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Two GC-rich boxes in *huC* promoter play distinct roles in controlling its neuronal specific expression in zebrafish embryos

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Abstract

HuC, a vertebrate ortholog of *Drosophila elav* gene, encodes an RNA binding protein and is involved in early neurogenesis. Zebrafish *huC* is expressed in distinct neurons, including Rohon-Beard (RB) sensory neurons, interneurons and motoneurons, during primary neurogenesis, and in all neurons later during secondary neurogenesis. In this study, we identify two GC-rich box elements, proximal GC (p-GC) box from −172 to −149 and distal GC (d-GC) box from −218 to −208, in zebrafish *huC* promoter. Using transgenic approach, we demonstrate that deletion of the p-GC box from the promoter results in loss of expression of the reporter GFP in neurons while deletion of the d-GC box leads to GFP expression only in dorsal RB sensory neurons. These results suggest that the p-GC box alone confers transcriptional activity of *huC* promoter in primary RB neurons and the d-GC is required for *huC* transcription in the full spectrum of spinal cord neurons. Further studies are needed to identify specific Sp1-like transcription factors that bind to these GC boxes and activate *huC* transcription.

Keywords: Zebrafish; *huC*; Neuron; GC-rich box; Sp1-like transcription factors

HuC is a vertebrate homolog of *Drosophila elav* gene that is first identified in an embryonic lethal abnormal visual system phenotype with early neural defects [1]. Hu proteins constitute a highly conserved Hu RNA binding proteins family, which is characterized by the presence of three copies of RNA binding domain called the RNA recognition motif (RRM) [2,3]. Members of this family have been reported to enhance the stabilization and/or translation of target mRNAs by binding to AU-rich elements in untranslated regions (UTRs) [4–8]. In mammals, four members of Hu protein family have been reported: HuA/HuR [9], HuB/Hel-N1 [10], HuC/Ple-21 [11], and HuD [12]. *HuB*, *Huc*, and *HuD* genes are specifically expressed in neurons and are involved in the neuronal differentiation and maintenance of the nervous system [13,14].

Zebrafish *huC* gene has a neuronal-specific expression pattern similar to that in other vertebrate species. Soon after gastrulation, zebrafish *huC* starts to be expressed in three longitudinal columns of scattering neurons on either side of the midline: primary Rohod-Beard (RB) sensory neurons in the lateral columns; primary motor neurons in the medial columns; and primary interneurons in between the lateral and medial columns [15,16]. The number of neuronal cells expressing *huC* increases sharply as the secondary neurogenesis occurs a few hours later. Although its specific functions are not known in zebrafish, *huC* has been widely used as an early neural marker for studying neurogenesis (for example [17–19]).

Using transgenic approach, Park et al. [20] demonstrate that a 2.8-kb 5′-upstream sequence of zebrafish *huC* gene is sufficient to confer neuronal-specificity of *huC* expression in developing embryos. They further reveal that a series of E-box are required for suppressing *huC* expression in non-neuronal cells and a proximal

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CCAAT box is essential for *huC* transcription. However, it remains unknown how *huC* expression is spatially controlled. During functional assays of Sp1-like family members, we noted that some of them could be involved in regulation of *huC* expression in zebrafish embryos. This observation prompted us to look for in the *huC* promoter GC-rich boxes, which are generally recognized and bound by Sp1-like transcription factors [21], and to study their roles in regulating *huC* expression. We report here that a distal GC box (d-GC) is required for *huC* expression in interneurons and motoneurons while a proximal GC box (p-GC) is an essential element of the core promoter for the *huC* gene.

Materials and methods

Zebrafish strains and maintenance. Wild-type embryos of the Tubingen strain were used. Zebrafish were raised and maintained under standard conditions.

Plasmid DNA constructs. According to Park et al.'s report [20] and related sequence AF173984, a 3.1-kb fragment, which contains the 2.8-kb 5′ upstream sequence and the first exon of zebrafish *huC* gene, was amplified by PCR from genomic DNA using primers Fp5 and Fp3. The PCR product was cloned into the *Xho*I/*Bam*HI sites of pBluescript II KS to obtain pKS-huC. A modified GFP reporter gene (GM2) [22] was inserted downstream of the *huC* promoter in pKS-huC to make the Full-GM2 construct. Deletional constructs were made by joining different PCR fragments from the promoter region and subcloning into expression vectors (Fig. 1A). Sequences of primers used for cloning were listed below, with added restriction sites in lower case and numbered positions in the *huC* promoter.

