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Zebrafish primary neurons initiate expression of the LIM homeodomain protein Isl-1 at the end of gastrulation

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SUMMARY

Isl-1 has previously been established as the earliest marker of developing chicken spinal motor neurons where it is regulated by inductive signals from the floor-plate and notochord. We now report that, in zebrafish, the expression of Isl-1 is initiated in Rohon-Beard cells, primary motor neurons, interneurons and cranial ganglia, hours before the neural tube itself is formed. The expression is initiated simultaneously in the Rohon-Beard cells and the primary motor neurons, at the axial level of the presumptive first somite. The Isl-1-expressing motor neurons appear on either side of the ventral midline whereas the interneurons and Rohon-Beard cells initiate expression while located at the edge of the germinal shield. Isl-1 expression is initiated in these cells before the formation of a differentiated notochord.

Isl-1 is expressed in the various functional classes of primary neurons at 24 hours postfertilization. This selective expression of a homeodomain protein in the primary neurons implies that these neurons share a common program of early development and that they have evolved and been selected for as a coordinated system. One of the functions of the primary neurons is to send long axons which pioneer the major axon tracts in the zebrafish embryo. An evolutionary conserved functional role for Isl-1 in the expression of the pioneering phenotype of the primary neurons is suggested.

Key words: LIM homeodomain, Islet-1, zebrafish, pioneering neurons, primary neurons

INTRODUCTION

During embryonic development of the vertebrate nervous system a variety of neurons, with characteristic morphology, axonal projections and connectivity, arise at predictable times and locations. For many neuronal types a wealth of information is available on the processes of axonal pathfinding and synapse formation, but less is known about the regulatory processes and molecules that are involved in the generation of different neurons. To understand the molecular mechanisms participating in the generation of specific neuronal cell types we need to identify regulatory proteins that are expressed at the initial stages of differentiation in these various neurons.

One such class of proteins are the homeodomain proteins and a number of homeobox genes are expressed during the formation of the vertebrate central nervous system (CNS; Kessel and Gruss, 1990). The *Hox* genes are expressed at high levels in the mouse spinal cord and hindbrain and the temporal and spatial patterns of expression of these genes are consistent with a role in the definition of spatial domains in the CNS (Kessel and Gruss, 1991; Hunt et al., 1991). In contrast to the broad expression patterns of the *Hox* genes, a few homeobox genes show a much more restricted pattern of expression in the adult brain including the mid- and forebrain. These include members of the *Dlx* (Price et al., 1991), *Pax* (Krauss et al., 1991), *Nkx* (Price et al., 1992), *Otx* (Simeone et al., 1992a), and *Emx* families of genes (Simeone et al., 1992b).

Islet-1 (Isl-1) is one of the three original members of the LIM homeodomain protein family (Way and Chalfie, 1988; Freyd et al., 1990; Karlsson et al., 1990). This family of proteins has now expanded and includes an increasing number of new members from many different species (Bourgouin et al., 1992, Cohen et al., 1992, Taira et al., 1992, Xu et al., 1993). A variety of proteins containing one or two LIM motifs but which lack a homeodomain have also been described (Greenberg et al., 1990). Isl-1 was originally isolated as a protein that could bind to the insulin gene enhancer. We have previously analyzed the pattern of expression of Isl-1 in the adult rat using anti-Isl-1 antiserum (Thor et al., 1991). Isl-1 was found to be expressed in a subset of endocrine cells and neurons. In the central nervous system (CNS), Isl-1 expression is restricted to the parts of the brain involved in autonomic and endocrine control. Isl-1 is not expressed in regions involved in processing of sensory information and neocortical areas. Thus, Isl-1 appears to be selectively expressed in the phylogenetically old parts of the brain.

In the chicken spinal cord Isl-1 has been identified as the earliest known marker of developing motor neurons and the onset of Isl-1 expression in the motor neurons can be

