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Evolutionary conservation of a cell fate specification gene: the *Hydra achaete-scute* homolog has proneural activity in *Drosophila*

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SUMMARY

Members of the Achaete-scute family of basic helix-loop-helix transcription factors are involved in cell fate specification in vertebrates and invertebrates. We have isolated and characterized a cnidarian *achaete-scute* homolog, *CnASH*, from *Hydra vulgaris*, a representative of an evolutionarily ancient branch of metazoans. There is a single *achaete-scute* gene in *Hydra*, and the bHLH domain of the predicted gene product shares a high degree of amino acid sequence similarity with those of vertebrate and *Drosophila* Achaete-scute proteins. In *Hydra*, *CnASH* is expressed in a subset of the interstitial cells as well as differentiation intermediates of the nematocyte pathways. In vitro translated CnASH protein can form heterodimers with the *Drosophila* bHLH protein Daughterless, and these dimers bind to consensus Achaete-scute DNA binding sites in a sequence-specific manner. Ectopic expression of *CnASH* in wild-type late third instar *Drosophila* larvae and early pupae leads to the formation of ectopic sensory organs, mimicking the effect of ectopic expression of the endogenous *achaete-scute* genes. Expression of *CnASH* in flies that are *achaete* and *scute* double mutants gives partial rescue of the mutant phenotype, comparable to the degree of rescue obtained by ectopic expression of the *Drosophila* genes. These results indicate that the *achaete-scute* type of bHLH genes for cell fate specification, as well as their mode of action, arose early and have been conserved during metazoan evolution.

Key words: basic helix-loop-helix proteins, *achaete-scute*, cell fate, *Hydra vulgaris*, nematocytes

INTRODUCTION

Identification of the mechanisms of cell fate specification is one of the fundamental questions of developmental biology. Genes that encode basic-helix-loop-helix (bHLH) transcriptional regulators are required for many types of cell fate decisions in a variety of organisms (reviewed by Murre and Baltimore, 1992). These proteins all share a common motif of a domain of basic amino acids required for DNA binding, followed by a helix-loop-helix domain involved in protein dimerization (Murre et al., 1989a,b; Davis et al., 1990; Voronova and Baltimore, 1990). Several key amino acids are shared by all bHLH proteins, and the class can be subdivided into families on the basis of conservation across the bHLH domain. These families include the myogenic determination genes such as *myoD*, the *myc* family, the *achaete-scute* genes and their homologs, and such widely expressed members as *Drosophila daughterless* and vertebrate *E12*. Except in extremely closely related homologs there is no conservation outside the bHLH domain.

The bHLH genes of the *achaete-scute* family play a role in a variety of different cell fate decisions in both vertebrates and invertebrates. In vertebrates, most of the *achaete-scute* homologs described to date are expressed during neural development (*MASH-1* in mice; Guillemot and Joyner, 1993), *XASH-1* and *XASH-3* in *Xenopus* (Ferreiro et al., 1992; Zimmerman et al., 1993), *CASH-1* in chicken (Jasoni et al., 1994) and *ZASH-1* in zebrafish (Allende and Weinberg, 1994). Also *MASH-1*-deficient mice exhibit deficiencies in both the central and peripheral nervous systems (Guillemot et al., 1993). Ectopic expression of the *Xenopus* gene *XASH-3* early in embryogenesis has been shown to convert ectodermal cells to a neural fate (Ferreiro et al., 1994; Turner and Weintraub, 1994). A second mammalian *achaete-scute* homolog, *MASH-2*, is expressed predominantly in extra-embryonic tissue, and analysis of *MASH-2*-deficient mice has demonstrated that this gene is required for the appropriate specification of trophoblasts and placental development (Guillemot et al., 1994).

Drosophila has four *achaete-scute* genes organized as the *achaete-scute* complex (*AS-C*). One of these genes, *scute*, is required at the syncitial blastoderm stage for chromosome counting and proper dosage compensation in females (Parkhurst et al., 1990; Belote, 1992; Parkhurst et al., 1993). *Scute* (*sc*) and *achaete* (*ac*) are both necessary for initiation of differentiation of both mesoderm and neuroectoderm during gastrulation (Gonzalez-Crespo and Levine, 1993), and all four

genes (*ac*, *sc*, *lethal of scute* and *asense*) are utilized in the specification of the larval central and peripheral nervous systems (reviewed by Campos-Ortega and Jan, 1991; Campuzano and Modolell, 1992). Later, during metamorphosis, *ac* and *sc* are required for the specification of sensory mother cells (SMCs) of the peripheral nervous system (PNS), which are the precursors of a variety of sensory neurons and their support cells (Ghysen and Dambly-Chaudiere, 1989).

The high degree of amino acid identity among the bHLH domains of the Achaete-scute proteins and their ability to recognize similar binding sites (Ferreiro et al., 1992; Johnson et al., 1992) leads to the speculation that the *achaete-scute* family of genes may represent an evolutionarily conserved approach to cell fate specification. If so, one might expect to find *achaete-scute* homologs in multicellular organisms that appeared early in metazoan evolution. *Hydra vulgaris* is a simple cnidarian, a group of animals that diverged from the main line of metazoan evolution before the pre-Cambrian radiation.

Here we provide evidence that *Hydra* contains a single *achaete-scute* homolog (named *CnASH* for *Cnidarian Achaete-Scute Homolog*) and that this gene is expressed in a subset of the interstitial cells and in cells of the nematocyte differentiation pathway, suggesting that it plays a role in cell fate decisions in this lineage. The predicted CnASH protein shows substantial amino acid sequence conservation with both the vertebrate and invertebrate members of the Achaete-scute family in the bHLH region. CnASH has in vitro dimerization and DNA binding properties similar to those of other Achaete-scute proteins, and has cell fate specification activity when ectopically expressed in *Drosophila*. Ectopic expression in wild-type *Drosophila* gives a phenotype indistinguishable from that obtained by ectopic expression of any one of the endogenous *AS-C* genes, and expression in an *AS-C* mutant background gives the same degree of rescue as that obtained with the fly *scute*, *lethal of scute*, or *asense* genes (Rodriguez et al., 1990; Brand et al., 1993; Dominguez and Campuzano, 1993; Hinz et al., 1994). These results suggest that although cnidarians and arthropods are separated by at least 600 million years of evolution, a fundamental developmental mechanism has been conserved.

