basis of neurogenesis in *Drosophila melanogaster*. *Ann. Rev. Neurosci.* **14,** 399-420.

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

Cubas, P. and Modolell, J. (1992). The *extramacrochaetae* gene provides information for sensory organ patterning. *EMBO J.* **11,** 3385-3393.

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

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

Ellis, H. M., Spann, D. R. and Posakony, J. W. (1990). *extramacrochaetae*, a negative regulator of sensory organ development in *Drosophila*, defines a new class of helix-loop-helix proteins. *Cell* **61,** 27-38.

Ferguson, E. L. and Anderson, K. V. (1992). *decapentaplegic* acts as a morphogen to organize dorsal-ventral pattern in the *Drosophila* embryo. *Cell* **71,** 451-461.

Ferreiro, B., Skoglund, P., Bailey, A., Dorsky, R. and Harris, W. A. (1993). *XASH-1*, a Xenopus homolog of *achaete-scute*: a proneural gene in anterior regions of the vertebrate CNS. *Mech. Dev.* **40,** 25-36.

Garrell, J. and Modolell, J. (1990). The *Drosophila extramacrochaetae* locus, an antagonist of proneural genes that, like these genes, encodes a helix-loop-helix protein. *Cell* **61,** 39-48.

Ghysen, A. and Dambly, C. C. (1988). From DNA to form: the *achaete-scute* complex. *Genes Dev.* **2,** 495-501.

Green, J. B. and Smith, J. C. (1991). Growth factors as morphogens: do gradients and thresholds establish body plan? *Trends Genet.* **7,** 245-250.

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

Harland, R. M. (1991). In situ hybridization: an improved whole-mount method for *Xenopus* embryos. *Methods Cell Biol.* **36,** 685-95.

Hopwood, N. D. and Gurdon, J. B. (1990). Activation of muscle genes without myogenesis by ectopic expression of *MyoD* in frog embryo cells. *Nature* **347,** 197-200.

Hopwood, N. D., Pluck, A. and Gurdon, J. B. (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.

Jarman, A., Grau, Y., Jan, L. and Jan, Y. (1993). *atonal* is a proneural gene that directs chordotonal organ formation in the *Drosophila* peripheral nervous system. *Cell* **73,** 1307-1321.

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

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

Kintner, C. (1992). Molecular bases of early neural development in *Xenopus* embryos. *Ann. Rev. Neurosci.* **15,** 251-284.

Kintner, C. R. and Melton, D. M. (1987). Expression of *Xenopus N-CAM* RNA is an early response of ectoderm to induction. *Development* **99,** 311-325.

Kosman, D., Ip, Y. T., Levine, M. and Arora, K. (1991). Establishment of the mesoderm-neuroectoderm boundary in the *Drosophila* embryo. *Science* **254,** 118-122.

Krieg, P. A. and Melton, D. A. (1987). *In vitro* RNA synthesis with SP6 RNA polymerase. In *Methods in Enzymology*. (ed. Abelson, J. N. and Simon, M. I.), pp. 397-415. New York: Academic Press.

Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Economides, A. N., Stahl, N., Yancopolous, G. D. and Harland, R. M. (1993). Neural induction by the secreted polypeptide *noggin*. *Science* **262,** 713-718.

Lassar, A. B., Buskin, J. N., Lockshon, D., Davis, R. L., Apone, S., Hauschka, S. D. and Weintraub, H. (1989). *MyoD* is a sequence-specific DNA binding protein requiring a region of *myc* homology to bind to the muscle creatine kinase enhancer. *Cell* **58,** 823-31.

Martinez Arias, A. (1993). Development and patterning of the larval epidermis of *Drosophila*. In *The Development of Drosophila*. (ed. Bate, M. and Martinez Arias, A.), pp. 517-608.Cold Spring Harbor, New York: Cold Spring Harbor Laborotory Press.

Murre, C., McCaw, P. S. and Baltimore, D. (1989a). A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, *MyoD*, and *myc* proteins. *Cell* **56,** 777-783.

Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D. and Lassar, A. B. (1989b). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **58,** 537-44.

Parkhurst, S. M., Bopp, D. and IshHorowicz, D. (1990). X:A ratio, the primary sex-determining signal in Drosophila, is transduced by helix-loop-helix proteins. *Cell* **63,** 1179-1191.

Rashbass, J., Taylor, M. and Gurdon, J. (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., Hernandez, R., Modolell, J. and Ruiz Gomez, M. (1990). Competence to develop sensory organs is temporally and spatially regulated in *Drosophila* epidermal primordia. *EMBO J.* **9,** 3583-3592.

Ruezinsky, D., Beckmann, H. and Kadesch, T. (1991). Modulation of the IgH enhancer's cell type specificity through a genetic switch. *Genes Dev.* **5,** 29-37.

Sharpe, C. R., Fritz, A., DeRobertis, E. M. and Gurdon, J. B. (1987). A homoeobox-containing marker of posterior neural differentiation shows the importance of predetermination in neural induction. *Cell* **50,** 749-758.

Slack, J. M. and Tannahill, D. (1992). Mechanism of anteroposterior axis specification in vertebrates. Lessons from the amphibians. *Development* **114,** 285-302.

Taira, M., Jamrich, M., Good, P. and Dawid, I. (1992). The LIM domain-containing homeobox gene *Xlim-1* is expressed specifically in the organizer region of *Xenopus* gastrula embryos. *Genes Dev.* **6,** 356-366.

Turner, D. L. and Weintraub, H. (1994). Expression of *achaete-scute* homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8,** 1434-1447.

Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R. and Hollenberg, S. (1991a). The myoD gene family: nodal point during specification of the muscle cell lineage. *Science* **251,** 761-766.

Weintraub, H., Dwarki, V. J., Verma, I., Davis, R., Hollenberg, S., Snider, L., Lassar, A. and Tapscott, S. J. (1991b). Muscle-specific transcriptional activation by *MyoD*. *Genes Dev.* **5,** 1377-1386.

Wieschaus, E., Nusslein-Vollhard, C. and Jurgens, G. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. *Roux's Arch Dev. Biol.* **193,** 296-307.

Zimmerman, K., Shih, J., Bars, J., Collazo, A. and Anderson, D. J. (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.

(Accepted 19 August 1994)