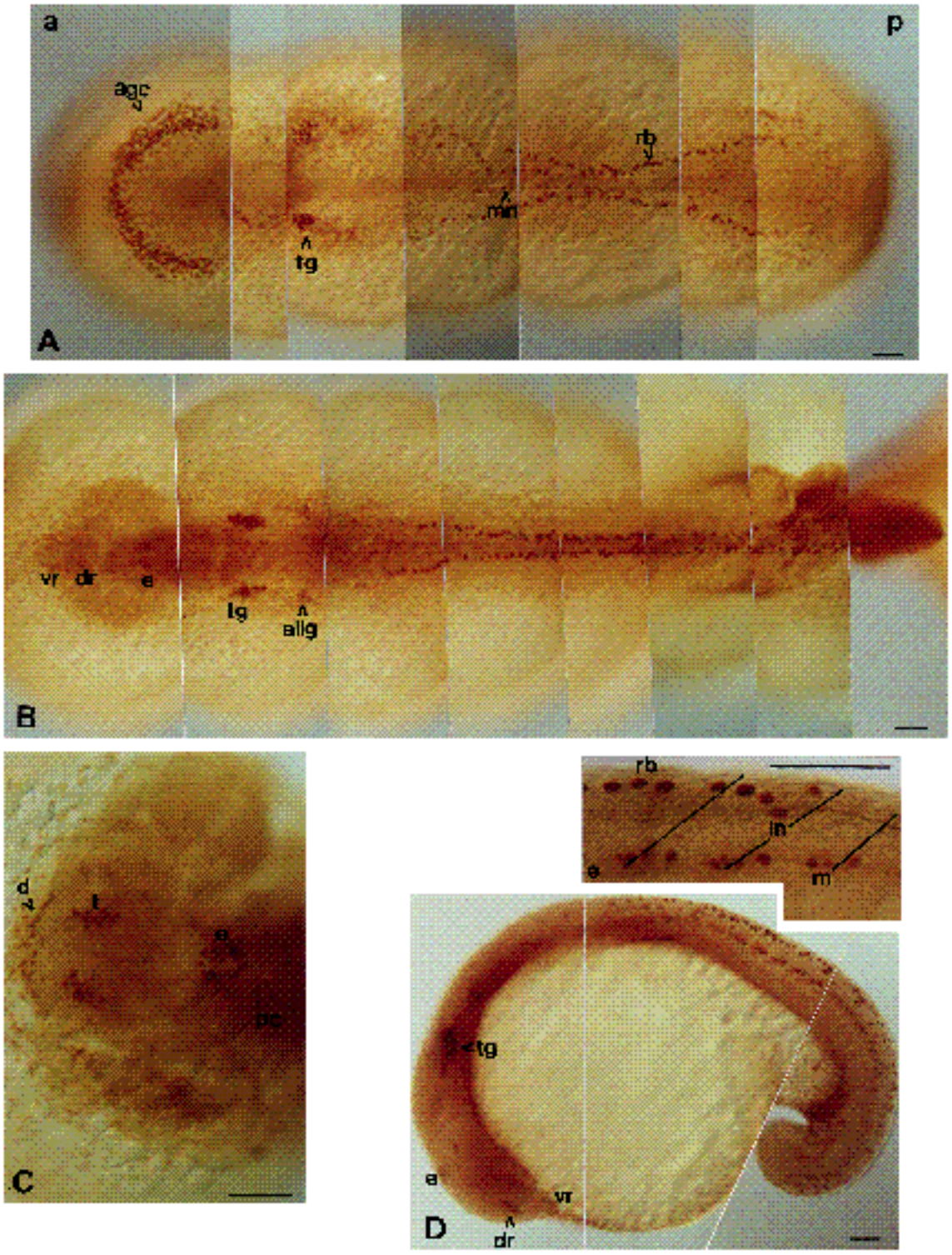

Fig. 1. Patterns of expression of Isl-1 immunoreactivity in early zebrafish embryos. (A) 13 hpf; (B) 17 hpf; (C-E) 18 hpf. a, anterior; p, posterior; agc, anterior group of cells; tg, trigeminal ganglia; mn, primary motor neurons; rb, Rohon-Beard cells; in, interneurons; vr, ventrorostral cluster; dr, dorsorostral cluster; e, epiphysis; allg, anterior lateral line ganglia; d, diencephalon; t, telencephalon; pc, neurons of nucleus of posterior commissure. In E the solid lines represent segment borders. Bars, 50 μm.

observed soon after their final mitotic division (Ericson et al., 1992). It has also been shown that the expression of Isl-1 in the motor neurons is regulated by inductive signals from the floorplate and notochord. These studies suggest that Isl-1 may have a function in the establishment of motor neuron fate. In general, this temporal pattern of expression