MATERIALS AND METHODS

General molecular biology procedures

Molecular biology techniques not detailed below were carried out by standard procedures as described by Sambrook et al. (1989).

Cloning and sequencing of *CnASH*

Construction of a cDNA library in λZapII (Stratagene, La Jolla, CA) by simultaneous random and oligo(dT) priming of poly(A)$^+$ RNA from adult *Hydra vulgaris* has been described in detail by Sarras et al. (1994). An aliquot of the unamplified portion of this library was screened at low stringency by the method of Burglin et al. (1989) with a fully degenerate oligonucleotide corresponding to the amino acid sequence N(E/A)RERNRVK(L/Q)VN; to reduce the degeneracy of the oligonucleotide, all positions of 4-fold degeneracy were substituted with inosines. Filters were washed at 48°C in 3 M tetramethylammonium chloride, a temperature which should allow hybridization of the oligonucleotide to targets containing as many as 5 mismatches (Wood et al., 1985). The resulting clones were released into pBluescript by the λZAP in vivo excision process (Stratagene). Nested unidirectional deletions were constructed using the Erase-a-Base kit from Promega (Madison, WI) and sequenced using the Sequenase system from United States Biochemicals (Cleveland, OH).

In situ hybridization

Digoxigenin-labeled RNA probes corresponding to the sense and antisense strands of the 5′ portion of the *CnASH* cDNA were prepared using the Boehringer Mannheim RNA Labeling Kit for in vitro transcription. In situ hybridizations on whole mounts were carried out using a method based on that of Harland (1991) with modifications described by Nardelli-Haeflinger and Shankland (1992) and Wilkinson (1992). The procedure was additionally modified in the following ways for use with hydra. Samples were fixed overnight at 4°C, and following postfixation treatments and refixation were heated at 80°C for 30 minutes to inactivate endogenous alkaline phosphatases. Hybridization of the probe was performed at 55°C for approximately 60 hours, and posthybridization washes were carried out at the same temperature. A detailed protocol is available on request and will be published in Grens, Gee, Fisher and Bode (manuscript in preparation).

To identify *CnASH*-expressing cells with more precision, *Hydra* were macerated as described by David (1973). Animals were placed in maceration fluid (1:1:13 acetic acid: glycerol: water) for 30 minutes, and then sharply shaken once to disperse the cells. The suspension of fixed cells were more extensively fixed by being exposed to 4% paraformaldehyde for 30 minutes, after which they were spread on glass slides. In situ hybridization on macerates was performed by a modified version of the procedure described in Kurz et al. (1991). Probes and detection were the same as those used for whole mounts.

Hydroxyurea treatment of *Hydra*

Populations of dividing cells of the interstitial cell lineage were eliminated or sharply reduced by treating *Hydra* with 10 mM hydroxyurea for 3 days (Sacks and Davis, 1979). After recovery in *Hydra* medium for 4 days, a sample of animals was macerated (David, 1973), and the cell composition of the animals was determined.

Electrophoretic mobility shift assay

In vitro translated proteins for electrophoretic mobility shift assays were produced using the Promega TNT Coupled Reticulocyte Lysate System for in vitro transcription and translation of cloned cDNAs. CnASH protein was produced from the cDNA clone the sequence of which is shown in Fig. 1A, which was cloned into pBluescript under the control of the T3 promoter. The pβGda clone, a cDNA of *Drosophila daughterless* (*da*) which has been described by Van Doren et al. (1991), was a generous gift from M. Van Doren and J. Posakony.

The T5E3 probe, containing a consensus E-box sequence CAGCTG and its flanking DNA from the *Drosophila achaete* (*AS-C T5*) promoter, was as described in Van Doren et al. (1991). The T5XX3 competitor, which is identical to the T5E3 probe with the exception of 2 base changes in the E box sequence, from **C**AGC**T**G to **A**AGC**G**G, was a gift from J. Posakony. Probes were labeled and electrophoretic mobility shift assays were performed exactly as described in Van Doren et al. (1991).

Transformed *Drosophila* lines

The full-length *CnASH* cDNA shown in Fig. 1A was cloned into a CaSpeR P-element vector (Pirrota, 1988) under the control of the *Drosophila heat shock 70* (*hsp70*) promoter. Germline transformants were obtained by P-element mediated transformation of syncitial Oregon R *white*^{1} embryos as described in Rubin and Spradling (1982). Two independent homozygous *CnASH*/*CnASH* lines were established and all subsequent experiments were performed with both lines. All flies were maintained and crossed under standard conditions at 22°-25°C except during heat shock periods.

Heat shock induction of *CnASH*

Embryos were collected from *CnASH*/*CnASH* transformants and control flies for a 24-hour period and maintained at 25°C until they had reached the late third larval instar. When the first larvae had begun to form pupae, a series of four heat shocks was performed. Each heat shock consisted of a 1- hour incubation at 37°C, followed by a 2-hour recovery period at 25°C. After the final heat shock the animals were returned to 25°C until adult flies had emerged from the pupal cases.

$Sc^{10\text{-}1}$ flies, containing a chromosomal breakpoint near the transcription start site of the *ac* gene and a nonsense mutation in the *sc* coding sequence, have been described in detail previously (Campuzano et al., 1985; Villares and Cabrera, 1987). $Sc^{10\text{-}1}$/*CnASH* flies were generated and back-crossed to obtain embryos carrying two copies of *hsp70-CnASH*. These embryos were maintained and heat shocked as described above. Because $sc^{10\text{-}1}$ flies generally fail to fully emerge from the pupal cases, partially emerged flies were dissected out by hand.

