A chicken achaete-scute homolog (CASH-1) is expressed in a temporally and spatially discrete manner in the developing nervous system

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SUMMARY

We have identified a basic helix-loop-helix encoding cDNA from embryonic chicken retina which shares sequence similarity with the achaete-scute family of genes of Drosophila. The deduced amino acid sequence of this chicken achaete-scute homolog (CASH-1) is identical, over the region encoding the basic helix-loop-helix domain, to the recently identified mammalian achaete-scute homolog (MASH-1) and to the Xenopus homolog (XASH1), and 70% identical, over the same region, to Drosophila achaete-scute complex members. The expression of CASH-1 is restricted to subsets of neuronal progenitor cells in the developing chicken nervous system, similar in distribution to that reported for MASH-1 and XASH1. In addition, in situ localization in the retina reveals a dynamic character of expression of the gene in a particular region of the CNS, and suggests that the expression of CASH-1 may be important in defining a particular stage in the progenitor cell necessary for the differentiation of particular neuronal phenotypes.

Key words: retina, neural progenitors, achaete-scute homolog

INTRODUCTION

During the development of the vertebrate central nervous system, a large number of different neuronal and glial phenotypes are generated by the progenitor cells of the germinal neuroepithelium. The mechanisms that control the generation of this phenotypic diversity are not well understood. Several lines of evidence indicate that the ultimate choice of fate by a progenitor cell comes about as a complex interplay between factors within the surrounding microenvironment and factors intrinsic to the progenitor cell that restrict its ability to respond to the extrinsic cues (Anderson, 1989; Reh, 1992b). In the retina, for example, cell ablation experiments (Reh and Tully, 1986; Reh, 1987; Negishi et al., 1983) and heterochronic co-culture experiments (Watanabe and Raff, 1990, 1992; Reh, 1992a; Altschuler and Cepko, 1992) have shown that the differentiation of particular types of retinal neurons is regulated by the microenvironment. Nevertheless, there is also evidence that progenitor cells change during development in their response to differentiating factors (Taylor and Reh, 1989) and to different mitotic agents (Lillien and Cepko, 1992).

In order to identify potential factors intrinsic to a progenitor cell that restrict its ability to respond to the extrinsic cues, we have looked to invertebrate developing systems where molecules with similar functions have already been identified. One such class of molecules, that is of particular interest in this context, is the Drosophila achaete-scute complex (AS-C), involved in sense organ development (see Ghysen and Dambly-Chaudière, 1988, or Campuzano and Modolell, 1992, for reviews). The cuticle of Drosophila is endowed with a number of sensory organs, both external and internal, which occupy defined, sometimes precise, locations in the embryo, larva and adult. All of the cells of a sensory organ (SO), both neuronal and non-neuronal, are derived from a single precursor cell, the sensory mother cell (SMC). Some sensory organs develop in precise positions, while others form at a particular density in a given region. Mutational analysis has revealed that members of the AS-C and related proteins play a role in endowing cells with the competence to become SMCs (Cabrera et al., 1987; Cubas et al., 1991; deCelis et al., 1991). The expression of AS-C members is correlated with a particular state of a sensory organ-forming progenitor cell, which may be acted upon subsequently, through cell-cell interactions, to bring about overt differentiation of a particular SO-forming SMC.

The AS-C encodes four proteins — scute (sc or T4), achaete (ac or T5), asense (ase or T8) and lethal of scute (l’sc or T3). The AS-C products, as well as modulators of their activity — daughterless, hairy and extramacrochaete — all share sequence similarity over a 57- to 71-amino acid region, the region encoding the (basic) helix-loop-helix motif (b-hlh). This motif is also similar to those of the proto-oncogene c-myc (Villares and Cabrera, 1987) and the muscle determination genes, including MyoD and myogenin (Murre et al., 1989a,b). The helix-loop-helix region mediates the protein-protein interactions giving rise to DNA-binding dimers, while the basic region functions in binding DNA (Murre et al., 1989b).

Although the level at which AS-C acts in SO formation is not obviously analogous to the restriction of neuroepithelial cell potential in the vertebrate retina, the AS-C does act to define a particular, transient, state of a progenitor. It confers upon the prospective SMC the ability to differentiate as a SMC, and perhaps restricts the range of phenotypes the SMC can produce, to yield specific SO types. Thus, we sought to identify...
chicken homologs of the AS-C which may be expressed during the development of the retina.

MATERIALS AND METHODS

PCR cloning, cDNA isolation and sequencing
Stage 24 (embryonic day 4) chicken retinae were dissected in DEPC-treated PBS. Total cellular RNA was extracted by homogenizing the tissue and centrifuging through a 0%-25% sucrose gradient to pellet nuclei. Isolated cytoplasmic nucleic acids were treated with 400 μg/ml proteinase K, phenol/chloroform extracted, and ethanol precipitated. 5 μg of total cellular RNA was reverse transcribed using 5 pmol/μl random hexamers (Pharmacia) to prime DNA synthesis. Polymerase chain reaction (PCR) was carried out using 2 μg (each end) modestly (2-fold) degenerate primers per 50 μl PCR (see Fig. 1 for primer positions). For the first four cycles, annealing was carried out at 45°C, followed by 35 cycles with an annealing temperature of 60°C. Products of the PCR were examined by electrophoresing 10 μl (20%) of the reaction through a 3% Nusieve agarose (FMC) gel. Total PCRs were blunt-end-ligated into the pBluescript II (SK−) vector (Stratagene) for 24 hours at 12˚C and ligation products were used to first binding to oligo(dT)-biotin followed by separation was isolated from extracts of total cellular RNA by hybridization (10 7 cts/minute/ml) overnight at 42˚C in stringency (0.1× the presence of 50% formamide, were washed at high −70˚C.