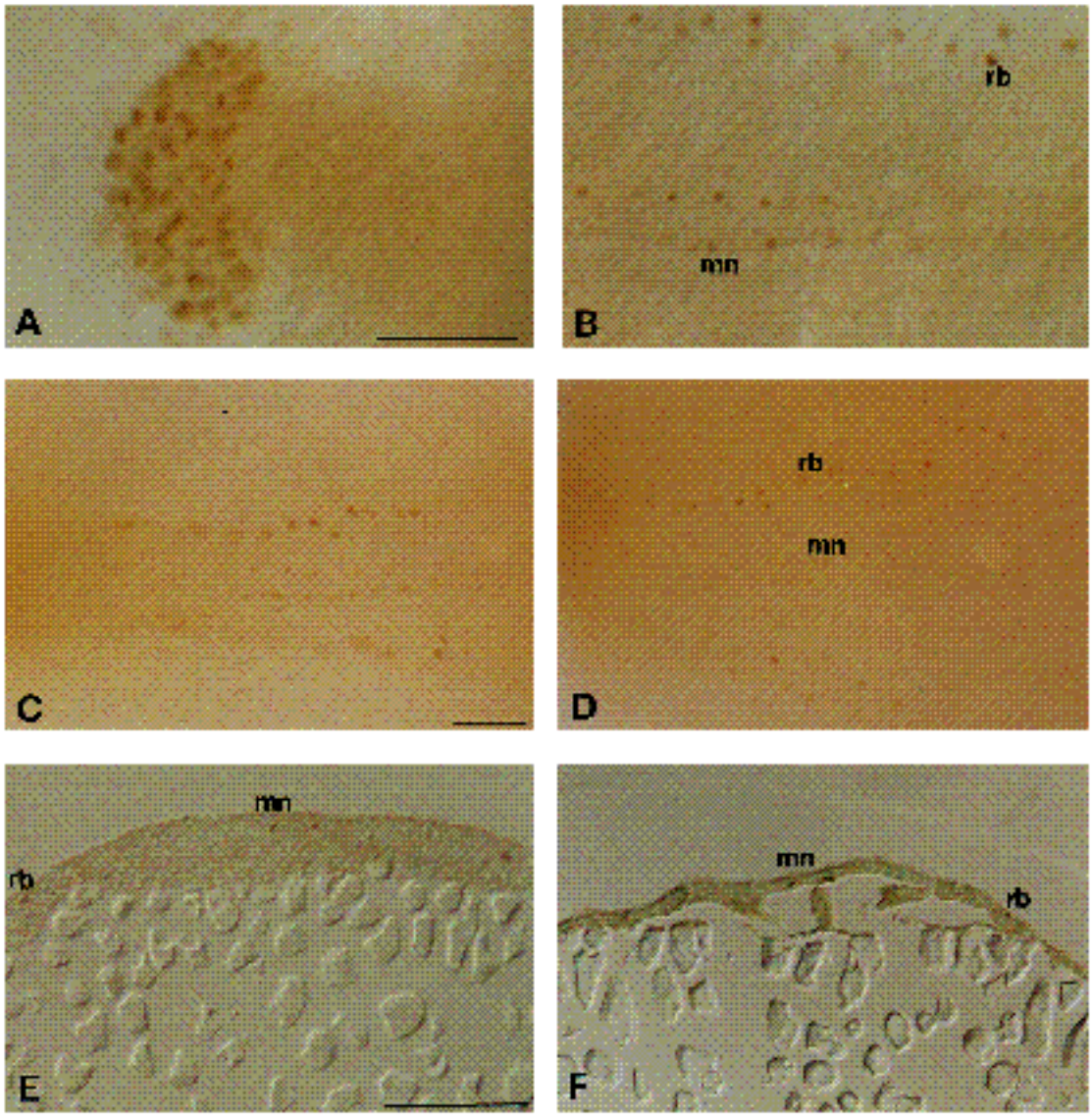

Fig. 2. Expression pattern of Isl-1 at the early neurulation stage. (A) The anterior group of cells at 12 hpf; (B) the region where the first somites will form, 12 hpf; (C) the same region as in B but with a lower magnification; (D) as in C but more posteriorly; (E) cross section through midpart of embryo at 10 hpf; (F) cross section through midpart of embryo at 12 hpf. mn, primary motor neurons; rb, Rohon-Beard cells. Bars, 50 μm.

is also observed in developing rat and mouse embryos where onset of Isl-1 expression is correlated with the early differentiation of the Isl-1-expressing endocrine and neural cells (unpublished observation).

To extend this analysis we chose to study the zebrafish, taking advantage of the small size and optic clarity of the embryo and the accessibility and the relative simplicity of the developing nervous system. During the first day of development of the zebrafish an ordered and relatively small and simple network of neurons develop (Kimmel and Westerfield, 1990). These neurons pioneer central and peripherial axonal pathways in a stereotyped manner (Kuwada and Bernhardt, 1990). These earliest neurons to develop are termed the primary neurons and are distinguished from the later secondary neurons by their small number, large size and long axons which are sent out before 24 hours post fertilization (hpf). These neurons arise at diverse locations within the nervous system such as the spinal cord, peripherial ganglia and brain, and include neurons of all functional classes (Wilson and Easter, 1992).

The primary neurons are best characterized in the spinal cord and hindbrain (Myers, 1985; Mendelson, 1986). In the spinal cord the Rohon-Beard sensory neurons, three identified spinal motor neurons and some interneurons are part of this system. The Mauthner cell and other reticulospinal neurons in the hindbrain are defined as primary as are the trigeminal and the lateral line ganglionic neurons (Kimmel and Westerfield, 1990). It is less obvious if cells in the anterior brain regions can be subdivided into primary and secondary neurons (Wilson and Easter, 1992).

Since the zebrafish does not develop by a unique and

invariant cell lineage, it is very unlikely that these neurons are related by lineage. On the other hand in homozygous *ned-1* mutants, where degeneration of the nervous system is observed, the primary neurons and the coordinated spontaneous motility and avoidance responses are saved suggesting that these neurons can be genetically distinguished from the later developing neurons (Grunwald et al., 1988).

At present very few markers are available for the earliest stages of commitment and differentiation of neurons in zebrafish. We now show that expression of Isl-1 is initiated in different sets of primary neurons at the end of gastrulation preceeding any other known neural marker. The selective expression of Isl-1 in the primary nervous system provides evidence that the primary neurons can be regarded as a system with a common early developmental regulatory program.

MATERIAL AND METHODS

Zebrafish embryos were obtained from the colony of zebrafish in our laboratory. The different stages of development are presented as hours postfertilisation (hpf) at 28.5°C as described by Westerfield (1989).