RESULTS

Isolation and characterization of *CnASH*

A fully degenerate oligonucleotide encoding the amino acids N(E/A)RERNRVK(L/Q)VN, which have been conserved in the DNA binding domain and first helix of all known *achaete-scute* genes (see Fig. 1B), was used to screen an unamplified cDNA library derived from adult *Hydra vulgaris*. Six independent, overlapping *CnASH* cDNAs were isolated, one of which was an essentially full length clone. The complete cDNA sequence and predicted protein product are shown in Fig. 1A. The 875 bp cDNA encodes a deduced protein of 173 amino acids, with a predicted relative molecular mass of 19.8×10^3. While this gene product is the smallest member of the Achaete-scute family so far identified, it contains a complete bHLH domain with the amino acid sequence characteristic of all Achaete-scute proteins.

Comparison of the amino acid sequence of the basic region and the two helices, shown in Fig. 1B, demonstrates that CnASH is equally related to the Achaete-scute proteins found in vertebrates and in *Drosophila*. As has been previously observed in other comparisons of *achaete-scute* gene products, outside of the bHLH region CnASH diverges from all other members of this family (Johnson et al., 1990; Zimmerman et al., 1993; Allende and Weinberg, 1994; Jasoni et al., 1994). The loop portion of the bHLH domain is also not conserved, varying in both length and amino acid sequence among the Achaete-scute family of proteins. *CnASH* is clearly an *achaete-scute* homolog, as comparison of its bHLH region with that of other families of bHLH proteins shows a much lower degree of amino acid sequence identity. The only conserved amino acids are those found in the general bHLH consensus sequence (residues indicated by * in Fig. 1B).

Southern analysis shows that there is only one *achaete-scute* homolog in *Hydra* (data not shown), in contrast to the multiple genes observed in other species (shown in Fig. 1B). This gene gives rise to a single transcript of approx. 950 bases (data not shown), which is consistent with the fact that all six of the *CnASH* cDNA clones had identical sequences.

CnASH is expressed in the nematocyte differentiation pathway in *Hydra*

A *Hydra* is composed of two epithelial layers, the ectoderm and endoderm that are separated by the mesoglea, a typical basement membrane. The cells of *Hydra* fall into three lineages that correspond closely with the structure of the animal. The cell types of each epithelial layer constitute a lineage, thereby accounting for two of three lineages. All the remaining cells reside in the interstices among the epithelial cells, and are part of the interstitial cell lineage. To determine the overall expression pattern of *CnASH*, as well as which cell lineage it is expressed in, in situ hybridization was carried out on whole mounts using a probe that excluded the conserved bHLH region. As shown in Fig 2A, the gene is expressed in isolated cells or groups of cells in the body column, but not in the head or foot. The size and location of the labeled cells suggest they are part of interstitial cell lineage.

The interstitial cell lineage consists of interstitial cells and three classes of somatic differentiation products: neurons (of which there are several types), nematocytes (four types) and secretory cells (two types) (e.g. Bode and David, 1978). A subset of the interstitial cells are multipotent stem cells that give rise to the differentiation products (David and Murphy, 1977), while the remainder are early differentiation intermediates. Because of the tissue dynamics of the adult *Hydra*, the stem cells of this lineage are continuously producing cells of each class to compensate for their continuous loss. At the same time, the stem cells of the two epithelial lineages also continue to divide and generate epithelial differentiation products located at the extremities of the animal (David and Campbell, 1972; Dubel et al., 1987).

The regional distribution of the labeled cells reduces the range of cell types that could be expressing *CnASH*. The large majority of the interstitial cells and the differentiation intermediates are in the ectoderm of the body column, while the majority of the mature neurons, nematocytes and one type of secretory cells are in the head and foot (Bode et al., 1973). The other secretory cell type, the gland cell, is found in the body column in the endoderm. The absence of labeled cells in the extremities suggests that the differentiation products are not expressing the *CnASH* gene. Instead, the labeled cells may be interstitial cells or differentiation intermediates.

One approach to determining whether the labeled cells in the whole mounts are interstitial cells or differentiation intermediates is to take advantage of the fact that all these cell types are in the mitotic cycle. These cells have cell cycle times of 18-24 hours, and 50-75% of their cell cycle is composed of S-phase (Campbell and David, 1974). Continuous treatment of animals with hydroxyurea sharply reduces, and within 3-4 days eliminates, these cell populations (Sacks and Davis, 1979). This treatment also affects epithelial cells but to a much lesser extent, as they have a cell cycle time of 3-4 days (David and Campbell, 1972). Animals were treated with hydroxyurea for 3 days, which reduced the interstitial cell populations to <5% of normal and the differentiation intermediates to <1%, and were subsequently examined as whole mounts for *CnASH* expression. As shown in Fig. 2B, the cells capable of expressing *CnASH* were absent, or in other samples reduced to a few labeled cells per animal. This indicated that they were cell types of the interstitial cell lineage capable of division. Analysis of RNA isolated from hydroxyurea-treated animals provided additional evidence (data not shown). Both northern analysis and RT-PCR demonstrated that *CnASH* RNA, which is readily detectable in untreated animals, was severely reduced in hydroxyurea-treated animals.

