The functional specificity of NeuroD is defined by a single amino acid residue (N11) in the basic domain

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Abstract In zebrafish, the basic helix-loop-helix (bHLH) gene *neuroD* specifies distinct neurons in the spinal cord. A preliminary experiment indicated that a related bHLH gene, *ndr1a*, normally expressed only in the olfactory organ in late embryos, also functions as *neuroD* to induce ectopic formation of spinal cord neurons in early embryos after introduction of its mRNA into early embryos. To define the functional specificity of these bHLH proteins, several mutant forms with selected point mutations in the basic domain were constructed and tested for inducing sensory neurons in the spinal cord. Our data indicate that the functional specificity of NeuroD to define sensory neurons is mainly due to a single residue (asparagine 11) in its basic domain. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Zebrafish; Basic helix-loop-helix; Basic domain; Neurogenesis; Neuron; *isl-1*

1. Introduction

Basic helix-loop-helix (bHLH) domain proteins play crucial roles in controlling the differentiation of various tissues such as the nervous system and muscles [1,2]. It has been documented that similar bHLH proteins could often substitute for each other in promoting specific cell types. For example, members of the *achaete-scute* complex, including *achaete*, *scute* and *asense*, can promote the ectopic development of external sensory organs in *Drosophila* imaginal wing discs [3–5], while another proneural bHLH gene, *atonal*, is able to promote the formation of chordotonal organ (CHO) [6]. Interestingly, the ability of Atonal to induce CHO could be transfer to Scute by replacing the bHLH or just the basic domain of Scute with those from Atonal. However, replacement of the HLH domain alone was insufficient to promote the formation of CHO. Thus, it has been concluded that the basic domain of Atonal is responsible for its functional specificity [7]. Consistent with this, Amos, a closely related bHLH protein, has a near identical basic domain with that of Atonal (only a single amino acid difference at position 1) and can mimic the function of Atonal in misexpression experiments [8,9]. The importance of the basic domain for functional specificity has also been reported for myogenic bHLH proteins [10,11].

In zebrafish, several proneural bHLH genes have been identified in our previous studies [12–14]. Among them, several proteins that belong to the *neuroD* subfamily (*neuroD*, *ndr1a*, *ndr1b* and *ndr2*) could be of most interest because their encoded proteins share a highly conserved bHLH domain. However, their ability to induce neuronal fate varies substantially during zebrafish neurogenesis. For example, *neuroD* is expressed in subsets of neurons distributed throughout the central nervous system, while expression of *ndr1a* and *ndr1b* is quite late and is only in the olfactory organ. Nevertheless, injection of *ndr1a*, but not *ndr1b*, mRNA can induce the early neuronal marker, *islet-1* (*isl-1*), which is an immediate downstream target of NeuroD [15,16]. To dissect the functional specificities of these bHLH genes, we compared the basic domains of NeuroD, Ndr1a and Ndr1b (Fig. 1), and generated several point mutations in the basic domain of Ndr1b. Our functional data showed that the change of a single amino acid residue, asparagine 11 (N11), was sufficient to provide Ndr1b with a NeuroD-like activity. In addition, replacement of this residue in the basic domain of NeuroD protein dramatically reduced the ability of NeuroD to induce ectopic expression of *isl-1*.

2. Materials and methods

2.1. Zebrafish and embryos

Zebrafish were maintained in our laboratories of the Department of Biological Sciences, National University of Singapore, and the Institute of Molecular Agrobiology. The developmental stages were defined as hours post fertilization (hpf) at 28.5°C.

2.2. Site-directed mutagenesis by PCR

Site-directed mutagenesis was performed by a PCR–ligation–PCR approach [17]. As diagrammed in Fig. 2, for generation of mutations in the basic domain of Ndr1b protein, a pair of mutation primers (P1 and P2) was designed based on the altered basic domain. Using these primers and vector primers (SP6 and T7), two fragments, each containing half of the basic domain, were amplified with high fidelity *pfu* DNA polymerase that generated blunt-ended products. The fragments were then ligated and secondary PCR reaction was performed with two vector primers to amplify full-length *ndr1b* cDNAs containing mutations. The products were then cut with *Bam*HI and *Xho*I, and ligated into pCS2+ vector [18]. After transformation and colony screening, clones with inserts of the right size were selected for sequencing to confirm the mutations. The detailed description of the mutations is summarized in Fig. 3. Corrections have been made to the previously published *ndr1b* basic domain sequence (GenBank accession number AF115773) [13].

