cash4, a novel *achaete-scute* homolog induced by Hensen's node during generation of the posterior nervous system

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In vertebrate embryos, the precursor cells of the central nervous system (CNS) are induced by signaling from the organizer region. Here we report the isolation of a novel vertebrate *achaete-scute* homolog, *cash4*, which is expressed in the presumptive posterior nervous system in response to such signaling. *cash4* is first expressed in epiblast cells flanking the late-phase organizer (Hensen's node), which retains its ability to induce *cash4* during regression to the caudal end of the embryo. We show that these node-derived signals can be mimicked in vivo by the activity of fibroblast growth factor (FGF). We demonstrate that *cash4* can substitute for the *achaete/scute* genes in the fly and that it also has proneural activity in vertebrate embryos. Together these results suggest that *cash4* functions as a proneural gene downstream of node-derived signals (including FGF) to promote the formation of the neural precursors that will give rise to the posterior CNS in the chick embryo.

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During vertebrate gastrulation, neural-inducing signals emanate from a unique region of the embryo, the organizer (Spemann and Mangold 1924; Waddington 1932; Beddington 1994). A number of neural-inducing molecules are expressed in the organizer and have activities that support Spemann's original observation that head and trunk regions of the nervous system are induced in two distinct phases (Spemann 1927; also see Nieuwkoop 1952; Saxén and Toivonen 1961; after Hamburger 1988). Initially, neural inducing molecules such as Noggin, Follistatin, and Chordin induce anterior neural tissue, in part by inhibiting the activity of the epidermal inducing factor BMP4 (Lamb et al. 1993; Hemmati-Brivanlou et al. 1994; Sasai et al. 1995; Piccolo et al. 1996; Zimmerman et al. 1996; also see Kengaku and Okamoto 1995; Streit et al. 1995). Later, signals such as fibroblast growth factor (FGF) and retinoic acid (RA) transform part of this neural tissue to a more posterior character (Maden and Holder 1992; Slack and Tannahill 1992; Cox and Hem-

mati-Brivanlou 1995; Kengaku and Okamoto 1995; Lamb and Harland 1995; Crossley et al. 1996).

How the newly induced neural tissue integrates these successive sets of signals and acquires its final identity is still poorly understood. However, recent characterization of vertebrate homologs of *Drosophila* genes that control neural specification in the fly has shed light on the molecular events that are initiated by neural inducing signals and serve to implement neural cell fates in vertebrate embryos. In *Drosophila*, neural precursors are established by a cascade of genes, at the top of which lies the *Achaete-Scute Complex* (ASC) (for review, see Campuzano and Modolell 1992; Ghysen and Dambly-Chaudière 1993; Jan and Jan 1994; Skeath and Carroll 1994). The four ASC genes [*lethal-of-scute* (*l'sc*), *scute* (*sc*), *achaete* (*ac*), and *asense* (*ase*)] encode basic helix-loop-helix (bHLH) transcriptional regulators whose transient expression confers neural potential on groups of ectodermal cells (so-called proneural clusters). Subsequently, lateral inhibition, mediated by the activities of the neurogenic genes, restricts neural potential to a single cell in a cluster (for review, see Campos-Ortega 1993), which expresses higher levels of the ASC genes

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and will become a neuroblast, a founder cell of the fly nervous system. In embryos carrying mutations in the ASC genes, fewer neural precursors are formed, which leads to a reduction in the number of nerve cells in the central or peripheral nervous system (Dambly-Chaudière and Ghysen 1987; Ruiz-Gómez and Modolell 1987; Villares and Cabrera 1987; Jiménez and Campos-Ortega 1990).

Vertebrate homologs of the ASC genes have been isolated and found to be expressed in the developing nervous system (Johnson et al. 1990; Ferreiro et al. 1992; Guillemot and Joyner 1993; Zimmerman et al. 1993; Jasoni et al. 1994). Mutations in one of these, *mash1*, result in the loss of particular classes of neuron in the olfactory and peripheral nervous system (Guillemot et al. 1993). However, *mash1* is not expressed during the determination of neural precursors and is not required for their specification; instead, *mash1* is present in terminally dividing neuronal progenitors to control subsequent differentiation (Guillemot et al. 1993; Gordon et al. 1995; Sommer et al. 1995). Another vertebrate ASC homolog, *xash3*, which has been isolated only in *Xenopus* embryos, is expressed in subsets of neural precursors shortly after neural induction and is a better candidate for acting as a proneural gene (Zimmerman et al. 1993; Ferreiro et al. 1994; Turner and Weintraub 1994). Overexpression of *xash3* mRNA in the frog embryo results in the expansion of the neural plate, because of the conversion of presumptive neural crest and epidermis to a neural plate character (Turner and Weintraub 1994; Chitnis and Kintner 1996), indicating that *xash3* may promote the neural precursor state in a manner analogous to that of the ASC genes in the fly.

Another group of vertebrate genes encoding related bHLH proteins is expressed in the developing CNS and has homology with the *Drosophila* proneural gene *atonal* (Akazawa et al. 1995; Lee et al. 1995; Shimizu et al. 1995; Ma et al. 1996). One of these genes, *neuroD*, is expressed in postmitotic neurons and has been shown to drive neuronal differentiation when expressed ectopically in the amphibian embryo (Lee et al. 1995). Another *atonal* homolog, *neurogenin* (Ma et al. 1996), has an expression pattern in the developing mouse central nervous system (CNS) that is complementary to that of *mash1* and may thus have a similar function during neuronal differentiation. A related gene, *ngnr-1*, is expressed early during primary neurogenesis in *Xenopus* embryos and has been proposed to act as a neuronal determination gene (Ma et al. 1996). Ectopic *ngnr-1* expression also promotes the formation of primary neurons and is able to induce the expression of *neuroD*, suggesting that *ngnr-1* functions in the determination of neuronal progenitors during the genesis of the frog primary nervous system.