A
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  1  CTATTTTCTTAATAAAGGGTAAATACACTTTTTAAAAAGCTATCAATCACCAGGTTCATA

 61  CAACTACACAATCGCTGCCGGTAAAAATAAACTGTAAAAATAACAGGCGCCACCATGCAA
                                                            M  Q
121  CTTTTGTTTCCAAGGCCATTATTAAATGAGCATATGGTAATAAATAATCAGTTATTTACT
      L  L  F  P  R  P  L  L  N  E  H  M  V  I  N  N  Q  L  F  T
181  AAGTATGAACAAATTAATGGATATACCTCCACCGTCATCTGCCATCCATGGAGTTCGGAA
      K  Y  E  Q  I  N  E  Y  T  S  T  V  I  C  H  P  W  S  S  E
241  AACGGTGGTGGAAAATTTAGAAGAAGACGTTCTAGTCATAGTGTTGTCGCAAATATGGAT
      N  G  G  G  K  F  R  R  R  R  S  S  H  S  V  V  A  N  M  D
                                basic region
301  CCAGCTGCAGTGGCACGAAGAAACGAACGAGAAAGAAATCGTGTAAAGCAGGTCAATGAT
      P  A  A  V  A  R  R  N  E  R  E  R  N  R  V  K  Q  V  N  D
       helix                                          loop
361  GGCTTTGATGAACTTAGGCAGAGAGTACCTTTTTTACCAGATAAGAAAAAATTATCAAAA
      G  F  D  E  L  R  Q  R  V  P  F  L  P  D  K  K  K  L  S  K
                                helix
421  GTAGAAATTTTGCGCTGTGCCGCGCTCTACATTAGAGACTTAAAAGATATATTAGAAGAA
      V  E  I  L  R  C  A  A  L  Y  I  R  D  L  K  D  I  L  E  E
481  TATGACTGCAATAATTCTTCAAAAAATAAAAGAAGCAGTTCCGAATGCAACTCTAGCAGA
      Y  D  C  N  N  S  S  K  N  K  R  S  S  S  E  C  N  S  S  R
541  GATAGCAATAGTGGAGATGAAGATGATATATTAAGAACGGAAATGTTAAATTTATCAACG
      D  S  N  S  G  D  E  D  D  I  L  R  T  E  M  L  N  L  S  T
601  GACCAGCTTTTAGTAAAATCGAAACTCCGATGTCTTAATATAAATGACAGGAACACAAAA
      D  Q  L  L  V  K  S  K  L  R  C  L  N  I  N  D  R  N  T  K
661  TGCAGGAAGTAGACACAAAATTTAAAAAATGGTGTTTTGACGTTAACGTAAAAATGTTTG
      C  R  K  *
721  TAGTTTTCATGAAAAATTTTTGTTTAATTTTTTATAAATTATTTTTGTGTTCGTTTTTAT
781  ATACCTTTTTGTCCAAAGAAATTATGTGAGCATATTTTGTAATATAGATATTTTTAGTTT
841  AATAAATGACGTTTAATAATATTCAAATTAAA
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B
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                basic      helix                  loop               helix
CnASH      VARRNERERNRVKQVNDGFDELRQRVP.....FLPDK.........KKLSKVEILRCAALYIRDL
MASH-1     -------------L--L--AT--EH--........NG......AAN--M----T--S-VE---A-    69%
MASH-2     ------------------QA---H--........HG......GAN-------T--S-VE---A-    78%
XASH-1     -------------L--L--AT--EH--........NG......AAN--M----T--S-VE---A-    69%
XASH-3     SE-----------L--L--AK---H--Q.......AQ......GPN--M----T--S-VE---A-    72%
ZASH-1a    -------------L--L--AT--EH--........NG......AAN--M----T--S-VE---A-    69%
ZASH-1b    ----------------L--QT---H--........NG......AAN--M----T--S-VE---A-    75%
CASH-1     -------------L--L--AT--EH--........NG......AAN--M----T--S-VE---A-    69%
ascT3      -----A----------N--VN---HL-QTVVNSLSNG....GRGSS------DT--I-VE---G-    69%
ascT4      -Q---A----------NS-AR---HI-QSIITDLTKG..G.GRGPN--I---DT--I-VE---S-    67%
ascT5      -I---A----------N--SQ---HI-AAVIADLSNGRRGIGPGAN------ST-KM-VE---R-    67%
ascT8      -----A----------N--AL--KKI-EEVSEAFEAQ..GAGRGAS-------T--M-VE---S-    67%


             **   * *** *    *  *  *                         *   *  *  **  *
CnASH      VARR...NERERNRVKQVNDGFDELRQRVP.....FLPDK......KKLSKVEILRCAALYIRDL
c-myc      -K--.TH-VL--Q-RNELKRS-FA--DQI-.....E-EN......ME-AP--V--KK-TA--LSV    42%
myoD1      AD--KAATM---R-LSK--EA-ET-KRCTS.....SN-.......MQR-P--E---N-IR--EG-    47%
da         KK--QAN-A---I-IRDI-EALKK-GRMCM......THL-....SD-PQT-LG--MM-VEV-MT-    39%
E12        RK--VAN-A-R-V--RDI-EA-R--GRMCQ......MNL-....SD-AQT-LL--QQ-VQV-LG-    44%
hairy      SD--SNKPIMRKR-RARI-NCLNK-KTLIL.....DATK-DF.ARNS--E-AD--EKTVKHLQK-    28%
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Fig. 1. Deduced amino acid sequence of CnASH and its relationship to other bHLH proteins. (A) Nucleotide sequence and predicted protein product of the *CnASH* cDNA. The basic-helix-loop-helix domain is indicated and a polyadenylation signal is shown in bold. (B) Comparison of the basic domain and the two helices of CnASH with all known Achaete-scute proteins (Villares and Cabrera, 1987; Alonso and Cabrera, 1988; Johnson et al., 1990; Ferreiro et al., 1992; Zimmerman et al., 1993; Allende and Weinberg, 1994; Jasoni et al., 1994) and representatives of other bHLH families (from Fig. 1 in Johnson et al., 1990). The loop, which varies in length as well as sequence, is not included in the percent amino acid identity calculation. Dashes indicate amino acid identity with CnASH and dots indicate gaps in the protein alignment. The asterisk identifies consensus residues present in most or all members of the bHLH class. GenBank accession no. for the *CnASH* sequence is U36275.

To determine which cell types of this lineage were expressing *CnASH*, in situ hybridization was carried out on macerates. Whole *Hydra* can be macerated into suspensions of fixed cells (David, 1973). Because the morphology of the *Hydra* cell types are very distinct from one another, one can readily identify each type of cell in macerates (Fig. 3A). The differentiation pathways for all of the cell types are well understood, and differentiation intermediates can be easily identified (Slautterback and Fawcett, 1959; David, 1973; David and Gierer, 1974; Bode et al 1987; Bode et al, 1990). For example, the nematocyte differentiation pathway, which is the most complex, is as follows. Single interstitial cells (Fig. 3B,C), termed large interstitial cells, undergo two divisions to form first a pair (Fig. 3D,E) and then a cluster, or nest, of four cells (Fig. 3F). These cells continue to divide, becoming smaller and altering their nuclear morphology as they form nests of 8, 16, or 32 cells (Fig. 3G). All of the cells form a syncytium as cytokinesis at each step is incomplete, leaving them connected to one another by cytoplasmic bridges. Once cell division is complete the cells synchronously undergo differentiation elaborating the nematocyst, a complex organelle that is the characteristic hallmark of the Cnidaria. All cells within a nest differentiate into the same type of one of the four types of nematocytes. Formation of the capsule begins with a small vesicle (Fig. 3H), which then enlarges (Fig. 3I), and takes on the characteristic shape of the particular nematocyst type (Fig. 3J), and eventually fills a large fraction of the cell. When differentiation is complete the cells separate and migrate independently among the epithelial cells into the tentacles. Both the neuron and secretory cell pathways also start with single and/or pairs of large interstitial cells (David and Gierer, 1974; Bode et al, 1987; Bode et al, 1990). Thereafter the pathways diverge as the cells take on different morphologies. Furthermore, for these pathways, differentiation intermediates are found as single cells in macerates.