2.3. RNA synthesis and microinjection

All DNA constructs used for RNA synthesis have a pCS2+ expression vector backbone [18]. The plasmids were linearized with *Not*I. 5′-capped mRNAs were synthesized using the SP6 mMESSAGE

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mMACHINE kit according to the instruction manual from the manufacturer (Ambion, USA). The in vitro transcribed mRNAs were suspended in DEPC (diethyl pyrocarbonate)-treated water and then mixed with injection buffer containing phenol red. 4.4 nl of the mRNA solution (150 ng/μl) was injected into zebrafish embryos at the one-cell stage using a Nanoliter Injector (MPI, USA).

2.4. Whole mount in situ hybridization

Whole mount in situ hybridization using a digoxigenin (DIG)-labeled riboprobe was carried out as previously described [12]. All template plasmids were linearized with *Bam*HI, followed by in vitro transcription by T7 RNA polymerase for antisense RNA probe. Embryos were fixed with 4% paraformaldehyde and hybridized with the probe in hybridization buffer (50% formamide, 5×SSC [1×: 15 mM NaCl, 1.5 mM sodium citrate, pH 7.6], 50 μg/ml heparin, 500 μg/ml yeast tRNA and 0.1% Tween 20) at 70°C, followed by washes with 2×SSC and 0.2×SSC for 2 h each at 70°C. Embryos were then incubated with anti-DIG antibody conjugated with alkaline phosphatase. Signals were developed by staining with nitroblue tetrazolium and 5-bromo,4-chloro,3-indolyl phosphate.