All these results point to the existence of a cascade of bHLH proteins acting during primary neurogenesis in the frog embryo, including early determination factors such as XASH3 and NGNR-1, and later differentiation factors like *NeuroD*, in a manner akin to the cascade of myogenic bHLH factors that operate during myogenesis (Weintraub 1993; Lee et al. 1995). However, none of the

bHLH-encoding genes already isolated in vertebrates are expressed early enough during CNS development to play a role in neural determination (specification of neural precursors that give rise to both neurons and glia) as distinct from neuronal determination (specification of neuronal progenitors that give rise solely to neurons).

Here we report the isolation of a novel ASC gene homolog in the chick, *cash4* (chick achaete-scute homolog-4) whose early expression in neural precursors is initiated by signals from Hensen's node. We show that these signals persist in the node throughout the period during which the posterior CNS is laid down, and that this activity can be mimicked in vivo by a member of the FGF family. *cash4* can promote neural cell fates in vertebrate embryos and is a functional homolog of the fly ASC genes. These findings suggest that *cash4* functions as a proneural gene in the chick to specify precursor cells of the posterior CNS, and we show that this specification process takes place continuously in response to signals, including FGF, from the regressing node/anterior primitive streak.

Results

Isolation of *cash4* cDNA

We have identified a novel ASC homolog, *cash4*, using PCR and degenerate oligonucleotide primers that correspond to sequences conserved between the fly ASC proteins and the three vertebrate ASC homologs isolated previously (Johnson et al. 1990; Guillemot and Joyner 1993; Zimmerman et al. 1993). The *cash4* PCR fragment was used to isolate a 1.4-kb *cash4* cDNA that encodes a 167-amino-acid bHLH protein, related to the ASC family of proteins (Fig. 1A). These are characterized by the unique structure of their bHLH domain, with a basic region that differs from analogous motifs in other bHLH transcriptional regulators. In the case of CASH4, the basic region clearly resembles that of the *Drosophila* ASC proteins and is distinct from that of other classes of bHLH proteins (Fig. 1B). However, in common with vertebrate ASC proteins isolated previously, the loop region of the CASH4 bHLH domain is shorter than equivalent regions of the fly ASC proteins. CASH4 possesses two further conserved regions (Fig. 1A): a 15-amino-acid sequence just amino-terminal to the basic domain (region A), conserved between CASH4 and the vertebrate ASH1 and ASH2 proteins (less conserved in XASH3; Zimmerman et al. 1993), and another (region B, 25 amino acids) at the carboxyl terminus, which is almost identical in CASH1 and CASH4 but absent from the other two vertebrate homologs, MASH2 and XASH3. Both regions include serine residues that are potential sites of phosphorylation. CASH4 also contains proline and alanine-rich regions amino-terminal to the bHLH domain that could function in transcriptional modulation.

cash4 expression in the early chick embryo

cash4-expressing cells in the early chick embryo were localized by whole-mount in situ hybridization using di-

Figure 1. (A) Amino acid sequence of CASH4 and comparison with other vertebrate members of the ASC family. CASH4 shows the highest homology with the chick ASH1 protein (Jasoni et al. 1994), the ortholog of the mouse MASH1 (Guillemot and Joyner 1993). The bHLH domain is well conserved between all the vertebrate ASC proteins, with a shorter loop than that of the fly ASC proteins. Two other regions (A and B) are also conserved between the vertebrate ASC proteins, in particular between CASH4 and the vertebrate ASH1 proteins. (B) Alignment of the bHLH domain from the various ASC family members and members of other classes of bHLH proteins. The CASH4 bHLH domain shares the unique characteristics of the ASC bHLH domain, in particular a distinctive (3 amino acid) shorter basic region, as compared with other classes of bHLH proteins (Villares and Cabrera 1987; ASC, Alonso and Cabrera 1988; chick MyoD, Lin et al. 1989; Atonal, Jarman et al. 1993; CnASH, Grens et al. 1995; mouse NeuroD, Lee et al. 1995).

goxygenin (DIG)-labeled riboprobes. *cash4* transcripts are first detected at HH5 [stages according to Hamburger and Hamilton (1951)], at two major sites of expression: prospective blood islands in the extra-embryonic tissue of the *area opaca*, and the epiblast cells adjacent to Hensen's node and the anterior primitive streak that will give rise to the posterior CNS (Fig. 2A). *cash4* expression in the blood islands resembles that of *scl/tal1* and *gata2*, two genes that are expressed very early in the blood stem cells and that are essential to their normal development (Tsai et al. 1994; Shivdasani et al. 1995). *cash4* expression precedes that of *gata2* (data not shown), suggesting a very early role for *cash4* in the development of the primitive blood stem cells. Thus, like the fly ASC genes, which play roles in a range of developmental processes from sex determination to the formation of muscle, gut, and nervous system (for review, see Jan and Jan 1993), *cash4* may also be involved in the generation of a number of different cell types. In this paper, however, we focus only on the expression of *cash4* in the developing nervous system and its potential proneural function in this tissue.