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde in BT fix buffer (0.15 M $CaCl_2$, 4% sucrose in 0.1 M $NaPO_4$, pH 7.4) or 3.7% formaldehyde in 0.1 M MOPS buffer, pH 7.4, 1 mM $MgSO_4$, 2 mM EGTA (12-16 hours at 4°C or 2 hours at room temperature (RT)) washed 4× 15 minutes in TBS, 0.5% Triton X-100 (TBST), pH 7.4, treated 30-45 minutes in blocking solution (10% goat serum in TBST), incubated 3 hours at RT with primary antibody diluted 1:500 in TBST, washed 6× 15 minutes in TBST, incubated with secondary biotinylated antibody (goat anti-rabbit) 1:500 (Vector ABC kit), in blocking solution overnight at 4°C washed 6× 10 minutes at RT in TBST, incubated for 2 hours in ABC reagent diluted 1:6 in TBST at RT, washed in TBST 4× 15 minutes and 2× 5 minutes in 0.1 M $NaPO_4$. Staining after incubation (30 minutes at RT) in 0.75 ml DAB solution (0.03 mg/ml diaminobenzidine in 0.05 M $NaPO_4$, pH 7.4) by adding H_2O_2 up to 0.003% in 0.75 ml of DAB solution for 10 minutes or longer at RT. After dehydration in methanol, embryos were cleared in benzyl-benzoate:benzyl alcohol, 1:2 and embedded in Permount between two coverslips. For sectioning, embryos in methanol were slowly rehydrated, embedded in agar-sucrose blocks, frozen and sectioned using a cryostat. 10 μm sections were mounted on gelatinised ('subbed') slides, dehydrated, counterstained with methylene green and embedded in DPX under coverslips.

Double labelling with anti-Isl-1 and anti-HNK-1 or anti-acetylated α-tubulin antibodies

After staining with the anti-Isl-1 antibody, embryos were rehydrated in TBST and incubated with monoclonal anti-acetylated α-tubulin or HNK-1 antibodies as described above. Biotinylated goat anti-mouse IgM or IgG antibodies (Vector) were used as secondary antibodies. In the ABC reaction biotinylated β-galactosidase (Vector) was used. After washing in TBST, embryos were washed in PBS 2× 15 minutes and stained in darkness at 28-30°C in 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 1 mg/ml X-gal, 1× PBS for 1-2 days. The reaction was stopped in methanol and embryos were processed for sectioning and photographing as before.

RESULTS

Isl-1 expression is initiated in subsets of neurons at the end of gastrulation

We have previously shown that antiserum raised against bacterially produced rat Isl-1 protein crossreacts with chicken Isl-1. To test if the anti-Isl-1 antibodies crossreacted with zebrafish Isl-1 we performed whole-mount immunohistochemistry on 13 hour postfertilization (hpf) embryos. A very distinct and reproducible pattern of nuclear immunoreactivity was evident at this stage of development (Fig. 1A). For simplicity we call the detected protein(s) Isl-1 although at the moment we do not know if these affinity purified antibodies detect more than one structurally similar nuclear protein. The time of appearance of Isl-1 expression in different cell types was studied in more detail. Isl-1 expression starts to appear in the region of the head primordium at the end of gastrulation (stage 90% of epiboly or 9 hpf). About 0.5-1.0 hour later (10 hpf) the expression becomes more intense and a distinct group of cells starts to be visible at the anterior tip of the axial mesenchyme in front of the head primordium. This structure is called the 'pillow'. The exact function(s) and subsequent fate of these cells during the continued development of the embryo are not known, but there are indications that the 'pillow' contributes to the mesenchyme of the prechordal plate (Ballard, 1973; Kimmel et al., 1990). At this early stage of development Isl-1 expression is also initiated in two symmetrically located clusters of cells which will later become the trigeminal ganglia. Isl-1 expression is also observed in a few cells that are evenly spaced in two bilateral columns close to the midline and in two other symmetrically distributed lines of cells located in a very lateral position at the edge of the germinal shield. These two different populations of Isl-1-positive cells could at later stages of development be identified as primary motor neurons, interneurons and Rohon-Beard cells, respectively (Fig. 2C,D). The first Isl-1-positive cells of both of these subtypes appear at roughly the position where the first somites will form. From this initial site, additional Isl-1-positive primary motor neurons, interneurons and Rohon-Beard cells appear in both the anterior and the posterior directions (Fig. 2C,D). The appearance of these Isl-1-positive cells precedes somitogenesis in both directions.

In sections of 10 hpf embryos, the motor neurons at the midline and the laterally located interneurons and Rohon-Beard cells are clearly visible. In some but not all sections more than one laterally located Isl-1-positive cell is observed (data not shown), it is likely that these cells are interneurons. In these early embryos, the midline appears as two rows of polarized cells with no obvious signs of notochord differentiation (Fig. 2E). In sections of 12 hpf embryos the notochord is clearly separated from the notoplate (Fig. 2F). The organization of the primary motor neurons at the midline is in striking contrast to that of the interneurons and Rohon-Beard cells which start to express Isl-1 when they are still located in a very lateral position at the edge of the germinal shield (Figs 1A, 2C,D). The birth of primary motor neurons, using labelling by DNA precursors, have previously been mapped to between 10 and 16 hpf (Myers, 1985). The exact time when the Rohon-

Beard cells become postmitotic in zebrafish is not known. They start, however, to express the HNK-1 antigen as a sign of differentiation around 14 hpf (Metcalfe et al., 1990). In *Xenopus* these cells are believed to be specified during or shortly after gastrulation (Lamborghini, 1980)

In all of these neurons Isl-1 expression is detected before the appearance of any other neural marker and in at least some of these neurons the onset of Isl-1 expression is correlated with the previously determined time of withdrawal from the cell cycle. This early onset of Isl-1 expression strengthens the suggestion that Isl-1 is involved in the initiation of differentiation of specific neuronal subtypes.