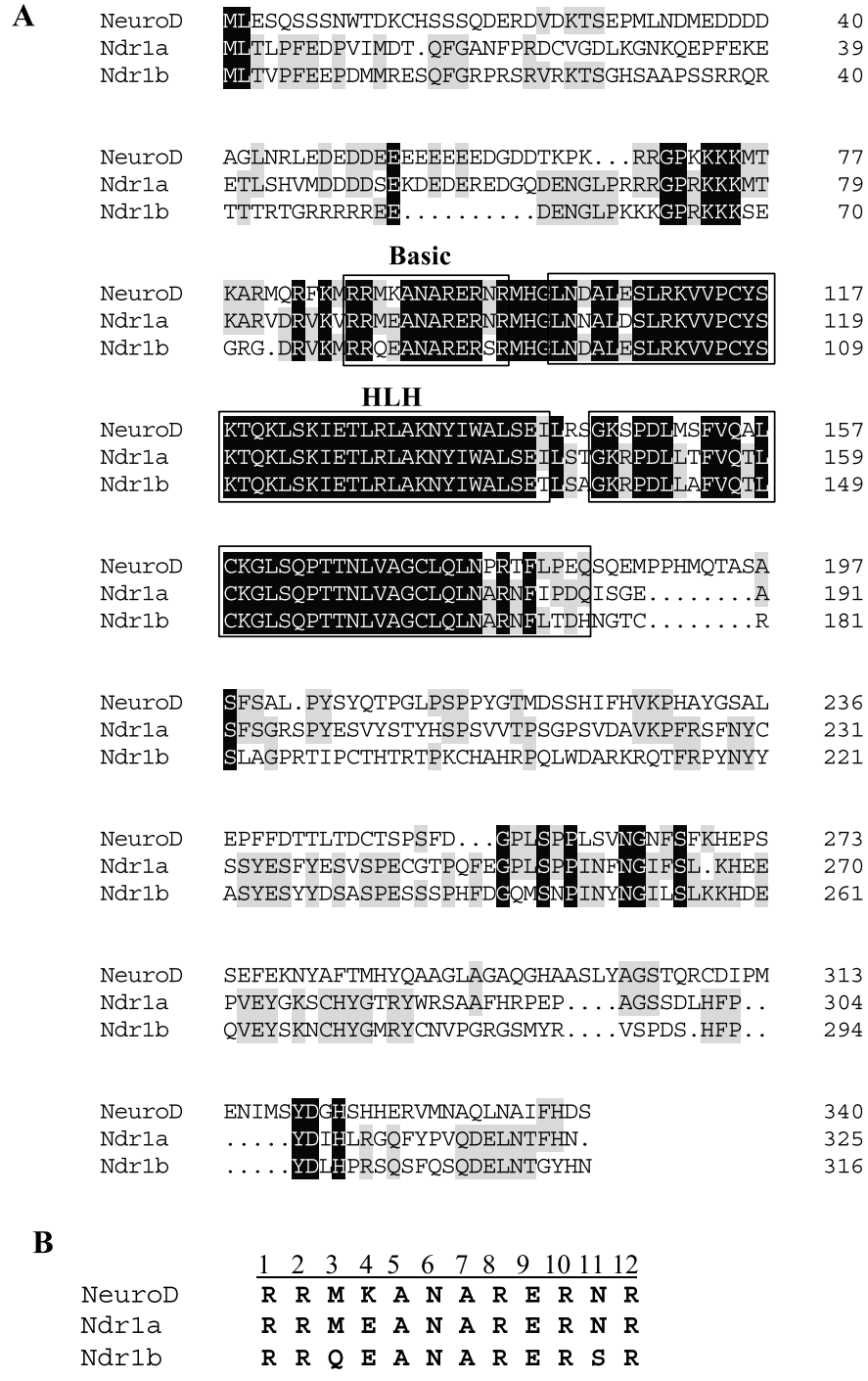

Fig. 1. Alignment of complete amino acid sequences (A) and basic domains (B) of zebrafish NeuroD, Ndr1a and Ndr1b. In A, the basic domain, HLH domain and another conserved region are indicated by boxes. Identical amino acid residues in all three sequences are dark-highlighted and in two of the three sequences are light-highlighted. Dots represent gaps inserted for maximal alignment. In B, the order numbers of residues in the basic domains are indicated.