cash4 expression in the developing CNS

In the developing chick CNS, *cash4* is expressed initially in epiblast cells on each side of the organizer region (i.e., next to Hensen's node and the anterior primitive streak) at HH5 (Fig. 2A,B). The boundaries of the *cash4* domain are not sharp and there is also heterogeneity in the levels of *cash4* expression within its domain; some cells express higher levels of *cash4* mRNA than their immediate neighbors (Fig. 2C,D). Fate maps of the epiblast adjacent and posterior to the node at HH5 show that cells in the *cash4* domain contribute extensively to the posterior CNS, comprising the posterior hindbrain and spinal cord (Spratt 1952; Schoenwolf and Alvarez 1989, 1991). We have confirmed this by using the lineage tracer 1,1'-dioctadecyl-3,3',3' tetramethyl indocarbocyanineperchlorate (DiI) to label groups of ~25–30 epiblast cells in the *cash4*-expressing domain at HH5, adjacent to the node and anterior primitive streak. After 12 hr (HH10), DiI-labeled cells are found unilaterally within the developing posterior CNS, commencing in the posterior hindbrain at the level of somites 2–3 and extending within

Figure 2. Initial pattern of *cash4* expression and the fate of the first *cash4*-expressing cells. (A) *cash4* mRNA is first detected in the epiblast adjacent and posterior to Hensen's node (small arrowhead) and in the developing blood islands (arrowhead) in extraembryonic tissue at HH5 (shown here slightly later at HH6–). (B) Transverse section through a stage HH5 Hensen's node. *cash4* expression is confined to the epiblast layer lateral to the node. Although a few cells expressing low levels of *cash4* may be present at the lateral edges of the node and anterior primitive streak, *cash4* transcripts are absent from the presumptive floor plate (arrowhead). (C) High magnification of A depicting anterior region of the *cash4* domain (primitive streak is to the right). (D) High-power view of box in C. Single epiblast cells express *cash4* at higher levels than their immediate neighbors. Bar, 20 μ m. (E) DiI-labeling of groups of 25–30 epiblast cells adjacent to Hensen's node/anterior primitive streak at HH5–6 reveals that cells in the initial *cash4* domain proliferate and rearrange so that they are distributed along the length of the posterior CNS including the open neural plate at HH10.

the spinal cord. In most cases (15/20), labeled cells are present along the entire length of the spinal cord and in the open neural plate at the posterior-most end of the CNS (Fig. 2E), confirming that cells in the initial *cash4* domain include precursors whose descendants become distributed throughout the posterior CNS.

At HH5–6, the primitive streak begins to regress to the caudal end of the embryo and the domain of *cash4* expression retains its spatial relationship to the node (Fig. 3). This displacement of the *cash4* domain reflects the rostrocaudal sequence in which the posterior CNS is laid down (Schoenwolf and Alvarez 1989; Schoenwolf 1992).

As neurulation proceeds and the neural plate becomes morphologically distinct adjacent to Hensen's node, *cash4* transcripts are always present in the open neural plate but decrease as the neural folds elevate (Fig. 3A–D). When the posterior neuropore closes and primary neurulation is completed (HH11–12), the expression of *cash4* ceases in the developing CNS. Thus, *cash4* is specifically expressed during the expansion of the posterior nervous system, preceding the first signs of neuronal differentiation.

cash4 is induced by Hensen's node

The expression of *cash4* in presumptive posterior neural tissue close to Hensen's node at HH5 suggests that it may be induced by signals from the node, which is the source of neural-inducing signals in the chick embryo (Waddington 1932; Gallera 1971; Dias and Schoenwolf 1990; Storey et al. 1992). To test this possibility, we grafted nodes derived from HH5–6 quail embryos into the presumptive anterior neural plate of HH3–3+ chick embryos, a region that does not normally generate cells that express *cash4*. As a control, we carried out similar experiments in which we grafted quail-derived pieces of the posterior primitive streak, a region not normally flanked by *cash4*-expressing cells (Fig. 4A). All host embryos were maintained in New culture (New 1955) until they had developed to HH8–10, when they were analyzed using a chick-specific *cash4* riboprobe. The species specificity of the probe was confirmed by combining in situ hybridization with immunocytochemistry, using the QCPN antibody to identify grafted quail cells (Fig. 4D). In all cases (15/15), the grafted node induces *cash4* expression in host tissue, whereas control grafts of posterior primitive streak do not (5/5) (Table 1; Fig. 4B–E). This result indicates that *cash4* expression can be induced by signals that emanate from Hensen's node at HH5–6.

As *cash4* expression moves in concert with the regressing node, it is possible that the node continues to provide *cash4*-inducing signals throughout regression. We tested this possibility by repeating the above quail/chick grafting experiments with nodes from progressively older embryos (HH7+–11). Nodes explanted from even the oldest stage (HH11) induce a dome-shaped region of raised host epithelium that has a diffuse, speckled pattern of *cash4*-expressing cells (Fig. 4F). Although the oldest nodes are the weakest inducers of *cash4*, they clearly retain the ability to elicit expression of this gene in the host epiblast (Table 1), demonstrating that *cash4*-inducing signals are continually present in the node during its regression and for as long as *cash4* is normally expressed in the presumptive neural plate (up to HH11).

Signals from the regressing node induce cash4 expression in the extraembryonic epiblast

Our DiI-labeling study suggests that some descendants of the initial *cash4*-expressing cells retain expression of this gene as they shift posteriorly. Thus, signals provided by older nodes may serve largely to maintain *cash4* ex-

Figure 3. The dynamic pattern of *cash4* expression during development of the posterior CNS. (A) HH6 embryo. The *cash4* domain of expression accompanies the regressing node/anterior primitive streak. (B) HH8 embryo. *cash4* is now expressed in broadening domains on either side of the streak that include the nascent neural plate and more laterally prospective epidermis (after Spratt 1952). At HH8–9, *cash4* is also expressed transiently in the neural plate just anterior to the first forming somite. This patch of expression is just beginning to appear in this embryo (arrowhead). (C) *cash4* expression domain continues to shift posteriorly with the regressing node/primitive streak at HH9. (D) Reduced levels of *cash4* are detected in the elevating neural plate at HH11.