Isl-1 is selectively expressed in neurons of the primary nervous system

Both the Rohon-Beard cells and the primary motor neurons are classified as primary neurons (Kimmel and Westerfield, 1990). The primary nervous system includes many additional neurons of all functional classes, which arise at diverse locations within the brain, the spinal cord and peripherial ganglia. To analyze if Isl-1 is expressed in the various types of primary neurons we analyzed the pattern of Isl-1 expression in the nervous system of embryos at later stages of development.

In whole mounts of the spinal cord, at 18 hpf, Isl-1 expression is observed in Rohon-Beard cells, in all three primary motor neurons, and in a small and variable number of interneurons per hemisegment including at least CoPA, DoLA and VeLD interneurons (Fig. 1D). The classification of spinal interneurons into primary and secondary neurons is not obvious, although the CoPA cell meets the criteria of a primary neuron due to the large size of the somata and the long and thick axon. The DoLA interneuron has most of these characteristics, in addition this neuron seems to be specific for the embryonic stage of the zebrafish. Later in development Isl-1 starts to appear in secondary neurons spreading from anterior to posterior.

The presence of Isl-1-positive cells in the region of presumptive anterior brain can already be observed at the 13 hpf stage (Fig. 1A). These cells are located in close proximity to the midline between the anterior group of cells in the pillow and the trigeminal ganglia. At around 17 hpf (Fig. 1B) Isl-1 expression starts to be visible in small bilaterally symmetrical clusters of neurons in the brain. These cells are more clearly seen at 18 hpf (Fig. 1C). To map the Isl-1-expressing nuclei in the embryonic brain, we analyzed the pattern of Isl-1 expression in cryosections of embryos at 22 hpf. The two most anterior located clusters were identified as the ventrorostral cluster in the diencephalon and the dorsorostral cluster in the telencephalon, respectively (Figs 1C, 3A). More posteriorly located dorsal clusters correspond to the epiphysis (Figs 1C, 3B) and to the nuclei of posterior commissure (Figs 1C, 3C). Isl-1-positive cells in the midbrain are most likely located in the ventrocaudal cluster (Fig. 3D). Isl-1 expression starts to appear at 13 hpf in the hindbrain and at 22 hpf the expression is evident in ventrally located reticulospinal neurons. The Isl-1-positive clusters are confined to all hindbrain segments and the largest numbers of positive cells are found in the 3rd, the 4th (Fig. 3E) and the 6th hindbrain segments.

Isl-1 is also expressed in a number of cranial ganglia at 22 hpf. Isl-1 expression starts around 10 hpf in a few cells that will give rise to the trigeminal ganglia and at 13 hpf the trigeminal nuclei are distinctly visible (Fig. 1A). At 13 hpf Isl-1-positive cells also start to occur in the positions of the anterior and posterior lateral line ganglia and the acoustic ganglia (Fig. 1A,B). Taken together these analyses show that Isl-1 is expressed in various neuronal subtypes of the primary nervous system and that Isl-1 expression is initiated prior to axogenesis in all of these neurons.

The primary neurons are known to send long axons which pioneer the major axon tracts of the embryonic zebrafish. To show that Isl-1 is, in fact, expressed in neurons that send long axons in the brain, spinal cord and cranial ganglia, we performed double whole-mount labelling with anti-Isl-1 and anti-HNK-1 or anti-acetylated α-tubulin antibodies. As examples, Isl-1-expressing neurons in the ventrorostral and dorsorostral clusters are stained with the anti-acetylated α-tubulin antibody and these axons make up the supraoptic tract and the tract of the postoptic commissure (Fig. 4C). The axons of the Isl-1-stained sensory neurons in the trigeminal ganglia express the HNK-1 epitope and descend into the anterior part of dorsolateral fascicle (DLF; Fig. 4A) The HNK-1-expressing axons of the Isl-1-positive Rohon-Beard cells contribute to the posterior part of DLF in the spinal cord (Fig. 4B).

To show that Isl-1 is just not a general marker for neuronal differentiation, we analyzed the pattern of Isl-1 expression in the brain and the spinal cord at a later stage, when the secondary nervous system has been formed (Fig. 5A,B). In the brain, at 72 hpf, a highly restricted pattern of expression is observed. Although we have not attempted to map the individual Isl-1-positive nuclei in the anterior brain it is clear that Isl-1 is also expressed in secondary neurons. These Isl-1-positive secondary neurons mainly arise in association with the early clusters of primary neurons. In the retina (Fig. 5A), all the Isl-1-positive neurons appear after 24 hpf. In general, this pattern of expression is reminiscent of what is seen in the adult rat brain and eye (Thor et al., 1991). In the spinal cord, expression is evident in the dorsal Rohon-Beard cells and in both primary and secondary motor neurons.