We screened an approx. stage 29 (embryonic day 6) chick head cDNA library (a kind gift from Dr D. Johnston, University of California, San Francisco), using the cloned PCR fragment as a probe. The probe was prepared by random-primed synthesis (Boehringer-Mannheim Biochemicals), following the manufacturers procedure, with the incorporation of 32P-dCTP to a specific activity of ≥106 cts/minute/μg. A single positive plaque was isolated, purified, and its 2-kb insert cloned into the EcoRI site of the pBluescript II vector (SK−). Single-stranded DNA was produced and complementary strands were sequenced, in both directions, either with the dyeoxy chain termination method (Sequenase, USB) or by using an automated sequencer (Applied Biosystems, Model 373A).

Northern blots
15 μg of total cellular RNA (extracted by the procedure described above) was fractionated by electrophoresis on a 1.5% agarose/3% formaldehyde gel, as described by Sambrook et al. (1989), transferred to a nylon membrane and immobilized by UV cross-linking. Blots were probed with the PCR product, labelled by random-primed synthesis incorporating 32P-dCTP to a specific activity of ≥106 cts/minute/μg. Blots were hybridized (105 cts/minute/ml) overnight at 42°C in the presence of 50% formamide, were washed at high stringency (0.1× SSC, 1% SDS at 65°C, 2×15 minutes), and exposed to X-ray film for 72 hours at −70°C.

For several stages of development, poly(A)+ RNA was isolated from extracts of total cellular RNA by first binding to oligo(dT)-biotin followed by separation with avidin-conjugated magnetic beads (poly(A)-tract mRNA isolation system II, Promega). 1 μg of poly(A)+ RNA was electrophoresed and blotted as described for total RNA. Blots were probed with a 32P-end-labelled 21-base oligonucleotide, complementary to CASH-1 in a region outside of and N-terminal to the bHLH-encoding region. Blots were hybridized for 4 hours at a temperature equal to the calculated Tm minus 5 to 10°C, washed at high stringency (6×SSC at 65°C, 2×15 minutes), and exposed to X-ray film for 72 hours at −70°C.

In situ hybridization
RNA probes were synthesized, using the CASH-1 cDNA as a template, from T7 (sense) and T3 (anti-sense) promoters of the pBluescript II (SK−) vector, incorporating approx. 37% digoxigenin-11-rUTP (Boehringer-Mannheim Biochemicals). Whole embryos or whole retinas were fixed in 0.1 M MOPS, 2 mM EGTA, 1 mM MgSO4 and 3.7% paraformaldehyde for 1-2 hours at room temperature or overnight at 4°C. Fixed tissue was hybridized in solution at 60°C at least 12 hours (for details see Harland, 1991). Bound probe was visualized using an alkaline phosphatase-conjugated antibody to digoxigenin (Boehringer-Mannheim Biochemicals). Alkaline phosphatase reactions were allowed to proceed for various times up to 18 hours. Reacted tissue was fixed in 4% paraformaldehyde/4% sucrose for 2 hours (4°C), frozen and sectioned at 10 μm. To analyze the distribution of CASH-1-expressing cells in the retina, sections were digitized using an MTI DS2000 integrating CCD camera, and the distances between clusters were measured using an Apple Quadra 950 with Image 1.49 software (NIH).

Immunocytochemical analyses
Following whole-mount in situ hybridization, sectioned embryos and retinas from various developmental stages were further analyzed with immunohistochemistry using the following antibodies to identify particular types of neurons: (1) anti-NF-160 (a generous gift from Dr G. Bennett, University of Florida, Gainesville), which is only expressed in ganglion cells, in the retina and in differentiated neurons throughout the rest of the CNS; (2) HPC-1, which is present in a subpopulation of amacrine cells in the retina (a gift from Dr C. Barnstable, Yale University; Barnstable, 1980); (3) Rho-4D2 (a gift from Dr R. Molday, University of British Colombia, Canada and Dr D. Hicks, Strasbourg, France), which labels rod photoreceptors and (4) Visinin (a gift from Dr A. Polans, Dow Neurological Institute, Portland, OR), which labels cone receptors. Tissue sections were prepared for antibody binding by blocking with 1% BSA in PBS. Primary antibody was allowed to bind to sections overnight at 4°C and visualized using a FITC-conjugated secondary antibody, diluted 1:300 in PBS (Cappel).

To determine the number and distribution of progenitor cells at various stages, whole retina explants were cultured for 18 hours in F12/DMEM (Gibco) containing 10% fetal bovine serum and 0.01 mM F12/DMEM (Gibco) containing 10% fetal bovine serum and 0.01 mM

Fig. 1. The deduced amino acid sequence of the bHLH region of the CASH-1 PCR product. Modestly degenerate primers (bold) were based on conserved residues in the basic region and second helix of the bHLH domain. The nucleotide sequence of the primers (5′-3′) is: upstream (1) GCCGTGCGAGAGAAACCGG (2) GCCGTGCGAGAGAAACCGG downstream (1) CTGCAAGGCTCTGTAGTACTC (2) CTGCAAGGCTCTGTAGTACTC. The deduced amino acid sequence of CASH-1 is identical to MASH-1 and XASH-1 over this region, but exhibits less similarity (approx. 65-70%) to MASH-2 and the Drosophila AS-C products.
bromodeoxyuridine (BrdU, Boehringer-Mannheim). After 24 hours, the retinas were fixed in 4% paraformaldehyde for 2 hours (4°C), frozen and sectioned at 10 μm. Cells that had taken up BrdU during the culture period were visualized using an anti-BrdU antibody (Becton-Dickenson).