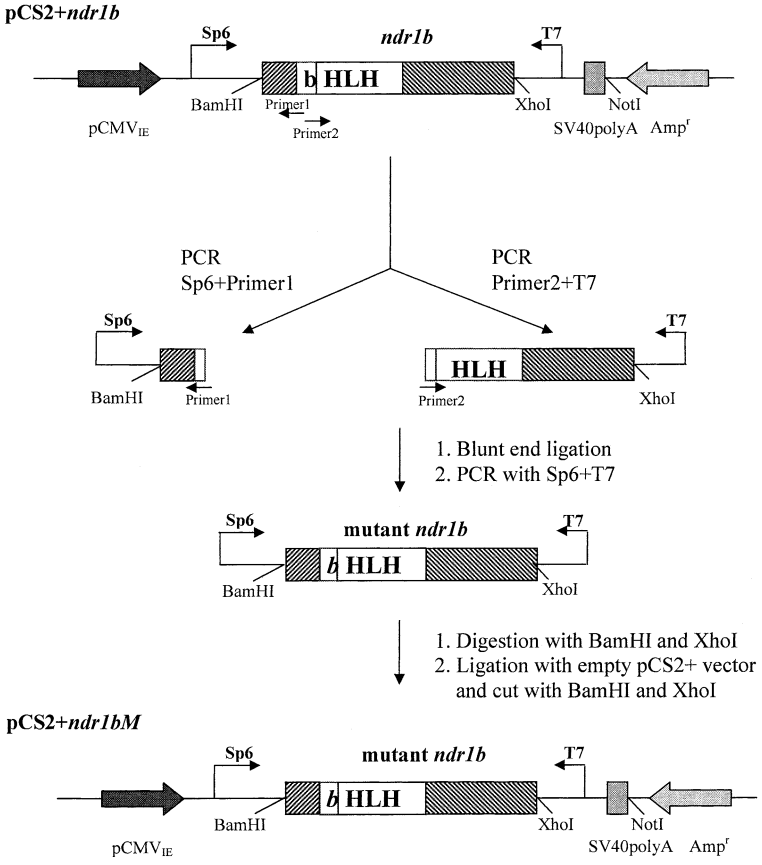

Fig. 2. Schematic representation of the procedure to introduce mutations in the basic domain of Ndr1b protein. Amp^r, ampicillin-resistant gene; pCMV$_{IE}$, cytomegalovirus (CMV) promoters; SV40polyA, simian virus 40 (SV40) polyA signal; P1 and P2 are two PCR primers containing substituted nucleotides for site-directed mutagenesis.

3. Results and discussion

3.1. Basic domain of NeuroD family members

Fig. 1A shows the sequence alignment of three closely related members of the NeuroD family from zebrafish: NeuroD, Ndr1a and Ndr1b. As indicated by boxes, the central one third of the sequence including the bHLH domain and its immediate downstream region is highly conserved. The remaining two-thirds of the sequence are quite divergent among the three proteins. The overall sequence identities range from 40.8% to 63.5%.

In Fig. 1B, the basic domains of the three bHLH proteins are aligned and the amino acid residues are defined by numbers. The three proteins have nine invariant residues (R1, R2, A5, N6, A7, R8, E9, R10 and R12), seven of which (except for A5 and A7) were thought to contact DNA based on the crystal model of MyoD and a computer model of the Atonal/Daughterless heterodimer [7]. Only the residues at positions 3, 4 and 11 vary among the three proteins. Ndr1a has only one amino acid (E4) different from NeuroD, while Ndr1b has three amino acids (Q3, E4, S11) different.

3.2. The ability of NeuroD to induce ectopic isl-1 expression may be determined by N11 in the basic domain

Isl-1 is a LIM homeodomain transcription factor and is expressed in primary motoneurons and sensory neurons in the spinal cord in zebrafish [19–22]. As described in our previous study, the expression of zebrafish *neuroD* and *isl-1* overlaps in the early neural plate [12], implying that *neuroD* is probably an upstream regulator of *isl-1*. Recently, we demonstrated in zebrafish that mis-expression of *neuroD* induces ectopic expression of *isl-1* [16]. With the isolation of more *neuroD*-related bHLH genes such as *ndr1a*, *ndr1b* and *ndr2*, evaluation of their developmental roles in specification of various types of neurons was conducted. The expression of *ndr1a* and *ndr1b* ($\sim$24 hpf) starts much later than that of *isl-1* ($\sim$10 hpf). Nevertheless, over-expression of *ndr1a* induces formation of ectopic *isl-1*-expressing cells (Fig. 4C), just like over-expression of *neuroD* (Fig. 4B). In comparison, the control wild-type embryo has more or less a single line of *isl-1*+ Rohon–Beard sensory neurons on each side of the neural plate (Fig. 4A), while increased numbers of *isl-1*+ cells are observed

Table 1
Summary of embryos injected with mRNAs from bHLH genes and mutants

mRNA injected	Number of embryos injected	Embryos with ectopic *isl-1* expression at 12 hpf (percentage)
nrd	27	21 (77.8)
ndr1a	33	8 (24.2)
ndr1b	31	0 (0)
ndr1b-M1	28	4 (14.3)
ndr1b-M2	26	3 (11.5)
ndr1b-M3	24	3 (12.5)
ndr1b-M4	22	0 (0)
nrd-M	30	9 (30.0)