pression in those cells, rather than to induce de novo expression of this gene. To assess whether the regressing node retains the ability to induce *cash4* de novo, we therefore transplanted older nodes into the extraembryonic epiblast, a region that normally gives rise to extraembryonic membranes and that is unlikely to have been exposed to the early neural-inducing signals from Hensen's node (Waddington 1932; Gallera 1971; Storey et al. 1992, 1995). Nodes were explanted from quail embryos of HH5–10 and placed against the extraembryonic epiblast of chick HH3–3+ embryos, which were then allowed to develop to HH8–10 (Fig. 5A). In the majority of cases, HH5–6 nodes are able to induce *cash4* expression in host epithelial cells overlying the graft (Table 1; Fig. 6B). Hensen's nodes derived from older embryos were also found to elicit *cash4* expression in the extraembryonic epiblast (Table 1; Fig. 5C).

As *cash4* is expressed in non-neural tissue in extraembryonic regions (see above), we confirmed that the *cash4*-expressing cells induced by older nodes are indeed neural by also assaying for the ectopic expression of the neural specific marker *Sax1* (an NK-1 class of homeodomain transcription factor expressed in the spinal cord; Spann et al. 1994). In most cases (12/19) nodes of HH7+–11 induce ectopic *Sax1* expression, which like *cash4* is found in a strip of thickened epithelial cells overlying the graft. By combining *Sax1* in situ hybridization with immunocytochemistry for the quail specific antibody QCPN, we confirmed that *Sax1* is induced in chick epithelial cells (9/9 cases; Fig. 5D and E).

Together, these findings demonstrate that signals from the regressing node can induce de novo expression of both *cash4* and the neural specific gene *Sax1* in epiblast cells that do not normally give rise to neural tissue.

Thus, the organizer appears to remain a source of neural inducing signals during regression.

FGF can induce cash4

The confinement of *cash4* expression to the presumptive posterior regions of the CNS suggests that *cash4* might be induced by a posteriorizing factor. We therefore tested whether FGF signaling, which has been demonstrated to have a posteriorizing effect during neural induction (for review, see Doniach 1995), may contribute to induction of *cash4* by the regressing Hensen's node. Heparin-coated acrylic beads soaked in FGF4 or control beads (washed in PBS) were implanted beneath and in contact with the presumptive anterior neural plate of HH3+ embryos (Fig. 6A,B). These embryos were cultured until HH9–10 and *cash4* expression was monitored by whole-mount in situ hybridization. Embryos implanted with FGF4 beads are characterized by the proliferation of tissue around the bead and the loss of anterior structures, such as optic lobes (a more detailed analysis of this phenotype will be presented elsewhere). In these embryos, *cash4* expression is strongly induced in the cells closest to the implanted FGF4 bead (Table 2; Fig. 6C,D), and no ectopic *cash4* is induced in response to control PBS-soaked beads (Fig. 6E). These results suggest that a member of the FGF family of signaling molecules is involved in the activation of *cash4* expression in the presumptive posterior CNS.

cash4 has proneural activity in fly and amphibian embryos

The sequence conservation between *cash4* and the fly ASC proteins, in particular in their bHLH domains, and

Figure 4. Hensen's node induces ectopic *cash4* expression in the presumptive anterior neural plate. (A) Schematic of grafting experiments: Quail nodes or pieces of posterior primitive streak derived from HH5–6 embryos were juxtaposed with the presumptive anterior neural plate of young chick host embryos. (B) Hensen's node (HH5–6) induces ectopic *cash4* expression following transplantation into the presumptive anterior neural plate (arrowhead). Node tissue is present beneath this region of ectopic *cash4* and extends from the host embryo, seen here in whole mount. (C) Higher magnification of whole mount seen in B showing ectopic *cash4*. (D) Simultaneous detection of *cash4* mRNA and a quail-specific perinuclear antigen (recognized by Mab QCPN detected with a peroxidase conjugated secondary antibody reacted with diaminobenzidine to give a brown precipitate) confirmed the species-specificity of the 3' *cash4* probe. In this transverse section quail tissues (arrowhead) derived from a HH7+ Hensen's node (in this case placed in contact with the extraembryonic epiblast; see below) can be distinguished from chick host epiblast expressing *cash4*. (E) Posterior primitive streak does not induce ectopic *cash4* in presumptive anterior neural tissue (whole mount). (F) HH11 Hensen's node induces scattered ectopic *cash4* expression following grafting into presumptive anterior neural tissue (whole mount).

the striking pattern of *cash4* expression in the developing nervous system may reflect a conserved function of these proteins as proneural factors during embryonic development. We investigated this possibility by testing whether CASH4 is able to promote neural development in *Drosophila*. We used the GAL4/UAS (upstream activating sequence) system (Brand and Perrimon 1993) to drive *cash4* expression during larval development. Flies

expressing *cash4* in the scutellar primordium develop numerous ectopic bristles (Fig. 7A,B). This phenotype is indistinguishable from that obtained by equivalent expression of the *Drosophila* *l'sc* or *sc* genes (cf. Fig. 7B and Hinz et al. 1994, Fig. 2) showing that CASH4 can mimic the proneural activity of related fly ASC proteins.

To test whether CASH4 promotes bristle development directly or by activating the expression of endogenous proneural genes, we asked whether the proneural activity of CASH4 depends on *ac* or *sc*, the endogenous proneural genes that normally drive the formation of sensory organs in the fly scutellum (Cubas et al. 1991; Skeath and Carroll 1991). Flies carrying *Df(1)sc¹⁰⁻¹*, a deletion that inactivates both *ac* and *sc*, normally lack all sensory bristles (Fig. 7C; Villares and Cabrera 1987). However, expression of *cash4* in this genetic background is still able to induce ectopic scutellar bristles (Fig. 7D), indicating that the proneural activity of CASH4 in flies does not depend on the genes that normally specify adult bristles.