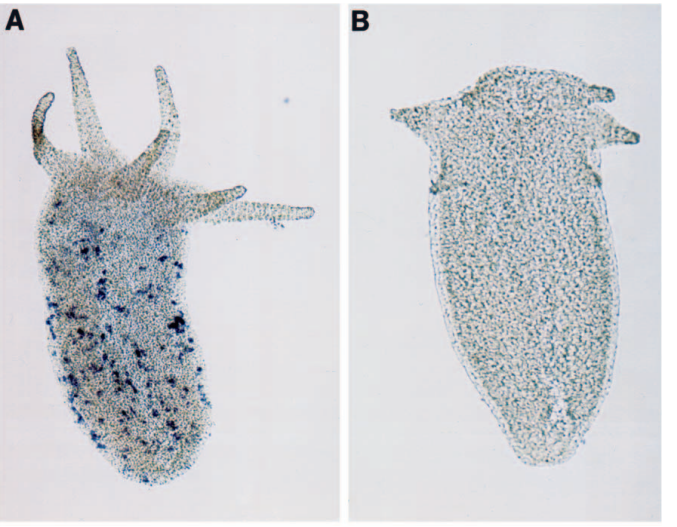

Fig. 2. In situ hybridization of *Hydra* using a *CnASH*-specific probe. (A) Whole mount showing the regional distribution of *CnASH*-expressing cells. The head extends from the apex to immediately below the tentacles. The foot is the basal 2% of the column, and the body column is the remainder. (B) Whole mount of an animal after 3 days treatment with hydroxyurea and 4 days recovery. Magnification 10×.

Examination of macerates following in situ hybridization with the *CnASH* probe showed that 10-15% of the single and 25-30% of the pairs of large interstitial cells were labeled (Fig. 3B-E). In contrast, cells that were clearly part of the nematocyte pathway were labeled to a much greater extent (85-95%). These included nests of large interstitial cells (Fig. 3F), of small interstitial cells (Fig. 3G), and of nematoblasts (Fig 3H and I). Late stage nematoblasts (Fig. 3J) and mature nematocytes were not labeled. No other cell types were labeled. The results of both the whole mount and macerate in situ hybridization analysis are consistent with *CnASH* being expressed in the nematocyte pathway.

CnASH shows in vitro DNA binding specificity

All bHLH proteins bind DNA sequences known as E boxes, which have the consensus sequence CANNTG (Murre et al., 1989a,b; Murre and Baltimore, 1992). In *Drosophila*, the consensus E box sequence for binding of AS-C proteins has been shown to be CAGCTG (Van Doren et al., 1991). The *Drosophila* AS-C proteins bind to DNA as heterodimers with the ubiquitous bHLH protein Daughterless (Da) (Van Doren et al., 1991). The structural homology between CnASH and the *Drosophila* AS-C proteins implied that CnASH could be capable of forming heterodimers with Da, and that such a dimer might bind to DNA in a sequence-specific manner. To examine the ability of a CnASH-Da dimer to bind an appropriate E box sequence we performed electrophoretic mobility shift assays using in vitro translated CnASH and Da proteins and a T5E3 oligonucleotide as a probe. The T5E3 probe is a 30 bp DNA fragment containing an E box and flanking sequences from the *Drosophila achaete* (*AS-C T5*) promoter (Van Doren et al., 1991). As shown in Fig. 4, neither CnASH nor Da alone is capable of binding to the T5E3 probe and causing a shift in its electrophoretic mobility. However, when the two proteins are combined in equimolar amounts, binding and retardation of the probe is evident (band indicated by arrow in Fig. 4). When a 100-fold molar excess of unlabeled competitor T5E3 fragment is added, the amount of labeled T5E3 probe which is shifted is greatly reduced. Competition with excess cold T5XX3, a fragment identical to T5E3 except for 2 base changes in the E box sequence, from **C**AGC**T**G to **A**AGC**G**G,