RESULTS

Identification and sequencing

We used a degenerate polymerase chain reaction (PCR) strategy to identify AS-C homologs in the chicken retina. In an effort to identify bHLH family DNA-binding transcription factors, the design of our upstream primer set was based on amino acids in the basic region, while the downstream primer set was based on the second helix of the bHLH (Fig. 1). This approach has been successful in the identification of achaete-scute homologs in the chicken retina. In an effort to use a PCR strategy to identify AS-C homologs in the chicken retina, we screened a stage 29 (embryonic day 6) chicken head cDNA library. A single positive plaque containing one AS-C clone was isolated, which contained an insert of approx. 1.9 kb. We believe that our 1.9 kb cDNA includes the entirety of the AS-C protein coding region as well as several hundred base pairs of 5' and 3' untranslated sequence, since northern blot analysis reveals a species of approx. 2 kb (see below). The CASH-1 cDNA has a 657-nt open reading frame encoding two in-frame methionines, spaced 36 nt apart, and a single stop codon. Of the two potential translation initiation sites, the first better fits the consensus for eukaryotic translation initiation.

Fig. 2. Sequence of the 1.9 kb CASH-1 encoding cDNA determined by the dyeodeoxy chain termination method (Sanger et al., 1977). The deduced amino acid sequence of the coding region is also shown. The basic region of the bHLH domain is shown in bold. This cDNA also encodes several additional potential functional domains. Near the N terminus, there is a stretch of basic residues, which have appropriately sized inserts and, of these, seven were randomly selected and sequenced. One of the seven was identical to Drosophila AS-C family members over various HLH-containing proteins, and provides a means for assessing product heterogeneity based on differential electrotheric mobility.

PCR, of random hexamer reverse-transcribed total RNA extracted from stage 24 (embryonic day 4) retina, yielded a single band of approx. 140 bp, which was cloned into pBluescript II (described in Materials and Methods). Thirty clones were selected, all of which had appropriately sized inserts and, of these, seven were randomly selected and sequenced. One of the seven was identical to MASH-1 and XASH1 and 70% identical to AS-C products, at the deduced amino acid level (Fig. 1), while others were unrelated. The nucleotide sequence of our PCR product exhibits 88% identity with MASH-1, 85% identity with XASH1, and 65-69% identity with Drosophila AS-C family members over the corresponding bHLH-encoding region (Fig. 2).

Using our PCR product as a probe, we screened a stage 29 (embryonic day 6) chicken head cDNA library. A single positive plaque was isolated, which contained an insert of approx. 1.9 kb. We believe that our 1.9 kb cDNA includes the entirety of the CASH-1 protein coding region as well as several hundred base pairs of 5' and 3' untranslated sequence, since northern blot analysis reveals a species of approx. 2 kb (see below). The CASH-1 cDNA has a 657-nt open reading frame encoding two in-frame methionines, spaced 36 nt apart, and a single stop codon. Of the two potential translation initiation sites, the first better fits the consensus for eukaryotic translation initiation.

CASH in the nervous system

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A best-fit sequence analysis of the coding region revealed 81% identity with MASH-1 at the nucleotide level (over 727 bp; Fig. 2). There is no homology between CASH-1 and members of the AS-C or MASH-2 beyond the bHLH-encoding region. We have also sequenced approx. 1 kb of 3’ untruncated sequence. There is no significant homology between MASH-1 and CASH-1 in this 3’ region. In addition to the bHLH, CASH-1 possesses several other potential functional domains. Amino-terminal to the bHLH there is a proline-rich region and an alanine-rich region, both of which may function in transcriptional modulation (Mitchell and Tjian, 1989). Although the alanine-rich region is also present in MASH-1, the proline-rich region of CASH-1 substitutes for a glutamic acid-rich region found in MASH-1. The XASH-1 cDNA does not encode either of these domains. The CASH-1 cDNA also encodes a potential nuclear localization signal (Fig. 2), which is similar to that found in XASH-1, and which loosely fits the consensus described by Garcia-Bustos et al. (1991). C-terminal to the bHLH domain there are serine, threonine and tyrosine residues which represent potential phosphorylation sites. One of the serine residues lies within a consensus sequence which conforms to the serine/threonine kinase consensus sequence shared by c-myc and c-jun (Pearson and Kemp, 1991; Alvarez et al., 1991), while several others fit the consensus for phosphorylation by casein kinase I (Pearson and Kemp, 1991). There are several tyrosine residues which represent potential sites for phosphorylation, similar to those described by Villares and Cabrera (1987) for AS-C proteins. However, since tyrosine kinase substrate sequences do not exhibit consensus recognition motifs (Pearson and Kemp, 1991) it is difficult to determine the significance of these residues. Thus, although MASH-1 and XASH1 share considerable sequence identity with CASH-1, each of these genes has unique domains outside the bHLH. The different domains may reflect species differences in regulation and/or usage (e.g. different effectors, downstream genes) of achaete-scute homologs (ASHs), but it is difficult to determine, from our current level of understanding, whether they all perform the same (homologous) function in the cells in which they are expressed.

Analysis of CASH-1 expression

We used both northern blot and in situ hybridization to examine CASH-1 expression. Northern blot analysis, with a random-primed probe containing bHLH-encoding sequences (Fig. 3A) or an oligonucleotide probe outside the bHLH region (Fig. 3B), reveals the presence of a single major transcript of approx. 2 kb and a minor species of 1.2 kb in several different brain regions, including diencephalon, midbrain and telencephalon at stage 28 (embryonic day 5.5). The highest level of expression was present in the midbrain. Although message for CASH-1 was not detectable in the eye at stage 28 from total cellular RNA blots, we were able to detect message for CASH-1 by in situ hybridization at this stage and in retina at later stages using poly(A)+-selected RNA (Fig. 3B). The same two species were found in the blots of retinal RNA as we had observed in blots from other CNS regions (Fig. 3A).