We also tested whether *cash4* can promote neurogenesis in vertebrate embryos by expressing this gene during early amphibian development. We injected *cash4* mRNA into one blastomere of two-cell *Xenopus* embryos and assayed effects on neural development using a pan-neural marker, *NCAM* (Kintner and Melton 1987), and *N-tubulin*, which labels early differentiating neurons (Chitnis et al. 1995). Ectopic expression of *cash4* leads to enlargement of the neural plate and an increase in neural precursor cells as measured by the expansion of the domain of *NCAM* expression (28/35 embryos; Fig. 7E). The number of subsequently differentiating cells expressing *N-tubulin* also is increased sharply, and extends outside the normal domains of neurogenesis (58/65 embryos; Fig. 7F). Thus, ectopic *cash4* expression is able to promote the formation of neural precursors and, ultimately, the production of differentiated neurons.

Discussion

We have isolated a novel chick ASC homolog, *cash4*, and investigated its function during neural development. *cash4* is the earliest known ASC homolog to be expressed in the chick nervous system, being first present in epiblast cells on either side of Hensen's node/anterior primitive streak at HH5. We show that these cells will

Table 1. Induction of chick *cash4* mRNA by quail Hensen's node

Stage of Hensen's node	Graft position	Frequency of <i>cash4</i> induction
HH5–6	presumptive anterior neural plate	15/15
HH7+–11	presumptive anterior neural plate	9/9
HH5–6	extraembryonic epiblast	7/10
HH6+–8+	extraembryonic epiblast	9/12
HH9–10	extraembryonic epiblast	5/10

Figure 5. Hensen's node induces *cash4* and *Sax1* expression in extraembryonic epiblast cells. (A) Schematic showing the position of Hensen's nodes derived from progressively older stages transplanted in contact with extraembryonic epiblast. (B) Hensen's nodes of HH6 induce ectopic *cash4* (arrowhead) when placed in contact with the extraembryonic epiblast (whole mount). (C) Nodes derived from later stages (HH9–10) also retain the ability to induce ectopic *cash4* (arrowhead) in extraembryonic epiblast (whole mount). (D) Old nodes (in this case HH8) induce ectopic expression of the neural specific marker *Sax1* (whole mount). (E) The induction of *Sax1* in the chick host cells was confirmed by simultaneous detection of this gene and a quail-specific antigen (arrowhead; detected as described in Fig. 5) shown here in a transverse section of one of these ectopic structures. No *Sax1*-expressing cells were found to contain the quail antigen. *cash4* and *Sax1* are induced in only a thickened epithelium and in most cases are present on the side of ectopic structures facing the host axis; this may reflect changes in competence to respond to neural-inducing signals observed across the lateral extent of the extraembryonic epiblast (Storey et al. 1992).

Figure 6. Induction of *cash4* by local expression of FGF4 in presumptive anterior neural tissue. (A) Schematic of FGF bead grafting experiments. (B) FGF4-soaked bead (arrowhead) grafted in contact with the presumptive anterior neural tissue. (C) Ectopic *cash4* expression is induced in the anterior CNS by FGF4 beads. This phenotype is also characterized by the loss of characteristic anterior neural morphology such as the optic lobes. (D) Transverse section through C. *cash4* expression is localized in cells in the immediate vicinity of the bead (dotted outline). (E) Control PBS beads (arrowhead) do not induce *cash4* or affect morphogenesis of the anterior CNS.

contribute to the posterior CNS and that expression of *cash4* is induced by signals emanating from Hensen's node, throughout its regression. We demonstrate that the product of *cash4* has proneural activity: It can function as an ASC gene product in the fly and can promote neural plate formation in vertebrate embryos. Together these results suggest a proneural function for *cash4* in the chick, providing a link between node-derived signals (including FGF) and the molecular machinery responsible for the implementation of the neural program in posterior regions of the chick embryo.

cash4 expression defines neural precursors of the chick posterior CNS

In the developing chick CNS, *cash4* expression is initiated at HH5, in the epiblast adjacent to Hensen's node and anterior primitive streak. Previous studies (Spratt

Table 2. Induction of *cash4* mRNA by local overexpression of FGF4 in the presumptive anterior neural plate

Implant	Loss of optic lobe(s)	Ectopic <i>cash4</i>
FGF4 beads	18/28	11/12
PBS beads	0/28	0/12

Figure 7. Proneural effects of *cash4* expression in *Drosophila* and *Xenopus* embryos. (A) Wild-type pattern of scutellar sensory organs consists of four bristles (arrowheads). (B) *cash4* expression in the prospective scutellum, driven by the *Gal4455.2* line, results in ectopic development of bristles. (C) Absence of bristles on the adult thorax including the scutellum in a *Df(1)sc¹⁰⁻¹* fly, in which ac and sc function are eliminated. (D) *cash4* expression in a *sc¹⁰⁻¹* background (*sc¹⁰⁻¹; Gal4455.2; UAS-cash4*) still results in the development of ectopic scutellar bristles. (E) Unilateral expression of *cash4* in *Xenopus* embryos, following injection of *cash4* mRNA in one cell of the two-cell stage embryo, leads to hypertrophy of the neural plate on the injected side, revealed by the expansion of the *NCAM* expression domain. (F) Ectopic primary neurons induced by *cash4*, as revealed by *N-tubulin* positive cells outside the normal domains of primary neurogenesis. In E and F, injected side of embryos is uppermost, and white arrows show mid-lines of the embryos.