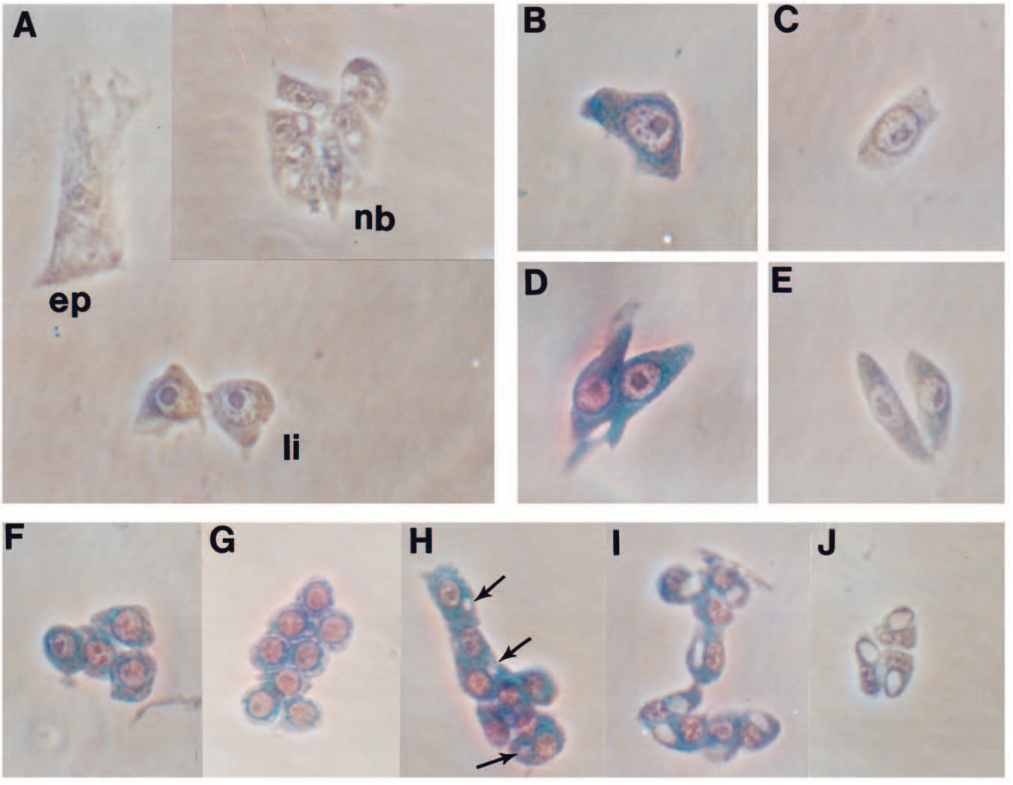

Fig. 3. In situ hybridization to macerates. (A) Unstained macerate showing epithelial (ep), large interstitial (li) and nematoblast (nb) cell types. (B,C) Single large interstitial cells. (D,E) Pairs of large interstitial cells. (F) Nest of large interstitial cells. (G) Nest of small interstitial cells. (H) Nest of early nematoblasts (developing capsules indicated by arrows). (I) Nest of mid-differentiation nematoblasts. (J) Partial nest of late-differentiation nematoblasts. Magnification (all panels) 200×.

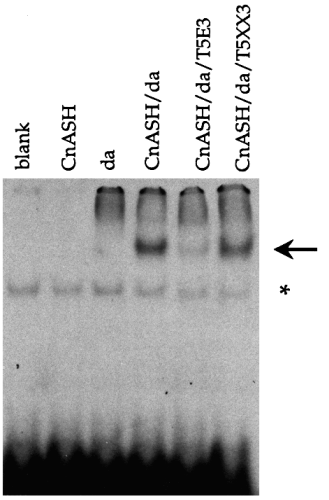

Fig. 4. Electrophoretic mobility shift assay of the dimerization and DNA binding properties of CnASH. Proteins were produced by coupled in vitro transcription-translation in a rabbit reticulocyte system. Each lane is labeled to indicate the cDNA(s) used to program the lysate(s), and the competitor oligonucleotide added, if any. The unprogrammed lysate ('blank') had no cDNA added but contains proteins which bind to the T5E3 oligonucleotide, generating the band indicated by an asterisk. The shifted T5E3 oligonucleotide resulting from binding of the CnASH-Da dimer is indicated by an arrow.

is ineffective, demonstrating that the observed shift is due to sequence-specific binding by the CnASH-Da dimer.

CnASH has cell fate specification activity in *Drosophila*

The ability of CnASH to dimerize with Da and of the dimer to bind to an appropriate E box sequence in vitro raised the possibility that *CnASH* might be capable of acting in vivo in the developing *Drosophila* nervous system. This would be a direct test of the possibility that *CnASH* is a functional as well as structural homolog of the fly *AS-C* genes. The effects of ectopic expression of the fly *scute*, *lethal of scute* or *asense* genes have been examined previously (Rodriguez et al., 1990; Brand et al., 1993; Dominguez and Campuzano, 1993; Hinz et al., 1994), and it has been shown that the bHLH domain alone is sufficient for proneural function (Hinz et al., 1994). Ectopic expression of any of these *AS-C* genes near the time of puparium formation and establishment of the adult PNS leads to the specification of extra sensory organ mother cells (SMCs), which results in the formation of ectopic sensory organs, most obviously bristles. To test the ability of *CnASH* to specify neural cell fates in the *Drosophila* PNS, we generated a transgene which would allow ectopic expression of *CnASH* under the control of the *Drosophila heat shock 70* promoter (see Materials and methods). Two independent homozygous transgenic lines were established by P-element mediated germline transformation and both showed similar effects of *CnASH* expression.

The phenotype produced by ectopic expression of *CnASH* is indistinguishable from that produced by ectopic expression of the fly genes (for comparison, see corresponding figures in Rodriguez et al., 1990; Brand et al., 1993; Dominguez and Campuzano, 1993; and Hinz et al., 1994). Ectopic bristles resulting from the expression of *CnASH* are observed on the notum of the thorax (Fig. 5, compare A and B), on the abdominal segments (Fig. 5C,D), and on the wings (Fig. 5E and G, F and H). Other sensory organs are also produced, such as ectopic chemoreceptors on the wings (see Fig. 5H). Certain proneural clusters of prospective SMCs are particularly susceptible to the effect of ectopic *AS-C* gene expression, and characteristic patterns of ectopic sense organs are commonly observed. For example, the clustering of ectopic bristles along the L2 vein of the wing is a frequent result. These same patterns are also produced in the *CnASH* transgenic flies after heat shock (see Fig. 5H).

One possible explanation for the observed effects of ectopic *CnASH* expression in a wild-type *AS-C* background could be activation of the endogenous *ac* and/or *sc* genes. Since *ac* and *sc* are known to auto- and cross-activate (Martinez and Modolell, 1991), a relatively small activation of one of these genes by CnASH could potentially give rise to the observed phenotype due to the resulting ectopic expression of the *Drosophila* genes themselves. To distinguish between this possibility and direct activation of target genes by CnASH, we crossed the *hsp70-CnASH* transgene into flies carrying the $sc^{10\text{-}1}$ chromosome. The $sc^{10\text{-}1}$ chromosome carries the most severe viable *AS-C* mutation, which eliminates both *ac* and *sc* functions (Campuzano et al., 1985; Villares and Cabrera, 1987). These mutations in the *ac* and *sc* genes result in a near-total failure to differentiate adult external sensory organs, as can be observed in the control $sc^{10\text{-}1}$ flies shown in Fig. 5I,K.