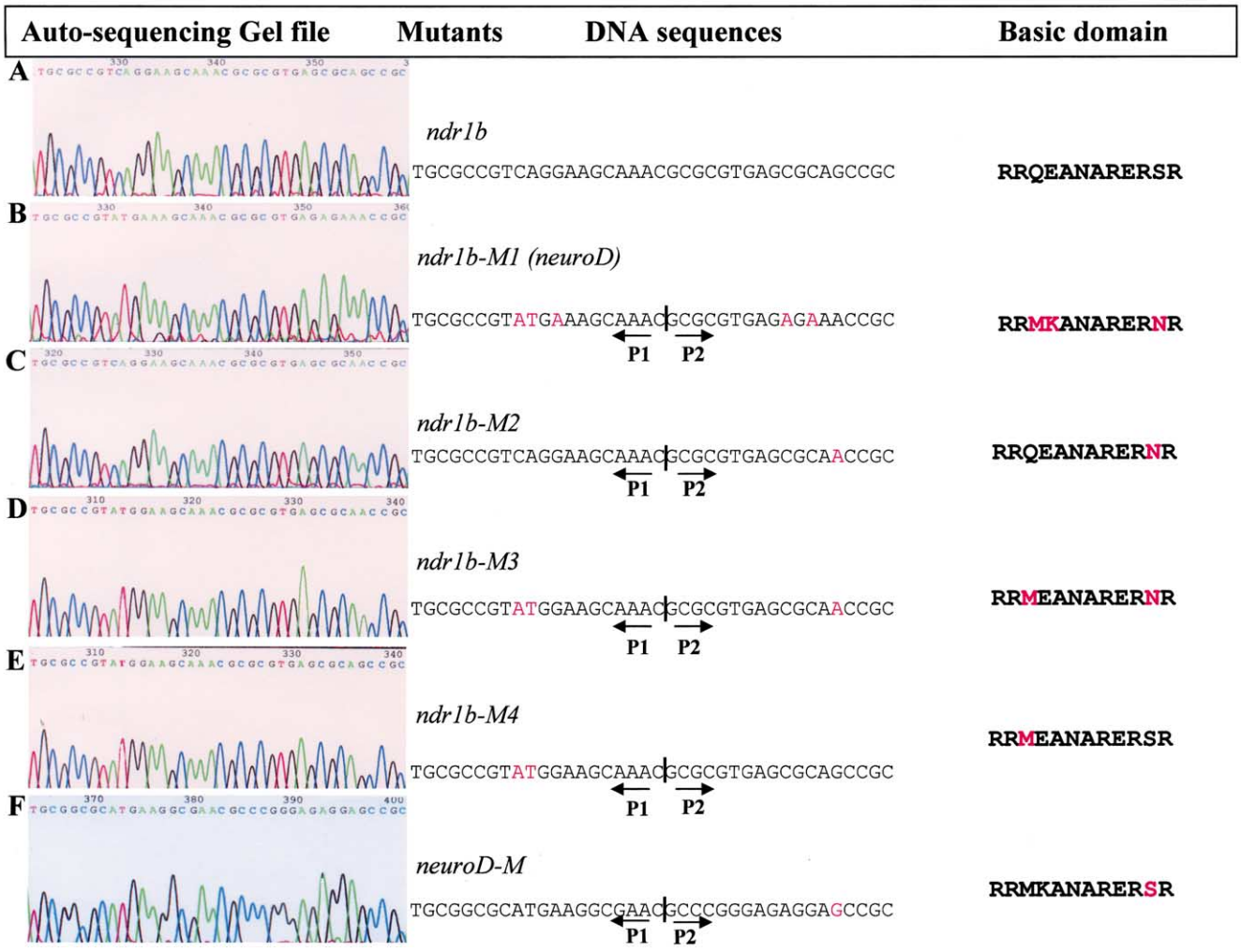

Fig. 3. Nucleotide and amino acid sequences of the basic domain regions of Ndr1b and mutants: *ndr1b* (A), *ndr1b-M1* (B), *ndr1b-M2* (C), *ndr1b-M3* (D), *ndr1b-M4* (E), and *neuroD-M* (F). The automatic sequencing gel files are shown on the left side to confirm the mutated nucleotides. P1 and P2 represent primer sequences used for creating mutations in the basic domain. Deduced amino acid sequences are shown on the right. Red letters indicate mutated nucleotides and amino acid residues.

in some embryos injected with *neuroD* or *nrd1a* RNA. This observation suggested that *ndr1a* may act as *neuroD*. However, over-expression of *ndr1b* failed to induce ectopic expression of *isl-1* (Table 1).