DISCUSSION

Isl-1 expression is first detected in different sets of neurons just when gastrulation has come to an end, hours before the neural tube itself forms and axonogenesis is initiated. The expression is initiated simultaneously in several groups of cells, spread on the surface of the yolk. At this stage the Isl-1-positive cells are not connected by any apparent morphological element. In the developing spinal cord both the primary motor neurons and the sensory Rohon-Beard cells start to express Isl-1 at 10 hpf. We find that the Rohon-Beard cells start to express Isl-1 at 10 hpf when they are still located in a very lateral position on the edge of the germinal shield, far from their final dorsal position in the spinal cord. The Rohon-Beard cells could reach this position either by migration within the neuroectoderm or, alternatively, their initial lateral position could demarcate the

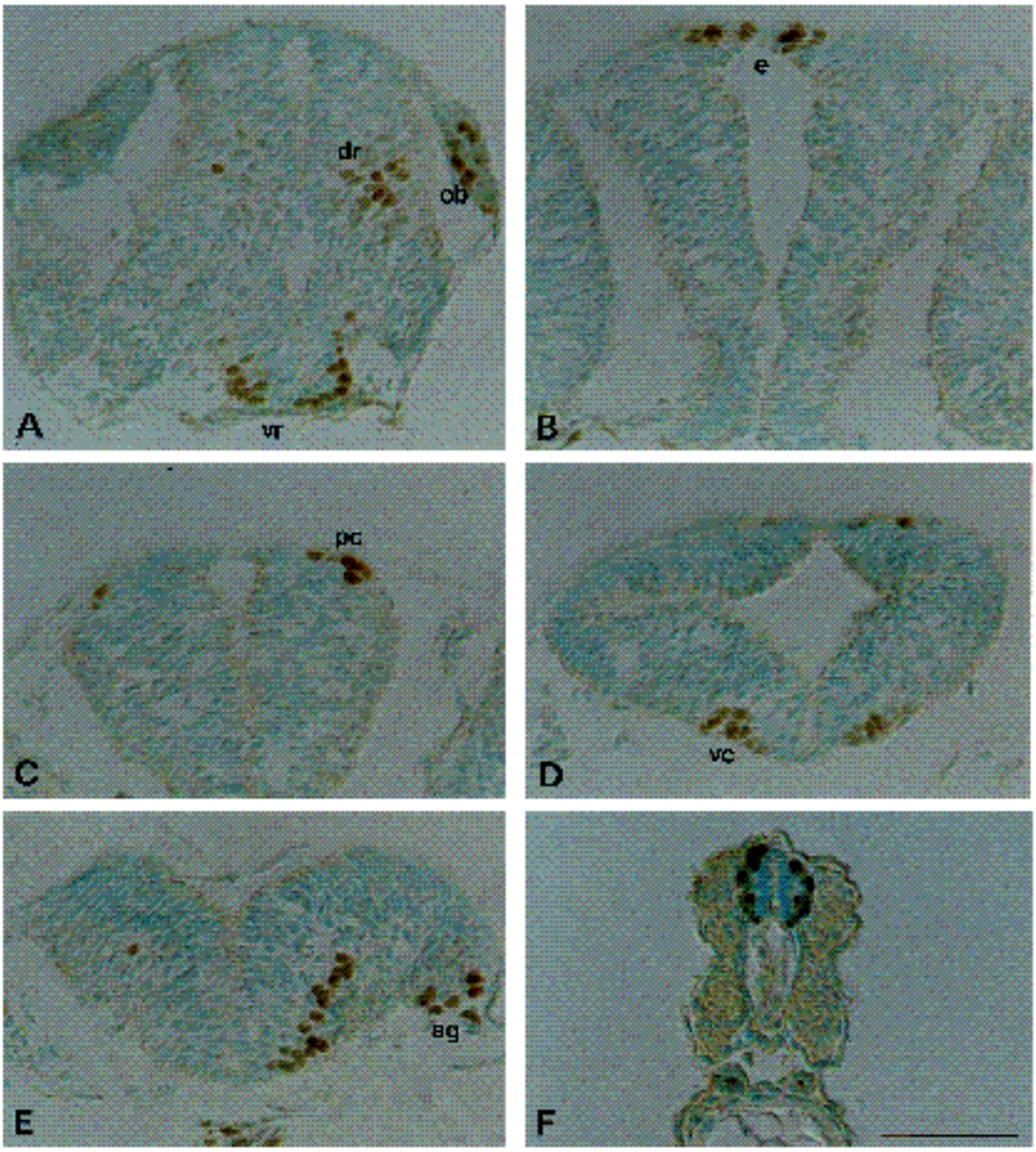

Fig. 3. Expression pattern of Isl-1 at 22 hpf. (A) Cross section through the anterior part of the brain in front of the eyes; (B) cross section through the anterior brain at the level of the epiphysis; (C) cross section at the level of the nucleus of posterior commissure; (D) cross section of the midbrain; (E) cross section of the 4th segment of the hindbrain; (F) cross section through the spinal cord of a double stained (anti-tubulin and anti-Isl-1) embryo. ag, acoustic ganglion; vr, ventrorostral cluster; dr, dorsorostral cluster; ob, olfactory bulb; pc, nucleus of posterior commissure; vc, ventrocaudal cluster. Bar, 50 μm.

border of the presumptive spinal cord. In contrast, Isl-1 expression in the primary motor neurons is initiated at 10 hpf in cells that are evenly spaced in two bilateral columns close to the midline, corresponding, more or less, to their final position in the ventral spinal cord. Although these two cell types are located at very different lateral positions relative to the midline, these two subtypes of neurons initiate Isl-1 expression at the same time and at a similar position along the anterior-posterior axis. In addition, the later forming Isl-1-expressing motoneurons and Rohon-Beard cells appear in an orderly manner, both anteriorly and posteriorly, relative to the initial site of expression.