Heat shock-induced expression of two copies of *CnASH* in a $sc^{10\text{-}1}$ background leads to a substantial rescue of the ability to form bristles (see Fig. 5, compare I and J, K and L). SMCs are specified in several different imaginal discs by the activity of *CnASH*, as evidenced by the variety of positions in which bristles are recovered. The degree of rescue resulting from ectopic *CnASH* expression is comparable to that obtained by ectopic expression of the *Drosophila AS-C* genes including *sc*, one of the endogenous genes normally responsible for the specification of SMCs (Rodriguez et al., 1990).

DISCUSSION

CnASH expression in *Hydra*

The early metazoan *Hydra* has a simple body plan and contains only 15-20 cell types (Bode et al., 1973). The presence of only a single *achaete-scute* gene may be a reflection of the relative simplicity of this organism. It has previously been observed that other genes that occur in large families in more complex organisms have fewer members in *Hydra*. For example, *Hydra* has only three actin genes (Fisher and Bode, 1989), two annexin genes (Schlaepfer et al., 1992) and one Na^+,K^+-ATPase gene (Canfield et al., 1992).

CnASH expression appears to be restricted to the nematocyte differentiation pathway. The evidence for this is as follows. In whole mounts *CnASH* is expressed in isolated groups of cells that are clearly not epithelial cells of either layer. Single and pairs of large interstitial cells derived from the multipotent stem cells are at the beginning of the pathway of each of the three classes of somatic differentiation products (David and Gierer, 1974; Bode et al., 1987).

If *CnASH* expression is restricted to only one of these differentiation pathways, one would expect that only a fraction of both single and pairs of large interstitial cells would be labeled. This is what was observed. Finally, intermediates in the nematocyte differentiation pathways, but not in the neuron or secretory cell pathways, were labeled. Hence, *CnASH* is expressed from the very beginning of the nematocyte pathway, the single large interstitial cell stage, through to the mid-nematoblast stage, which is close to the completion of differentiation. The fact that it is expressed in this differentiation pathway represents a novel cell type for the expression of members of the *achaete-scute* family of genes. They have been shown to be expressed in neuron, epithelial, gamete and trophoblast lineages in vertebrates and *Drosophila* (Parkhurst et al., 1990; Belote, 1992; Gonzalez-Crespo and Levine, 1993; Parkhurst et al., 1993; Guillemot et al., 1994).

The results also suggest a role for the *CnASH* gene. The population of single large interstitial cells is a heterogeneous one containing multipotent stem cells and committed cells at the beginning of the differentiation pathways. Since *CnASH* is expressed in a subset of these large interstitial cells, it is plausible that this gene is required for the specification of uncommitted stem cells to the nematocyte pathways. It is equally plausible that its prolonged expression in the differentiation intermediates may reflect a requirement for *CnASH* activity for successful traverse of the pathway. That is, *CnASH* could function to maintain rather than to initiate the cell fate specification. Other members of the *achaete-scute* family appear to have such a role.

During neurogenesis in *Drosophila*, one member of the *AS-C*, *asense*, is not expressed until after the neuroblasts have segregated from the neuroepithelium and appears to be required for differentiation and maintainence of the specified cell fate (Alonso and Cabrera, 1988; Gonzalez et al., 1989). Similarly, *XASH-1*, *MASH-1* and *CASH-1* are expressed relatively late in embryogenesis, being detectable only after neural progenitors have been specified, and may act as vertebrate *asense* homologs (Lo et al., 1991; Ferreiro et al., 1992; Jasoni et al., 1994). *CnASH* may have a similar function in the nematocyte lineage in *Hydra*.

Conservation of cell fate specification activity by *achaete-scute* genes

Given the 600 million year evolutionary separation between cnidarians and both vertebrates and arthropods, the degree of amino acid identity (67-78%), and thus structural homology, between the bHLH regions of CnASH and the other known Achaete-scute proteins is noteworthy. The degree of similarity is not substantially higher between the vertebrate and arthropod Achaete-scute proteins (72-81% amino acid identity), while