To characterize further the cells that express this gene during development of the chick, we undertook a series of in situ localization studies. The overall distribution of CASH-1 expression in stage 14 (50-53 hours of incubation), stage 17 (52-64 hours of incubation), and stage 20 (70-72 hours of incubation) embryos was examined with whole-mount in situ localization, followed by serial sectioning. At stage 14, CASH-1-expressing cells are present in the ventral telencephalon, the optic stalk, the diencephalon, the midbrain (not shown) and the rhombencephalon (Fig. 7A). In all of these regions, except midbrain, only a few expressing cells can be discerned; midbrain contains many CASH-1-expressing cells even at this early age. The number of CASH-1-expressing cells increases in all of these regions as development proceeds (see below), suggesting that in many regions of the CNS stage 14 represents the onset of CASH-1 expression. Fig. 4 shows the distribution of expressing cells in a sagittal section through the stage 17 embryo. At this stage there is a small patch of cells expressing CASH-1 in the ventral prosencephalon, a part of the basal prosencephalic plate (Fig. 4A, single arrowhead). A few cells in the anterior prosencephalon also express the gene. At this stage, however, there is no expression of CASH-1 in either the retina or the pigment epithelium (Fig. 4B). The dorsal mesencephalon has a large number of intensely labelled, densely packed CASH-1-expressing cells (Fig. 4C). The most anterior part of the rhombencephalon, probably the prospective metencephalon, contains cells that express CASH-1 (Fig. 4A, double arrowhead). Additionally, ventricular zone cells within and adjacent to the developing trigeminal nucleus express CASH-1 (Fig. 4A, open triangle). There are distinct groups of CASH-1-expressing cells in several of the rhombomeres (Fig. 4D, arrowheads). In a caudal to rostral sequence, CASH-1-expressing cells are present in the first, third (very intense), fourth, and fifth (slightly) rhombomeres, but not in the second or any rhombomeres rostral to the fifth. Throughout the rhomben-
cephalon, sagittal sections show two distinct stripes of labelled cells extending through the rhombencephalon and the full extent of the spinal cord (Fig. 4A, arrows). Transverse sections through the developing rhombencephalon and the spinal cord, which better show the distribution of these cells, reveal that the ventricular zone immediately adjacent to the general somatic afferent cell column possesses the highest density of CASH-1-expressing cells in the rhombencephalon (not shown for stage 17). There is a lower level of expression throughout both the alar and basal plates, both in the rhombencephalon and extending the length of the spinal cord. The cells that express this gene appear to stop abruptly at the margin of the floor plate, and immediately adjacent to the floor plate a column of cells that express CASH-1 at very high levels extends throughout the rhombencephalon and the spinal cord (Fig. 6G shows this for a stage 20 embryo, which exhibits an identical pattern to that of stage 17). Scattered cells in the preaortic ganglia express CASH-1 at this stage, but there is little or no expression of the gene in the dorsal root ganglia or other neural crest derivatives (not shown).

At stage 20, CASH-1-expressing cells persist in the elaborated derivatives of all stage 17-expressing regions and in areas in which CASH-1 expression was undetectable at stage 17. The ventral telencephalic plate has expanded laterally, and the CASH-1-expressing cells are present in the ventricular zone of the most lateral extent of the telencephalic vesicles (Fig. 5A). By contrast, labelled cells are not found in the dorsal telencephalon. The diencephalon has expanded considerably by this stage, and here cells in the infundibulum and ventral lateral diencephalic plates now express CASH-1. There is no expression in the retina by stage 20. Despite the paucity of CASH-1-expressing cells in the retina at these early embryonic stages, a few (approx. 3) expressing cells can be identified in the optic stalk as early as

Fig. 4. Stage 17 embryo. (A) Sagittal view of a stage 17 chick embryo which has been subjected to in situ hybridization in solution using a digoxigenin-labelled RNA probe derived from the 1.9 kb CASH-1 cDNA. (arrowhead, ventral telencephalon; double arrowhead, prospective metencephalon; open triangle, trigeminal ganglion; arrows, dorsal and ventral spinal cord expression). (B) A higher magnification of a stage 17 eye. CASH-1 is not expressed anywhere in the eye at this stage. (C) A higher magnification of a stage 17 midbrain. Midbrain possesses the highest density of CASH-1-expressing cells. Scale bar, 40 μm. (D) A higher magnification of stage 17 hindbrain showing differential expression of CASH-1 in the rhombomeres (arrowheads). This section of hindbrain is lateral to that shown in A, but was selected because it gives better rhombomere resolution. Scale bars, 110 μm (A), 40 μm (B,C) and 70 μm (D).
stage 14 and this number increases slightly by stage 20 (Fig. 5A). By stage 20, the dorsal mesencephalon is greatly enlarged, and virtually the entire tectal plate contains intensely labelled cells. This region of the CNS remains as one of the highest expressing areas. The \textit{CASH-1}-expressing cells reach the anterior pole of the developing tectum, while the ventricular cells at the posterior pole do not express the gene; the change in \textit{CASH-1} expression may represent the division

**Fig. 5.** Stage 20 embryo. (A) Sagittal section showing \textit{CASH-1}-expressing cells in the ventral telencephalic vesicles (T) as well as the optic stalk (OS, arrowheads). (B) Sagittal section showing \textit{CASH-1}-expressing cells in the rostral two-thirds of the midbrain. Cells that express \textit{CASH-1} are not present in the caudal third of the mesencephalon, which may represent a distinction between optic and auditory regions of the mesencephalon (arrowhead). Also, there is a paucity of \textit{CASH-1} expression in cells at the meso-metencephalic fold (arrow) and in metencephalic regions which will give rise to cerebellum. (C) Sagittal section showing \textit{CASH-1} expression in the ventricular zone underlying a large nuclear group in the ventral mesencephalon. The highly expressing cell population of the dorsal mesencephalon has been folded over on itself and is, therefore, not easily visible. (D) Cross-section of the hindbrain. \textit{CASH-1}-expressing cells are located in both ventral (arrows), adjacent to the general somatic efferent cell column, and dorsal (arrowheads), adjacent to the general somatic afferent cell column, positions. \textit{CASH-1}-expressing cells are not found in the floorplate. Scale bars, 45 µm (A), 60 µm (B,C,D).