To identify the element responsible for the functional specificity, various Ndr1b mutant proteins with altered basic domains were generated as summarized in Fig. 3. These mutant forms were tested in RNA injection experiments. As shown in Fig. 4D,F, both Ndr1b-M1 (with NeuroD basic domain) and Ndr1b-M3 (with Ndr1a basic domain) were able to induce *isl-1* expression, suggesting that the basic domains of NeuroD and Ndr1a may contain information for defining *isl-1*+ neurons. The basic domains of Ndr1a and Ndr1b differ at only two residues. To determine which one is responsible for the functional specificity, two mutant proteins were constructed. In Ndr1b-M2, S11 was replaced by N11, while in Ndr1b-M4, Q3 was substituted by M3. As shown in Fig. 4E,G, only Ndr1b-M2 has the ability to induce ectopic *isl-1* expression. Thus, the asparagine at position 11 in NeuroD may be necessary for its functional specificity. To further confirm the importance of this residue, a point mutation was made to substitute N11 with S11 (Ndr1b) in the basic domain of NeuroD and we found that the single amino acid substitution substantially reduced the ectopic *isl-1* expression from 77.8% to 30.0% (Table 1 and Fig. 4H), again indicating the importance of N11 in defining the functional specificity of NeuroD. Consistent with this, R11 in the basic domain of *Drosophila* Atonal is also one of the important residues identified to contribute to its functional specificity [7]. In *Xenopus*, replacement of S11 in the basic domain of XATH3, a homolog of *Drosophila* Atonal, also significantly decreased its ability to induce ectopic neurons [23].

3.3. The full function of NeuroD may also require other structural elements

As summarized in Table 1, although replacement of the basic domain of Ndr1b with that from NeuroD allowed the mutant Ndr1b to function like NeuroD in inducing ectopic neurons (mostly sensory neurons), it was not as efficient (Ndr1b-M1, 14.3%; Ndr1b-M2, 11.5%; Ndr1b-M3, 12.5%; Ndr1a, 24.2%) as NeuroD (77.8%). Moreover, the change of N11 in the basic domain of NeuroD still retains a relatively high ability to induce *isl-1*+ neurons (30.0%). Hence, there must be other structural elements that also contribute to Neu-