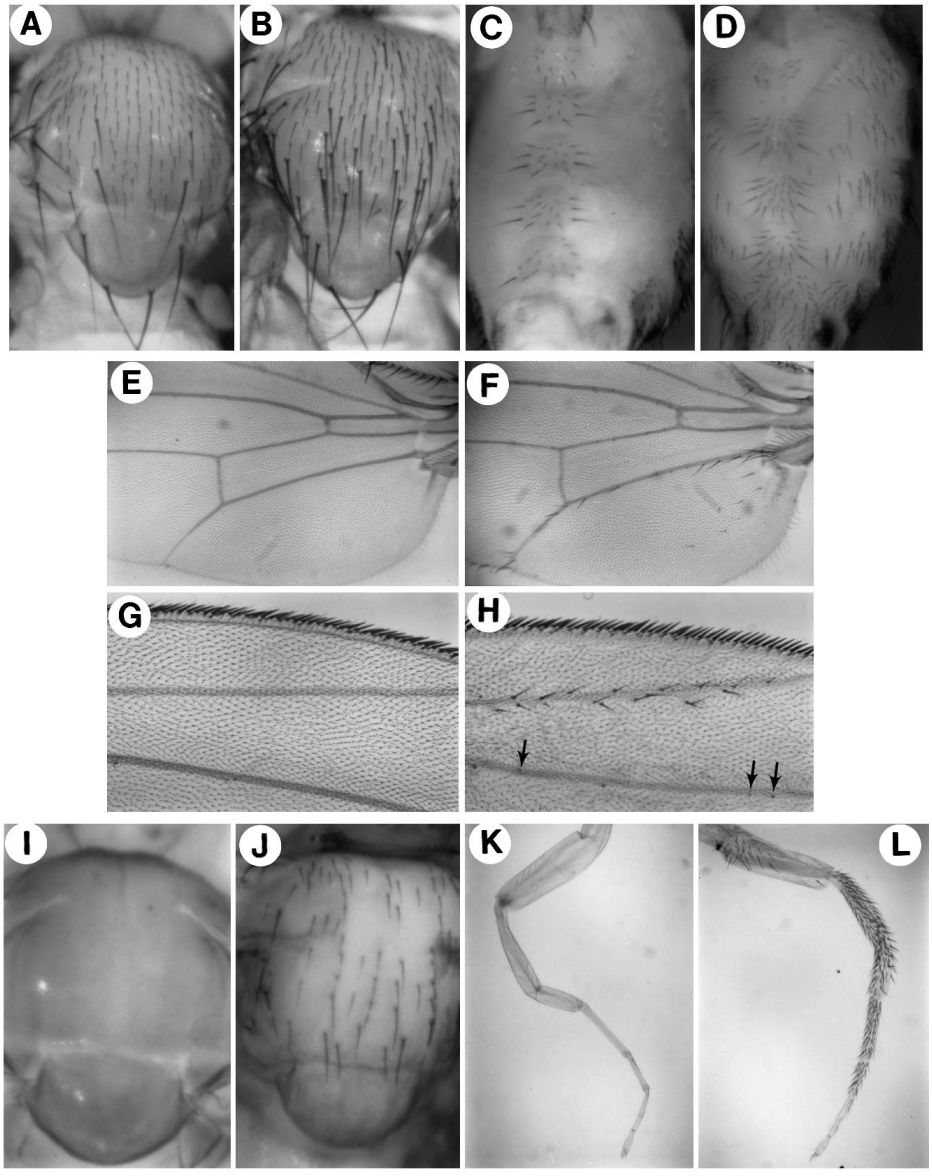

Fig. 5. Effects of heat shock induced ectopic expression of *CnASH* in *Drosophila*. (A,B) Notum of heat shocked control (A) and *CnASH*/*CnASH* (B) flies; both ectopic macrochetae (large bristles) and microchetae (small bristles) are present. (C,D) Abdominal sternites of *CnASH*/*CnASH* flies have ectopic bristles in the ventral margins (D) not seen in the control flies (C). Control (E,G) and *CnASH*/*CnASH* (F,H) wings following heat shock. (E,F) Anterior portion of wing blade showing ectopic bristles in *CnASH*/*CnASH* flies (F). (G,H) Clustering of ectopic bristles along the L2 vein of the wing. Ectopic chemoreceptors are indicated by arrows. (I,K) Control $sc^{10\text{-}1}$ flies, demonstrating the severely reduced ability of these flies to form bristles. (J) Bristle formation on the thorax in response to heat shock induction of *CnASH*. (L) Rescue of sensory organs on the distal segments of the legs is nearly complete, while fewer bristles are rescued on the proximal segments.

these phyla have been separated for a somewhat shorter period of time (500-540 million years). This suggests that the structure of these gene products has been highly conserved throughout evolution.

The ability of CnASH to dimerize with the *Drosophila* Achaete-scute binding partner Da, and for this heterodimer to bind DNA in a sequence-specific manner, demonstrates that in this case structural conservation is accompanied by a conserved biochemical activity. The fact that the CnASH-Da dimer can discriminate between two closely related DNA sequences, and bind only to the appropriate one, is particularly striking given that Da is widely expressed in *Drosophila* during development and has a role in several different processes (reviewed by Cronmiller and Cummings, 1993). The consensus DNA binding site for Achaete-scute proteins, and the ability of these proteins to distinguish this site, appears to have been extensively conserved. We have extended the observation of Ferreiro et al. (1992), that *Xenopus* XASH-1 protein recognizes the *Drosophila* E-box sequence in vitro, to the evolutionarily earlier homolog CnASH.

Because in vitro interactions can be misleadingly promiscuous, we also tested the ability of CnASH to dimerize, bind DNA and affect development in vivo. The results are unambiguous; *Hydra CnASH* can substitute for the fly *AS-C* genes during specification of cell fate in *Drosophila*. The effects of ectopic *CnASH* expression are virtually indistinguishable from those produced by ectopic expression of the endogenous genes (Rodriguez et al., 1990; Brand et al., 1993; Dominguez and Campuzano, 1993; Hinz et al., 1994). Furthermore, ectopic expression of *CnASH* can partially rescue *Drosophila* mutants in which the endogenous *achaete* and *scute* genes have been eliminated. This demonstrates that CnASH is capable of acting directly on appropriate *achaete-scute* target genes in vivo and activating the program of sensory organ commitment and differentiation. Among the possible target genes which may be activated by CnASH are *asense* and *lethal of scute*. These *AS-C* genes, which are still functional in the $sc^{10\text{-}1}$ mutant, have been shown to be capable of partial rescue of the $sc^{10\text{-}1}$ phenotype when ectopically expressed (Brand et al., 1993; Dominguez and Campuzano, 1993; Hinz et al., 1994). Jarman et al. (1993) have shown that *asense* contains E-box sequences in its regulatory region which make it a target for activation by Achaete-scute proteins. *CnASH* is therefore a true homolog of the *Drosophila achaete-scute* genes, having both a conserved protein structure and the ability to act as a functional substitute during development.

Conservation of structure as well as implied conservation of function of developmentally important regulatory genes has previously been shown between *Drosophila* and vertebrates. The *Hox* and *achaete-scute* gene families are two examples. In each case, the homologs have similar structure and perform similar functions during development. In some instances, functional conservation has been directly demonstrated. The role of one of the *Hox* genes in regional specification, for example, has been conserved to the extent that *HoxB4*, a vertebrate *deformed* homolog, can phenocopy *Drosophila deformed* in flies (McGinnis et al., 1990). That such functional conservation extends back to the beginning of metazoan evolution is implied by the presence of conserved structure and apparent function of some genes found in early metazoans such as *Hydra*. *Cnox-2*, a *deformed* homolog, has been isolated from *Hydra* (Shenk et al., 1993a) and appears to play a role in axial or regional patterning in this organism as well (Shenk et al., 1993b).

The results presented here are a direct demonstration that, in the case of the *achaete-scute* family, the function of a developmental regulatory gene has been conserved from the very beginning of metazoan evolution to the present. Furthermore, the fact that CnASH has the same in vitro DNA binding characteristics as the *Drosophila* and vertebrate Achaete-scute proteins indicates that the molecular basis of this mechanism for cell fate specification has been maintained, largely unaltered, throughout metazoan evolution. This is the most direct evidence to date for the unity of a developmental mechanism throughout the metazoans.

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