**Fig. 6.** \textit{CASH-1} in situ retina sections reacted with antibodies that recognize the 160×10^3 M_r neurofilament subunit reveal that although both \textit{CASH-1}-expressing and NF-160×10^3 M_r-immunoreactive cells may be present in a given region, the two cell types occupy discrete and non-overlapping positions. (A,B) Sagittal section of a stage 17 embryo showing otic vesicle. There are a few \textit{CASH-1}-expressing cells in otic vesicle at this time (B, arrowheads), although there are not any NF-160×10^3 M_r-immunoreactive cells (A). (C,D) Sagittal section of a stage 17 embryo showing the trigeminal ganglion. NF-immunoreactive cells and their processes outline the perimeter of the ganglion. In contrast, \textit{CASH-1}-expressing cells are present in the center of the ganglion, a region devoid of NF-immunoreactive cells. (E,F) Sagittal section of a stage 20 embryo showing the rhombomere (#3) which exhibits the highest density of \textit{CASH-1}-expressing cells. Caudal is up and ventral is to the right. NF-immunoreactive cells are restricted to the ventral surface of this structure (E), while \textit{CASH-1}-expressing cells occupy more dorsal positions (F). Interestingly, there appears to be heterogeneity in the level of \textit{CASH-1}-expression — rostral to the highest expressing cell cluster (arrowhead) is a patch of cells which appear to express an intermediate level of \textit{CASH-1} mRNA (arrow), while caudal to the highest expressing region is a zone of cells which do not express any detectable \textit{CASH-1} mRNA. (G,H) Cross-section of a stage 20 embryo at the level of the hindbrain. NF-160×10^3 M_r-immunoreactive cells and many of their processes are restricted to the ventral margins of the ventricular zone (G). \textit{CASH-1}-expressing cells are present in the ventral hindbrain, flanking, but conspicuously absent from, the floor plate. Scale bar, 45 µm (A-H).
between the developing optic and auditory tectal regions (Fig. 5B). At this stage, the meso-metencephalic fold has established a distinction between the midbrain and the developing metencephalon (Fig. 5B). The ventral and lateral regions of the metencephalon contain CASH-1-expressing cells, while the more dorsal regions contain few, if any, labelled cells. In addition, there are CASH-1-expressing cells in the ventricular zone underlying a large nuclear group in the ventral mesencephalon (Fig. 5C). The pattern of labelling in the rhombencephalon and spinal cord is essentially the same as that described for stage 17, with CASH-1-expressing cells in two major zones, which probably correspond to the progenitor cells that give rise to the general somatic afferent and general somatic efferent cell columns (Fig. 5D).

Chick embryos were also processed for combined in situ localization of CASH-1 and immunohistochemical localization of neurofilament (Bennett and DiLullo, 1985) to identify some differentiated neurons at stages 17 and 20. Fig. 6 demonstrates that at both stage 17 and 20, none of the neurofilament immunoreactive cells in any CNS region co-express CASH-1. At stage 17, very few scattered CASH-1-expressing cells are present in the otic vesicle (Fig. 6B), which possesses no neurofilament immunoreactive cells (Fig. 6A) at this stage. The stage 17 developing trigeminal ganglion contains cells that express CASH-1; however, the CASH-1-expressing cells are not immunoreactive for neurofilament proteins (Fig. 6C,D). At stage 20, neurofilament immunoreactive cells are abundant in the medulla, as are CASH-1-expressing cells (Fig. 6E,F), but CASH-1 and neurofilament expression are mutually exclusive. A cross-section of a stage 20 hindbrain is shown in Fig. 6G.H. Although many differentiating neurons are immunoreactive for neurofilament in this region, none of the CASH-1-expressing cells also express neurofilament (Fig. 6G,H).

Expression during retinogenesis

We were particularly interested in examining the distribution of CASH-1-expressing cells in the retina, since this was where we initially identified this gene and since the dynamic changes in the expression pattern of this gene might be better understood in the retina, where the details of the neurogenesis are more well characterized than in most other CASH-1-expressing areas. As stated above, there is no CASH-1 expression in the retina at stage 20. By stage 24 a few CASH-1-expressing cells are present in the central retina (Fig. 7C), although no expressing cells can be detected yet in the less mature periphery (Fig. 7B). As development proceeds, CASH-1-expressing cells become organized into radially arrayed clusters that span the neuroepithelium. As histogenesis continues, the number of CASH-1-expressing cells per cluster and the number of clusters across the retina increases. By stage 30 (embryonic day 6.5), the number of clusters, as well as the number of CASH-1-expressing cells per cluster, has increased substantially (Fig. 7D), and by stage 33-34 (embryonic day 7.5-8) the number of CASH-1-expressing cells has reached its peak (not shown). Importantly, while the number of CASH-1-expressing cells per cluster increases between stage 30 and stage 33-34, the cluster density does not change; the number of intervening non-CASH-1-expressing cells decreases. At stage 36 (embryonic day 10), individual CASH-1-expressing cells are scattered throughout the retinal inner nuclear layer and are no longer grouped into clusters (Fig. 7F). Cells in the pigment epithelial layer do not express the gene at this, or any, stage examined.