1952; Schoenwolf and Alvarez 1989; Schoenwolf and Sheard 1990; Schoenwolf and Alvarez 1991) indicate that this region of epiblast constitutes a primordium of the posterior nervous system, and it has been proposed that the posterior CNS is generated by the elongation of such primordia, through movement and proliferation of neural precursors present within this cell population (see Schoenwolf and Alvarez 1989; Storey et al. 1992). Our DiI-labeling study confirms that indeed cells in the initial *cash4* domain are neural precursors fated to contribute to extensive anteroposterior regions of the CNS, from the posterior hindbrain and along the length of the spinal cord.

Whereas lineage tracing of groups of cells within the initial *cash4* domain results in labeling confined almost exclusively to the neural tube, it is not clear whether all cells in the posterior CNS derive from the initial *cash4*-expressing domain, or whether there is an admixture of cells recruited later from elsewhere (see below). In any case, essentially all cells at each level of the prospective posterior hindbrain and spinal cord express *cash4* for a limited period, resulting in a rostrocaudal wave of *cash4* expression that foreshadows neurogenesis in the developing posterior CNS. Given the proneural activity of the *cash4* gene product in *Drosophila* and *Xenopus*, these observations strongly suggest that *cash4* plays a part in conferring neural potential on the precursor cells that will give rise to the posterior CNS. Indeed, the cells of the prospective floor plate do not express *cash4* (see Fig. 2B) and do not subsequently give rise to neurons.

The heterogeneous pattern of *cash4* expression within the newly formed neural plate may reflect the different developmental states of cells in this region. We favor a

model in which the cells expressing higher levels of *cash4* may become specified as the founder neural precursors of the posterior CNS, much as high levels of ASC expression direct *Drosophila* neuroectodermal cells to become neuroblasts, the founder stem cells of the fly nervous system (for review, see Skeath and Carroll 1994). Alternatively, higher levels of *cash4* expression could promote neuronal differentiation and drive those cells directly toward terminal differentiation. However, this hypothesis is unlikely because *cash4* expression does not correlate with the temporal sequence of neuronal production in the developing posterior CNS, as marked by the expression of *C-Delta-1* in newly born neurons (Sechrist and Bronner-Fraser 1991; Henrique et al. 1995). Further, cells with high *cash4* levels incorporate bromodeoxyuridine (data not shown) and therefore are not differentiating postmitotic neurons. Single-cell labeling to define early neural lineages arising adjacent to the node and anterior primitive streak will allow us to confirm our hypothesis.

As suggested previously (Lee et al. 1995; Chitnis and Kintner 1996; Ma et al. 1996), neurogenesis in vertebrate embryos may be controlled by a cascade of ASC homologs or functionally related genes whose sequential expression in neural cells regulates different steps in the progression toward their final fates. If so, the early and transient expression of *cash4* in neural precursors places it upstream of all related bHLH-encoding genes currently identified in higher vertebrates, including *mash1*, *neurogenin*, and *neuroD*. These are expressed later in descendant cells (*mash1*, *neurogenin* in neuronal progenitors; *neuroD* in differentiating neurons), and must regulate subsequent steps in neurogenesis.

cash4 is induced continuously by the regressing node/anterior primitive streak

cash4 expression is induced ectopically when Hensen's node from stage HH5–6 is grafted into the anterior neural plate, whereas grafts of posterior primitive streak do not have this effect. The late-phase node, therefore, is a source of *cash4*-inducing signals. Further, the rostrocaudal displacement of the *cash4* expression domain in concert with the regressing node suggests that *cash4*-inducing activity is present at all stages of node regression, while the posterior CNS is being laid down. This is confirmed by our finding that old nodes (up to stage HH10) retain the ability to induce *cash4* even in extraembryonic epiblast cells, which normally give rise only to extraembryonic membranes. In these experiments, induction of *cash4* expression correlates with induction of neural characteristics, as revealed by the ectopic expression of neural-specific marker *Sax1* in response to the explanted node. Our results thus show that neural-inducing signals are present in the node during regression, and persist for longer than reported in previous studies (Dias and Schoenwolf 1990; Storey et al. 1992; but see Kintner and Dodd 1991). These findings, together with our lineage data, indicate that during normal development, *cash4* expression could be maintained in cells that move posteriorly alongside the regressing node as well as being initiated in successive cell populations. Indeed, Selleck and Bronner-Fraser (1995) have shown that as late as HH10 the open neural plate contains pluripotent cells that can give rise to both epidermal and neural cell types, implying that continuing inductive signals are required to confer an appropriately neural character on all the cells of the posterior neural plate.

cash4 is induced by FGF

The restricted expression of *cash4* in the presumptive posterior CNS, and its onset when the node ceases to induce anterior neural tissue but retains the ability to induce genes characteristic of the posterior neural tissue (Storey et al., 1992; present results), suggest that *cash4* is activated by signals associated with the formation of the posterior CNS. FGFs are expressed in the regressing node and primitive streak of the chick embryo (Mahmood et al. 1995; Riese et al. 1995; K.G. Storey and J.K. Heath, unpubl.) and it is known that FGF signaling has both general posteriorizing effects (Slack and Tannahill 1992) as well as specific caudalizing activity in the amphibian CNS (Okamoto and Kengaku 1993; Cox and Hemmati-Brivanlou 1995; Kengaku and Okamoto 1995; Lamb and Harland 1995; Launay et al. 1996). Here we have shown that FGF4 induces ectopic expression of *cash4* when overexpressed in the presumptive anterior CNS of the chick and that it can therefore mimic the *cash4*-inducing signals of Hensen's node. The expression of *cash4* in cells in the immediate vicinity of FGF beads suggests that the induction of this transcription factor is a consequence of direct exposure to FGF. These findings support a role for FGF in the generation of the posterior CNS in

the chick embryo and are consistent with a function in both neural induction and anteroposterior patterning of the CNS, as has been reported recently in amphibian embryos (Okamoto and Kengaku 1993; Cox and Hemmati-Brivanlou 1995; Kengaku and Okamoto 1995; Lamb and Harland 1995; Launay et al. 1996).