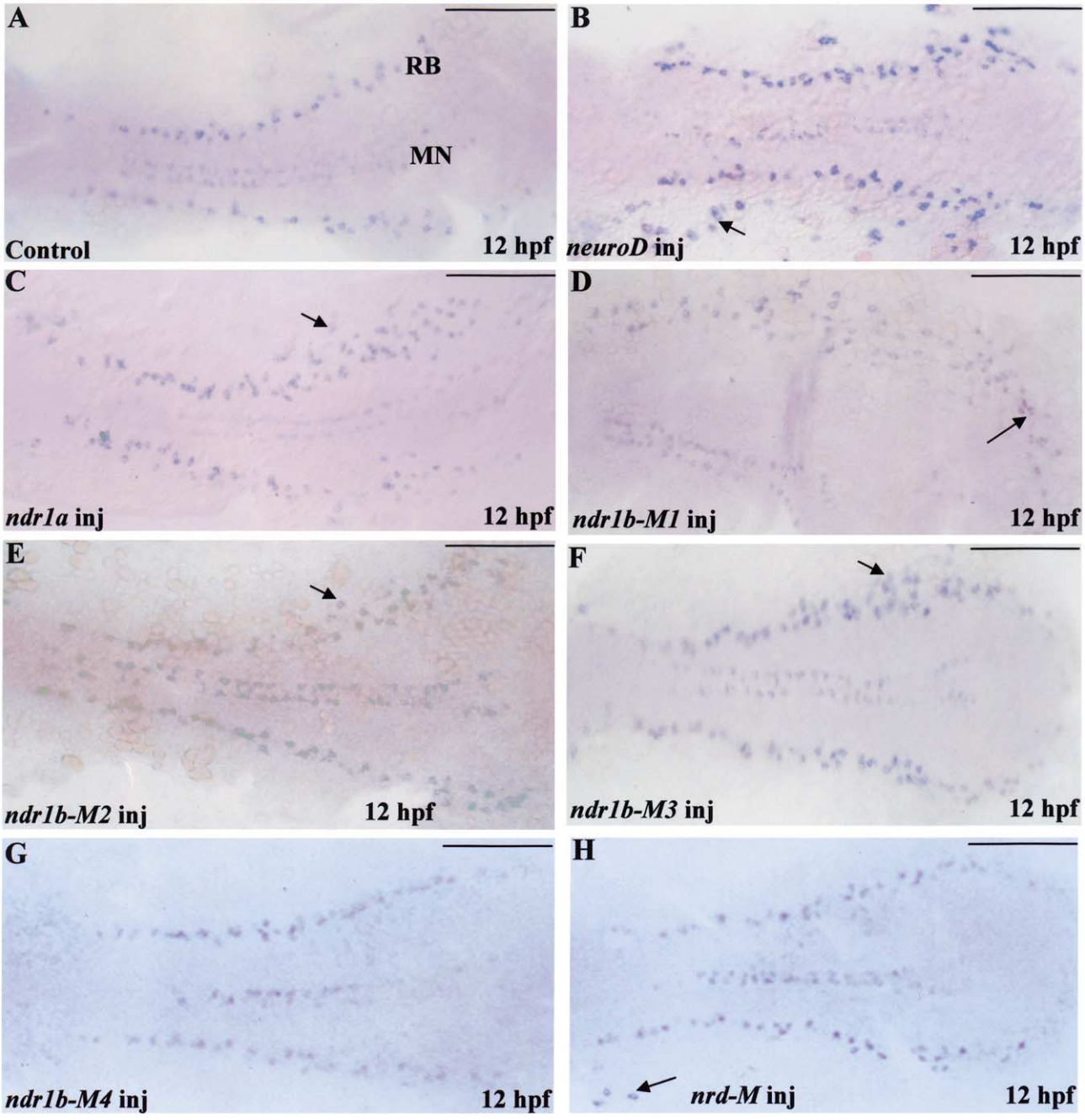

Fig. 4. Expression of *isl-1* in a control embryo (A) and embryos injected with synthetic mRNAs from *neuroD* (B), *ndr1a* (C), *ndr1b-M1* (D), *ndr1b-M2* (E), *ndr1b-M3* (F), *ndr1b-M4* (G) or *ndr-M* (H). All embryos were hybridized with the *isl-1* probe at 12 hpf. Only the spinal cord region is shown and all images are taken from the dorsal view of flat-mounted embryos. Arrows indicate positions of ectopic expression of *isl-1*. Abbreviations: MN, motoneurons; RB, Rohon–Beard sensory neurons. Scale bars show 100 μm.

roD specificity both qualitatively and quantitatively. Consistent with this, in a previous experiment in *Drosophila*, the Atonal (basic)–Scute (HLH) chimeric protein was much less efficient in promoting formation of CHO than the wild-type Atonal, again suggesting that the regions outside the basic domain may also contribute to the neuronal type specification [7].

Since the functional specificity of NeuroD is largely determined by a single residue in the basic domain that is well known for DNA binding, it is likely that N11 is critical to recognize specific *cis*-elements in the target genes. Change of residue 11 may change DNA binding affinity and/or specificity and thus change the expression of the target genes. Other unidentified structural elements may be required for optimal protein–protein interaction and thus to enhance the expression of the target genes. It is also possible that the observed activation of *isl-1* by Ndr1a and several mutants of Ndr1b is due to transactivation of endogenous *neuroD*, as discussed by Chien et al. [7] in the case of induction of external sensory organ by overexpression of Atonal. Finally, our observations in this study also indicate that caution may be required in interpretation of the data commonly generated by over- and

ectopic expression of a gene as the altered phenotype observed may be due to an alteration of a similar gene.

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