To investigate a possible correlation between the expression of CASH-1 and progenitor cell proliferation, retinas were cultured for 18 hours in the presence of BrdU. As shown in Fig. 7E, at stage 30, cells which have incorporated BrdU, and were therefore mitotically active during the culture period, are evenly distributed across the retina. This distribution is in contrast to the periodicity displayed by CASH-1-expressing cells at the same stage. From this experiment it appears that CASH-1 is expressed in a population of the mitotically active neuroepithelial cells in the retina at this stage.

To gain a better understanding of the temporal pattern of expression of CASH-1 in the retina, we counted the number of CASH-1-expressing cells in the neuroblastic layer (i.e. those cells outside the ganglion cell layer) as a percentage of cells at different ages (Fig. 8A). The percentage of cells expressing CASH-1 steadily increases from stage 24 and peaks three days later, at stage 33, when CASH-1-expressing cells make up approx. 65% of the total cells in the neuroblastic layer. These data can be directly compared to previous [3H]thymidine birth-dating studies of the developing chick retina (Fig. 8B; Prada et al., 1991). From this figure, it is apparent that CASH-1 expression occurs in the progenitor cells relatively late in the generation of the various retinal phenotypes. Although birth-dating analysis of the chick retina (Prada et al., 1991) has shown a great deal of overlap in the generation of retinal cell types, the peak of CASH-1 expression in the retina corresponds to a time when some of the later-appearing retinal cell classes are being generated.

Because the number of CASH-1-expressing cells increases in concert with an increase in the generation of some of the later-appearing retinal cell classes, we were interested in determining whether the differentiation of any retinal cell type more closely parallels the expression of CASH-1. Of the different retinal cell types that we examined with cell-type-specific antibodies, we found that the expression of CASH-1 in progenitor cells correlates best with the generation of amacrine cells. Fig. 9 shows sections from whole-mount CASH-1 in situ localizations which have also been processed by immunohistochemistry for HPC-1 (Fig. 9E,F), an amacrine cell-specific protein, and 160x10^3 M_i neurofilament subunit antibodies (Fig. 9A-D), specific for ganglion cells, in the chick retina. As noted above, the cells of the retinal neuroepithelium do not express CASH-1 at stage 17; however, already by stage 17, thymidine birthdating (Prada et al., 1991) and immunohistochemical labelling with neurofilament antibodies (Fig. 9A,B) indicate that the first ganglion cells have become postmitotic. Therefore, CASH-1 expression in the retinal neuroepithelium does not appear to be necessary for the generation of retinal ganglion cells. In central retina at stage 30, CASH-1-expressing progenitor cells are flanked by HPC-1-immunoreactive amacrine cells at the vitreal surface (Fig. 9E,F: arrows) and by newly generated, premitotary, HPC-1-immunoreactive amacrine cells at the scleral surface (Fig. 9E,F: arrowheads). Moreover, CASH-1-expressing cells are most abundant in central stage 30 retina, where amacrine cells are being actively generated, and appear to decrease in correspondence with a decrease in amacrine cell generation moving into the less mature peripheral retina (not shown). As noted above, there does not appear to be a similar correlation of CASH-1
Fig. 7. CASH-1-expressing cells are distributed in a unique spatial array in the developing retina and hindbrain, where they comprise only a subpopulation of BrdU-incorporating cells. (A) A cross section of a stage 14 hindbrain. CASH-1-expressing cells (arrows) flank the floor plate. Floor plate cells themselves do not express CASH-1 nor do those of the notochord (N). (B) Peripheral stage 24 retina. At this age there are not yet any CASH-1-expressing cells in the periphery of the retina. PE, pigment epithelium; NE, neuroepithelium. (C) Central stage 24 retina. The first CASH-1-expressing cells (arrows) are present and can be found at both the ventricular (pair of labeled cells adjacent to PE) and vitreal surfaces. PE, pigment epithelium; NE, neuroepithelium. (D) Central stage 30 retina. CASH-1-expressing cells are present throughout the neuroepithelium (NE), but are absent from the ganglion cell layer (GCL) and the pigment epithelium (PE). Expressing cells are organized in a repeating array of clusters (arrows). (E) Central stage 30 retina, which had been cultured as an explant for 18 hours in the presence of BrdU. BrdU-incorporating cells are localized almost exclusively to the neuroblast zone in which CASH-1-expressing cells are also located. (F) Central stage 36 retina. Individual CASH-1-expressing cells (arrows) are scattered through the inner nuclear layer. Scale bar, 45 µm for A,B,C; 25 µm for D,E,F.
expression and ganglion cell production; NF-immunoreactive ganglion cells are being generated in the far peripheral retina at stage 30, but there is only a low level of CASH-1 expression in the peripheral retina at this stage (Fig. 9C,D). In addition, neither of the photoreceptor cell-specific antibodies that we tested — Rho 4D2 and visinin — showed the presence of photoreceptors prior to the appearance of CASH-1-expressing cells (not shown), although it should be noted that neither of these stain normal retina until quite a few days after the time at which photoreceptor progenitor cells undergo their terminal mitosis, as judged by \(^{3}H\)thymidine incorporation birthdating studies (Prada et al., 1991).

An especially intriguing feature of CASH-1 expression in the retina is that clusters of CASH-1-expressing cells are periodically distributed across the retina and these clusters vary in size at different developmental ages. We have used video imaging (see Materials and Methods) to analyze the changing distribution of CASH-1-expressing cells across the retina during development; the results are shown in Fig. 10. At the peak expression ages (stage 30-34), the density or periodicity of clusters varies across the retina, such that in central retina clusters are closely apposed (Fig. 7D), while in mid-peripheral retina, clusters are spaced farther apart. Although CASH-1-expressing cells are still detectable near the ciliary margin, the distance between clusters is quite large in peripheral retina, such that there is no longer any discernible periodicity (Fig. 10). Also, peripheral ‘clusters’ consist of fewer cells than those in central and mid-peripheral locations (not shown). As development proceeds there is a change in the spacing of the clusters; clusters are widely spaced at the onset of expression and increase in density as expression peaks (Fig. 10). As expression declines, the periodicity breaks down, such that the remaining expressing cells appear to be randomly scattered across the retina.