cash4 has proneural activity in both vertebrate and *Drosophila* embryos

The ability of *cash4* to rescue sensory organ formation in *Drosophila* mutants lacking endogenous *ac* and *sc* genes represents the first example in which a vertebrate ASC homolog has been shown to substitute for a *Drosophila* proneural gene. Like *l'sc* (Hinz et al. 1994), *cash4* induces the formation of ectopic bristles in flies, independent of endogenous *ac* and *sc* genes. Thus, CASH4 appears to retain the transcriptional specificity that mediates the proneural function of fly ASC proteins. These results are consistent with the finding that the bHLH domain is a major determinant of the functional activity of the ASC family of proteins during neural development (Hinz et al. 1994). However, we cannot exclude the possibility that *cash4* recruits other endogenous proneural activities that do not normally function during imaginal bristle development.

Conservation of the proneural activity of *cash4* is demonstrated further by its ability to direct cells toward a neural fate during vertebrate development: When ectopically expressed in *Xenopus* embryos, *cash4* leads to an expansion of the neural plate and overproduction of primary neurons. This result differs from findings with *xash3*, another ASC homolog, and *ngnr-1* and *neuroD*, two bHLH-encoding genes related to the *Drosophila* proneural gene *atonal*. Although overexpression of *xash3* in *Xenopus* embryos also promotes the formation of neural precursors, leading to a similar expansion of the neural plate, the actual number of differentiated neurons in the neural tube is diminished (Ferreiro et al. 1994; Turner and Weintraub 1994), probably because *xash3* strongly activates the genes involved in lateral inhibition (Chitnis and Kintner 1996). In contrast, overexpression of *ngnr-1* and *neuroD* results in an overproduction of neurons, but without any enlargement of the neural tissue, suggesting that these genes function during neuronal differentiation, *ngnr-1* being upstream of *neuroD* (Lee et al. 1995; Ma et al. 1996). *cash4* in this assay, therefore, appears to behave both as determination and differentiation factor, as it mimics the action of *xash3*, *neuroD*, and *ngnr-1*.

Structural differences between CASH4 and XASH3, which are found mainly outside the bHLH domain, could account for the extra activity of CASH4. Alternatively, the avian protein CASH4 may not be recognized by the *Xenopus* inhibitors that act on the endogenous proneural proteins and for that reason may be able to promote neuronal differentiation in frog embryos. A similar situation occurs when the myogenic function of MyoD is tested by ectopic expression in early *Xenopus* embryos: Whereas mouse MyoD is translocated into the

nucleus and activates the myogenic program, *Xenopus* MyoD is sequestered in the cytoplasm by specific endogenous inhibitors and is thus unable to initiate transcription of the muscle-specific genes (Rupp et al. 1994). This second alternative is consistent with the proposed existence of a cascade of bHLH proteins acting during vertebrate neurogenesis and possessing different susceptibilities to specific inhibitors (Lee et al. 1995). The isolation of the *Xenopus* counterpart of *cash4* would allow us to distinguish between the two explanations, and help to elucidate the role of different bHLH proteins during vertebrate neural development. Nevertheless, from our experiments we can conclude that *cash4* is able to function as a proneural gene in that it is able to promote the formation of neural precursors in a vertebrate embryo.

Making the posterior CNS

The existence of independent molecular mechanisms for the generation of head and trunk regions of the vertebrate embryo is demonstrated dramatically by the phenotype of mouse embryos with a targeted mutation in the homeobox transcription factor Lim-1 (Shawlot and Behringer 1995). In these embryos, the head fails to form and the body axis begins in the hindbrain, apparently a result of a deficit in the activity of the node in the early phase of neural induction. However, an organized node is present later and posterior regions of CNS form correctly. Genesis of the posterior nervous system, therefore, does not depend on the presence of the rudiment of the anterior nervous system. These findings, together with our results, are consistent with the idea that the posterior CNS derives from an initial primordium (Schoenwolf and Alvarez 1989, 1991; Storey et al. 1992) that is induced by a specific combination of signals, including FGF, arising from the late-phase organizer. In addition, our results indicate that there could also be a progressive recruitment of new epiblast cells into the neural pathway in response to signals provided by the regressing node, indicating that neural induction may continue during node/primitive streak regression. The expression of *cash4* in precursor cells of the posterior nervous system reflects exposure to this later combination of signals and is an early manifestation of the distinct molecular mechanisms that underlie the formation of the posterior CNS.

We have argued that *cash4* has the characteristics of a vertebrate proneural gene. It is expressed transiently in neural precursors, before neuronal differentiation begins and prior to other known bHLH proteins, and it displays proneural activity in both *Drosophila* and *Xenopus*. Further, its expression is induced by Hensen's node, the in vivo source of neural-inducing signals, and by FGF, a candidate posteriorizing signal present in this region. We therefore suggest that *cash4* functions as a neural determination gene specifying the neural precursors that will generate the avian posterior nervous system, and that it may be the first of a cascade of bHLH factors (Lee et al. 1995; Chitnis and Kintner 1996) that would function se-

quentially to implement the neural program in the wake of the regressing node.