Recent studies in *Xenopus* suggest that some aspects of the anterior-posterior patterning of the neural ectoderm can occur without the involvement of involuted chordamesoderm (Dixon and Kintner, 1989; Doniach et al., 1992; Slack and Tannahill, 1992; Ruiz i Altaba, 1992). Analyses of neural differentiation in *Xenopus* exogastrulae embryos and in recombinates of ectoderm and mesoderm have specifically suggested that the development of both the primary motor neurons and the Rohon-Beard cells is not dependent on vertical mesodermal signals but instead on planar signals possibly emanating from the notoplate (Ruiz i Altaba, 1992). Our analysis of the temporal and spatial patterns of

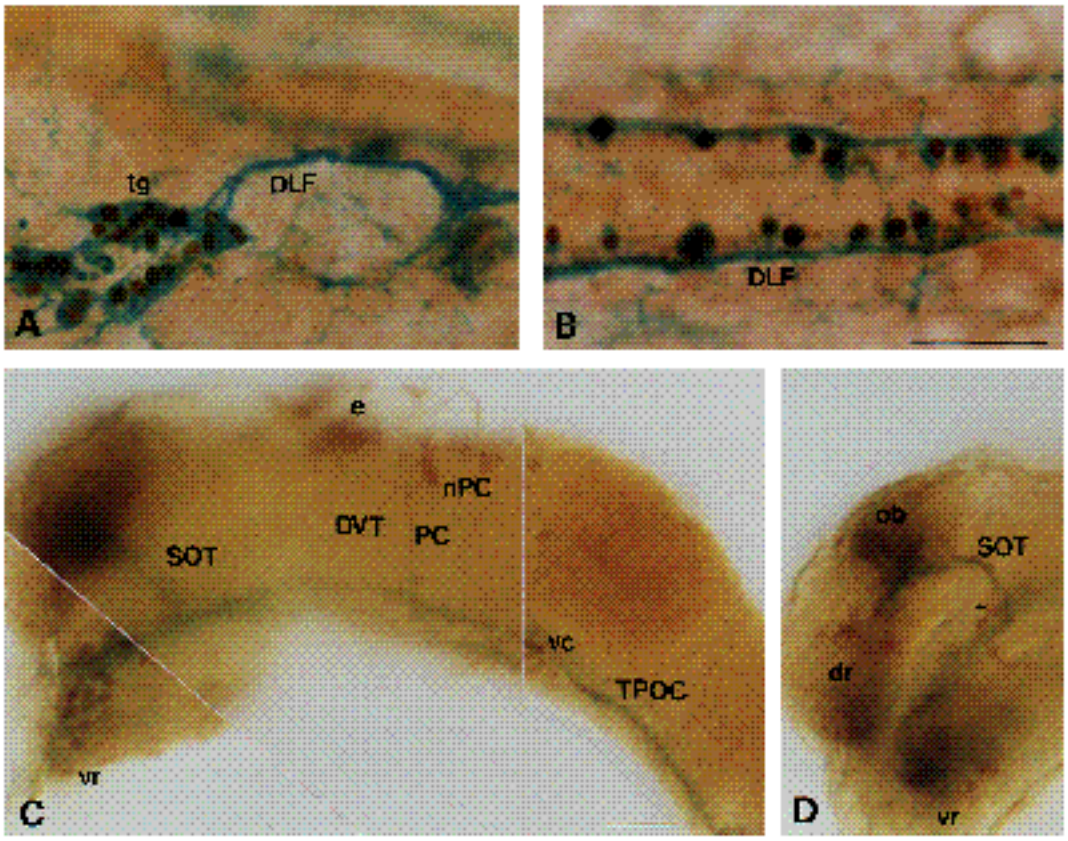

Fig. 4. Isl-1 is expressed in neurons with long axons. (A) Nuclei of the trigeminal ganglion and the anterior part of dorsolateral fascicle (DLF) visualized in a 28 hpf embryo by whole-mount immunostaining with anti-Isl-1 and anti-HNK-1 antibodies; (B) Rohon-Beard nuclei in the spinal cord and the posterior part of dorsolateral fascicle visualized in whole-mount immunostaing using anti-Isl-1 and anti-HNK-1 antibodies (28 hpf); (C,D) whole-mount immunostaining of a 22 hpf embryo using anti-Isl-1 and anti-acetylated α-tubulin antibodies (eyes and yolk were removed before staining). tg, trigeminal ganglion; dr, dorsorostral cluster; vr, ventrorostral cluster; vc, ventrocaudal cluster; ob, olfactory bulb; e, epiphysis; nPC, nucleus of posterior commissure; PC, posterior commissure; DVT, dorsoventral tract; SOP, supra-optic tract; TPOC, tract of postoptic commissure; +, Isl-1-positive cell nuclei in the region where SOT and TPOC fasciculate. Bars, 50 μm.