**DISCUSSION**

We have identified an achaete-scute homolog, CASH-1, a basic helix-loop-helix-containing protein in the chicken that is homologous to the Drosophila achaete-scute family of transcription factors. CASH-1 is expressed in the developing chick nervous system in a region- and stage-specific manner. CASH-1-expressing cells are most abundant in the midbrain, and are also present in the developing spinal cord, hindbrain, diencephalon and telencephalon. Moreover, throughout the CNS, CASH-1 is expressed in cells of the ventricular neuroepithelium and not in regions occupied by neurofilament-expressing differentiated neurons. The pattern of CASH-1 expression is similar to the pattern of expression observed for MASH-1, one of two mammalian achaete-scute homologs (Johnson et al., 1990; Lo et al., 1991) and XASH-1, a similar gene expressed in the Xenopus nervous system (Ferreiro et al., 1992). The high degree of sequence conservation, as well as the similarities in the anatomical and developmental localization of expression between the chicken, the rat and the frog, suggests that the functions of these genes may be conserved. Moreover, the pattern of expression of CASH-1 is distinctly different from that of the other achaete-scute homologs that have been described, namely MASH-2, which is not specific to the nervous system and XASH-3, which is expressed earlier in the developing nervous system, in cells near the sulcus limitans (Zimmerman et al., 1993).

**CASH-1 expression in the retina is restricted to a subpopulation of the dividing progenitor cell population**

In the retina, the anatomical location of CASH-1-expressing cells suggests that CASH-1 is expressed by a subpopulation of progenitor cells whose density varies both temporally and spatially across the retina. At early stages of retinal development, none of the progenitor cells express this gene; however, as development proceeds, an increasing fraction of the progenitor population expresses CASH-1, until at the peak of expression at stage 32-34, most if not all of the progenitor cells...
The expression of the gene. The fact that CASH-1 expression appears to be restricted to the latter part of retinal histogenesis, may provide some indication as to its function in neurogenesis in the retina, as well as in other areas of the CNS (see below). We have found that CASH-1 is not expressed in progenitor cells prior to, or concomitant with, the onset of ganglion cell commitment or differentiation in the chick retina. This is also likely to be the case in mammals; MASH-1 is not expressed in progenitor cells in the early embryonic rat retina (Lo et al., 1991), but is expressed at later developmental times (Jasoni and Reh, 1993). By contrast, XASH1, which is expressed in the frog retina, is present throughout the period of retinal neurogenesis. The difference in expression between species may be due to a difference in the function of this gene in neurogen-
esis, or alternatively, may simply reflect differences in the timing of cell generation between chicken and frog. Despite their temporal differences in expression, all three homologs are similar in that they appear to be expressed by a subpopulation of proliferating neuroepithelial cells.

**CASH 1 is expressed in a repetitive array in the developing retina**

Although CASH-1 is expressed in the mitotically active retinal progenitor cells, at any particular stage in development its expression is confined to a subset of the progenitor cells, which are organized in radially arrayed repeating clusters. The intriguing periodicity displayed by CASH-1-expressing cells is reminiscent of the distinct spatial array of AS-C-expressing cells seen in the CNS and thoracic imaginal discs (precursors to PNS) of Drosophila (Dambly-Chaudiere and Ghysen, 1986, Cabrera et al., 1987), a pattern which reflects the differentiation of SMCs. It is thought that the AS-C genes somehow ‘read’ the gridwork of positional information available to them from previously expressed positionally restricted genes, i.e. gap, pair rule, segment polarity and homeotic genes. The mechanisms by which this positional information is translated into the restricted expression of AS-C, in specific locations within the developing cuticle, are not understood.

Might CASH-1 be important in setting the periodicity of some aspect of the retinal mosaic, in a manner analogous to that of the Drosophila achaete-scute genes? Many of the cell types found in the mature vertebrate retina show a regular periodic arrangement; these repetitive arrays of differentiated neurons are known as mosaics. Both the pattern and precision...
of this mosaicism is unique for different cell types, and appears to be independent of cell density (Wassle and Riemann, 1978). Recent evidence on the development of the cone mosaic (Wikler and Rakic, 1991; Mack and Fernand, 1992) and the ganglion cell mosaic (M. W. Kelley and T. A. Reh, unpublished observations) suggests that the orderly arrangement of these cells occurs soon after their generation, rather than as a later recruitment or sharpening. Therefore it is possible that mosaicism is the result of a discrete pattern of progenitor differentiation across the retina. A mechanism that controls the differentiation of particular cell types at regularly spaced positions during development would be consistent with the periodicity of CASH-1 expression in a subset of the progenitor cells, and by analogy with Drosophila, this gene might be involved in the translation of axial positional information into regular arrays of cells.

**CASH 1 expression may define a particular stage of the retinal progenitor**

Another aspect of CASH-1 expression that is interesting in the light of a potential function for the protein, is that it is restricted to the latter half of retinogenesis. The generation of retinal cell types from neuroepithelial cells occurs in a precisely timed sequence, such that each cell type found in the adult retina is generated normally only at a particular period of developmental time (Sidman, 1961). Although the mechanisms responsible for orchestrating the developmentally timed appearance of retinal cell phenotypes remain to be elucidated, the expression of CASH-1 by subsets of retinal progenitor cells clearly shows, for the first time, that there are molecular differences among these cells.