Materials and methods

DNA cloning

The initial cDNA fragment from *cash4* was obtained by PCR using the following degenerate primers: 5'-(C/A)GIAACG(C/A)I(C/A)GIGAG(C/A)GNAA(C/T)(C/A)G-3' and 5'-AG-IGTCTC(G/C)AC(C/T)TT(T/G)CTCAT(C/T)TT-3' that correspond, respectively, to the sequences RN(E/A)RERNR and KM-SKVETL, located on helices 1 and 2 of the AS-C/MASH family of proteins. A fragment of 112 bp corresponding to a new ASC homolog was obtained by PCR [94°C for 30 sec, 50°C for 2 min, 72°C for 30 sec, 40 cycles] using cDNA prepared from stage 4–10 chicken embryos. This fragment was then used to screen a random-primed cDNA library in λ gt10, prepared from stage 10–14 chick embryos, and one positive clone was isolated from 10⁶ original plaques. This clone contained an insert of ~1.4 kb that was fully sequenced using the Sequenase kit (Amersham). The CASH4 sequence has been deposited in GenBank [accession no. U89138 (Bank It 95903)].

In situ hybridization

Chicken embryos were collected at the appropriate stages and fixed in 4% formaldehyde/PBS for 1 hr to overnight. Whole-mount in situ hybridization on these embryos was done according to Henrique et al. (1995). After the color reaction, some embryos were refixed in 4% formaldehyde/PBS with 0.1% glutaraldehyde, wax-embedded, and sectioned. A *cash4* 3' end fragment was subcloned in pKS (Stratagene) to generate an RNA probe that is specific for chick embryos. This probe did not give a detectable signal in quail embryos. *Sax1* cDNA was kindly provided by Dr. Yosef Gruenbaum (Spann et al. 1994).

Fly experiments

The entire *cash4* coding region (a 541-bp fragment from the *Bgl*I site just upstream of the initial ATG to the *Mbo*II site just after the stop codon) was subcloned into *pUAST* (Brand and Perrimon 1993), and germ-line transformants were generated by P element-mediated transformation (Rubin and Spradling 1982). Several independent transformant lines were established that behaved similarly, one of which is documented in this paper. The activator line *Gal4455.2* that is restricted to the presumptive scutellum (Hinz et al. 1994) was kindly provided by J. Campos-Ortega (University of Köln, Germany), and the *Df(1)sc¹⁰⁻¹* flies were obtained from F. Jiménez (Universidad Autónoma, Madrid, Spain). Crosses were maintained at 25°C.

Frog experiments

The entire *cash4* coding region was subcloned in the vector *pCS2+* (Turner and Weintraub 1994). After linearization with *Not*I, the vector was transcribed in vitro with SP6 polymerase in the presence of GpppG to produce capped *cash4* RNA. One nanogram of *cash4* RNA along with 0.2 ng of synthetic *lacZ* RNA was injected in a volume of 10 nl into one cell of a two-cell-stage embryo. As a negative control, embryos were similarly injected with just *lacZ* RNA. At early neurulae stages, the injected embryos were fixed, reacted with X-Gal to reveal the distribution of the *lacZ* tracer, and then double-stained for either NCAM or *N-tubulin* transcripts, using whole-mount in

situ hybridization (Chitnis et al. 1995). Double-stained embryos were postfixed and photographed in whole mount.

DiI-labeling

The use of DiI (Molecular Probes), as a dye for fate mapping studies has been described in detail elsewhere (e.g., Stern 1990). Groups of epiblast cells adjacent to node and anterior primitive streak were labeled at HH5–6. Following incubation, all DiI-labeled embryos were fixed in 4% buffered formol saline at pH 7.0 and viewed by epifluorescence (peak excitation 484 nm).

Grafting and culture techniques

Quail-derived nodes or pieces of posterior primitive streak (~150 × 150 µm) were excised and transplanted into chick host embryos of HH3–3+ prepared in New culture (New 1955) as described by Storey et al. (1992). Hensen's nodes from stages (5–11) were grafted either in contact with the presumptive anterior neural plate or with the extraembryonic epiblast of a host embryo and then maintained in a humid atmosphere at 38°C for up to 20 hr.

Immunocytochemistry after in situ hybridization

In situ hybridization techniques were combined with an anti-quail antibody (QCPN; supernatant was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, under contract N01HD-6-2915), to ascertain the origin of cells expressing *cash4* or *Sax1*. Following in situ hybridization embryos were washed in PBS and fixed overnight in formol saline at 4°C, washed in PBS, and placed in a blocking solution [PBS containing 3% BSA, 1% Triton X-100, 0.01% thimerosal, and 5% heat-inactivated normal goat serum (NGS)] for 4 hr at room temperature. QCPN supernatant was added 1:10 and embryos incubated for 3 days at 4°C. After extensive washing in PBS, embryos were incubated in blocking solution with peroxidase-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch) (1:500) overnight at 4°C. Embryos were then washed and underwent the usual diaminobenzidine tetrahydrochloride reaction, postfixed in 4% formol saline/0.1% glutaraldehyde, and wax-sectioned following standard methods.

Implanting FGF4 soaked beads

Heparin-coated acrylic beads (75–150 µm in diameter; Sigma H5263) were soaked in 780 µg/ml of Human FGF4 for 30 min at room temperature and washed in PBS prior to implantation. Heparin-coated beads just washed with PBS were used as controls. Embryos at HH3–3+ were set up in New culture and a small slit made in the hypoblast cell layer lateral and slightly anterior to the tip of the primitive streak. One bead was implanted in each embryo by pushing it through the slit in the hypoblast so that the bead made direct contact with underlying epiblast cells of the presumptive anterior neural plate. Embryos with implanted beads were allowed to develop for 20–24 hr prior to in situ hybridization procedures to detect *cash4* mRNA.

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