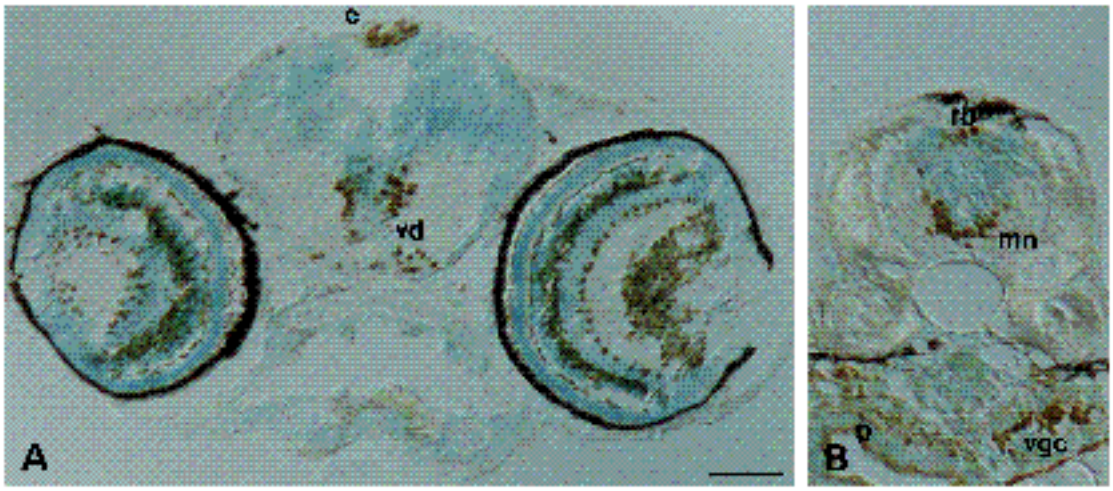

Fig. 5. Restricted pattern of Isl-1 expression in zebrafish larvae at 72 hpf.(A) Cross section through the brain at the level of eyes; (B) cross section through the spinal cord. e, epiphysis; vd, ventral diencephalon; vgc, ventral group of cells; p, putative enteroendocrine cells dispersed in the intestinal wall; rb, Rohon-Beard cells; mn, motor neurons. Bar, 50 μm.

Isl-1 expression in these two cell types support this hypothesis. However, even if these cells initiate Isl-1 expression prior to the formation of a differentiated notochord we cannot exclude the possibility that they respond to vertical neural inducing signals coming from the chordamesoderm. The organization of the Isl-1-expressing anterior group of cells suggests that these cells are also responding to a planar/lateral inducing signal coming from midline axial structures. In the chick, Isl-1 expression in the motor neurons is regulated by the notochord/floorplate signals. At later stages of zebrafish development Isl-1 is also turned on in the secondary motor neurons and most likely these cells respond, as in the chicken, to the notochord/floorplate signals. Using Isl-1 as a marker, it may be possible to identify the putative signal(s) that induce the differentiation of the primary motor neurons and to study the possible relationship between these putative signal(s) and the notochord/floorplate signals. The early onset of Isl-1 expression in the developing zebrafish spinal cord is very different from that observed in the chick, mouse and rat where there is no expression of Isl-1 prior to the closure of the neural tube (Ericson et al., 1992). This difference may be due to general differences in the process of neurulation between these two organisms and/or to the early specification of the primary spinal cord neurons in the zebrafish.

The selective expression of Isl-1 in all classes of neurons of the developing primary nervous system provide evidence that these neurons share a common program of early development. This in turn suggests that these neurons has evolved and been selected for as a coordinated system. Why the primary nervous system has evolved and its precise function(s) is not yet fully understood. These early neurons form a functional circuit which, in both the embryo and the adult, can respond to mechanosensory stimuli (Fetcho, 1992). In the adult this system seems to be used for a variety of rapid movements, but the function of this circuit before hatching is, however, not obvious.

A more structural function of this system has also been proposed since these early neurons pioneer central and peripherial axonal pathways (Chitnis and Kuwada, 1990; Kuwada and Bernhardt, 1990; Wilson et al., 1990; Ross et al., 1992). In all the different locations, pathfinding occurs by the growth cone of a single pioneering neuron and the routes taken are highly stereotyped. These first axons will soon be followed by other axons and small bundles of axons will be generated. This pattern has been described for many of the early developing neurons and collectively they will form a simple scaffold of axonal pathways at 24 hpf (Wilson and Easter, 1992). Thus, the major embryonic function of the primary nervous system may be to establish a framework of axon tracts which can be used as a scaffold for the continued development of the nervous system. The selective expression of Isl-1 in this system may indicate that Isl-1 is part of a developmental regulatory program that is needed for the expression of the pioneering phenotype of these neurons.

Comparative studies have suggested that the primary neurons are phylogenetically old. Assuming that Isl-1 is a marker for pioneering neurons it is striking that Isl-1 is selectively expressed in the phylogenetically old parts of the adult rat brain (Thor et al., 1991).

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