As noted in the Introduction, in the Drosophila cuticle, members of the AS-C gene family appear to confer competence to generate SMCs which then go on to generate the SOs. Moreover, individual members of the AS-C, which specify different SO types or regions of the central nervous system, are expressed in different subsets of the neuroblast population. Genetic analyses have revealed that members of the AS-C and related proteins play a role in endowing cells with the competence to become SMCs. In addition a recent study of a newly identified bHLH gene, atonal, indicates that proneural genes may also restrict the range of phenotypes the SMC can produce, to yield specific SO types. Ectopic expression of atonal preferentially gives rise to chordotonal organs rather than to external sense organs (Jarman et al., 1993). Thus, the AS-C proteins act to define a particular, transient, state of a progenitor. CASH-1 may be analogous to one of the AS-C members in that it is expressed at a progenitor population which is unique with respect to the phenotypes it is capable of generating. Moreover, CASH-1, by virtue of the complement of genes whose expression it modulates, may play a role in defining this state of progenitor differentiative ability.

Although the degree of overlap in the generation of the various retinal cell classes in the chick retina is too great to give a definitive prediction as to which cell classes are likely to relate to this gene, the data suggest that it would have to be one or more of the later-generated cell classes. Both the thymidine birthdating data, and the double labelling with various neuron-specific antibodies presented in this report, indicate that at least ganglion cells are generated prior to the expression of CASH-1 by the progenitor cells. A role for achaete-scute homologs (ASHs) in late-appearing phenotype generation is consistent with the late expression of MASH-1 in the developing rat retina, a time during which only about half of the major classes of retinal cell types are still being generated by the progenitor cells (Jasini and Reh, 1993). In frog, XASH1 is expressed throughout retinogenesis; however, birthdates of all of the various types of retinal cells roughly overlap in Xenopus (Holt et al., 1988). Thus, the hypothesis that ASHs function to restrict the progenitor population to the generation of a subset of retinal phenotypes is consistent with the observed patterns of ASH expression among the species in which they have been identified. XASH1 also differs from CASH-1 and MASH-1 in that it continues to be expressed, well after neurogenesis in central retina is complete (Ferreiro et al., 1992), by cells in the marginal zone. However, the adult frog retina retains a pool of progenitor cells (Wetts et al., 1989) at the peripheral margin, which are absent from chicken and rat retina.

Lineage studies of developing retina in mammals, chickens and frogs have shown that the progeny of single progenitor cells can be composed of any of the various major retinal cell classes (Wetts and Fraser, 1988; Turner and Cepko, 1990; Turner et al., 1990). On the other hand, it has been known for many years that the various retinal cell phenotypes are generated in a well-defined sequence, shared by most vertebrates (see Reh, 1992b for a review). These two facts have led to the general hypothesis that a series of cell inductions takes place during retinal histogenesis, such that the early differentiating cell types induce the multipotent progenitor to generate the later phenotypes. Cell ablation experiments and heterochronic co-culture experiments have both provided evidence in favor of this model (Reh and Tully, 1986; Reh, 1987, 1992a; Watanabe and Raff, 1992; Altschuler and Cepko, 1992). However, other lines of evidence have suggested that differences exist among the progenitor population from early embryonic retinas and late embryonic and postnatal retinas. First, in an attempt prematurely to induce the differentiation of progenitor cells by manipulation of their second messenger cascade, we found that while late embryonic and postnatal progenitor cells could be induced to differentiate in response to increasing levels of intracellular cyclic AMP, early embryonic progenitor cells did not respond to this treatment (Taylor and Reh, 1989). Second, progenitor cells from early embryonic retinas are stimulated to proliferate with bFGF, but not with TGFα or EGF, while late embryonic and postnatal progenitor cells respond to all of these factors (Anchan et al., 1991; Lilien and Cepko, 1992). Third, while heterochronic coculture studies have demonstrated that the developing microenvironment can influence the differentiation of rod photoreceptors in vitro, the ability of early embryonic cells to generate rods is more limited than postnatal progenitors (Watanabe and Raff, 1990). These studies taken together, support the idea that intrinsic differences exist between early and late retinal progenitors; the expression of CASH-1 appears to follow this functional distinction. Early retinal progenitors, competent to generate ganglion cells, horizontal cells and cone photoreceptors, do not require ASH-1 expression, while progenitors competent to generate later phenotypes express this gene. At intermediate stages of retinal development in the chicken, the two classes of progenitors appear to co-exist.
Transcriptional regulation of vertebrate achaete-scute homologs

Neither AS-C products (Cabrera et al., 1987; Romani et al., 1987) nor their chicken or mammalian homologs (Lo et al., 1991 and this report) are expressed by cells that are terminally differentiated. Furthermore, it appears that AS-C products are necessary, but not sufficient for progenitor cells to adopt a neuroblast fate. Results of experiments with MASH-1 point to a functional similarity between vertebrate and fly achaete-scute proteins. In mouse P19 embryonal carcinoma cells, MASH-1 expression is upregulated concomitant with differentiation along a neuronal pathway, in response to treatment with retinoic acid (Johnson et al., 1992) Misexpression of MASH-1 in undifferentiated P19 cells, however, fails to promote neuronal differentiation in the absence of retinoic acid. Additionally, transfection of fibroblasts with a MASH-1-encoding plasmid does not convert these cells to a neuronal phenotype (Johnson et al., 1990). Taken together, these data suggest that, like AS-C members, their vertebrate homologs may be necessary but not sufficient to lead to the acquisition of neuronal fate. Nevertheless, the high degree of conservation in sequence and pattern of expression suggests that these genes play an important role in neurogenesis. The results presented in this report show that for the retina, at least, this function is likely to be confined to a particular phase of neurogenesis, when specific neuronal phenotypes are generated.

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