Fp5: 5′-ccgctcgagTCTATTCCTAAAGACCTTGGGTG-3′, −2737 to −2715;
Fp3: 5′-cgggatccTAACCATTCTTGACGTACAAAG-3′, +368 to +390;
M1p3: 5′-ccggaattcAACACACATGCAGAGCTG-3′, −237 to −220;
M1p5: 5′-ccggaattcTCGGTGATCTCTCCTGAAGGG-3′, −145 to −125;
M2p3: 5′-ccggaattcCCTGCCCCCCCCAAACACACATGC-3′, −230 to −207;
M3p3: 5′-ccggaattcATCTCTCCACTCTTCCTTTTG-3′, −193 to −173;
M4p5: 5′- ccggaattcATGGTGGGGGCTTGGAGGGATGG-3′, −174 to −153;
M5p5: 5′-ccgctcgagCCTTCACACAGTTACAGCCGCTC-3′, −459 to −437.

Microinjection of zebrafish. To examine transcriptional activity of *huC* promoter elements, plasmid DNA of different constructs was injected into single-cell stage embryos at a concentration of 100 ng/μl. For generating germline transgenic fish, founder fish were crossed with wild-type fish and

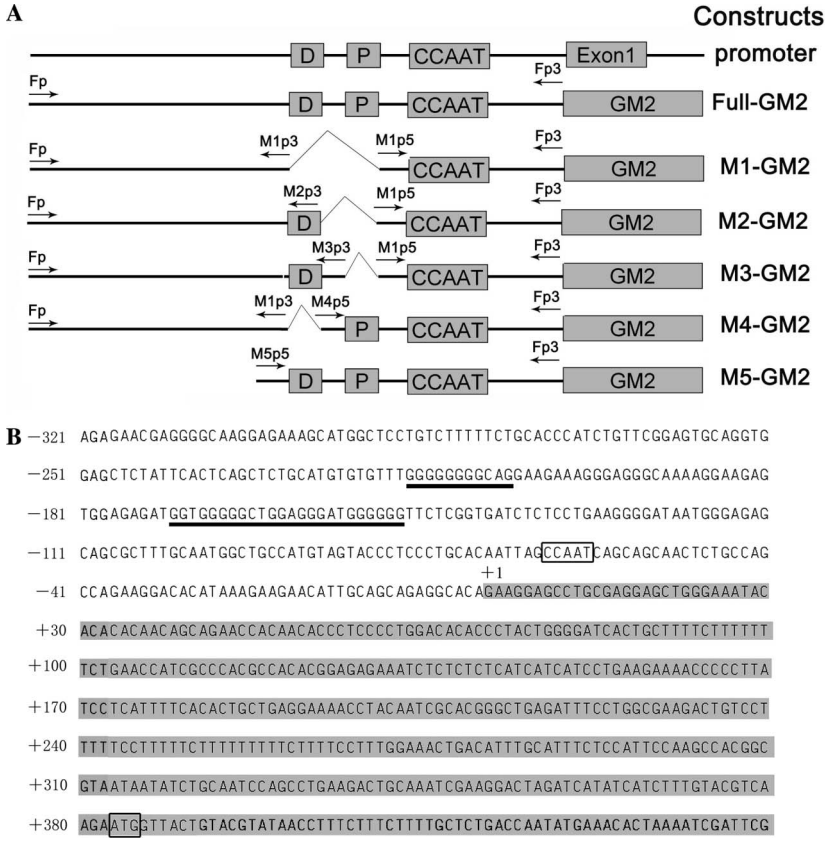

Fig. 1. Structures of transgene constructs and related *huC* promoter elements. (A) Key regulatory elements in *huC* promoter and cloning strategy. The positions and orientation of primers used for cloning were indicated. D, distal GC-rich box; P, proximal GC-rich box. (B) Promoter sequence of *huC* gene. The two GC-rich boxes were underlined. The CCAAT element and translation start codon were boxed. The transcription initiation site was numbered +1. Transcription sequence was shadowed.

the transgenic progeny was identified according to GFP expression. Microinjection was performed as described before [23]. Injected or transgenic embryos were observed for GFP expression by fluorescent microscopy.

In situ hybridization. Digoxigenin- or fluorescein-labeled antisense RNA probes were generated by in vitro transcription using linearized GFP or huC plasmid as template. Whole-mount in situ hybridization essentially followed the protocol described before [24].

Results and discussion

Two GC- rich boxes are located in the huC promoter

By analyzing the 2.8-kb upstream sequence of the *huC* promoter that was reported before [20], we found two GC-rich boxes that are potential binding sites for

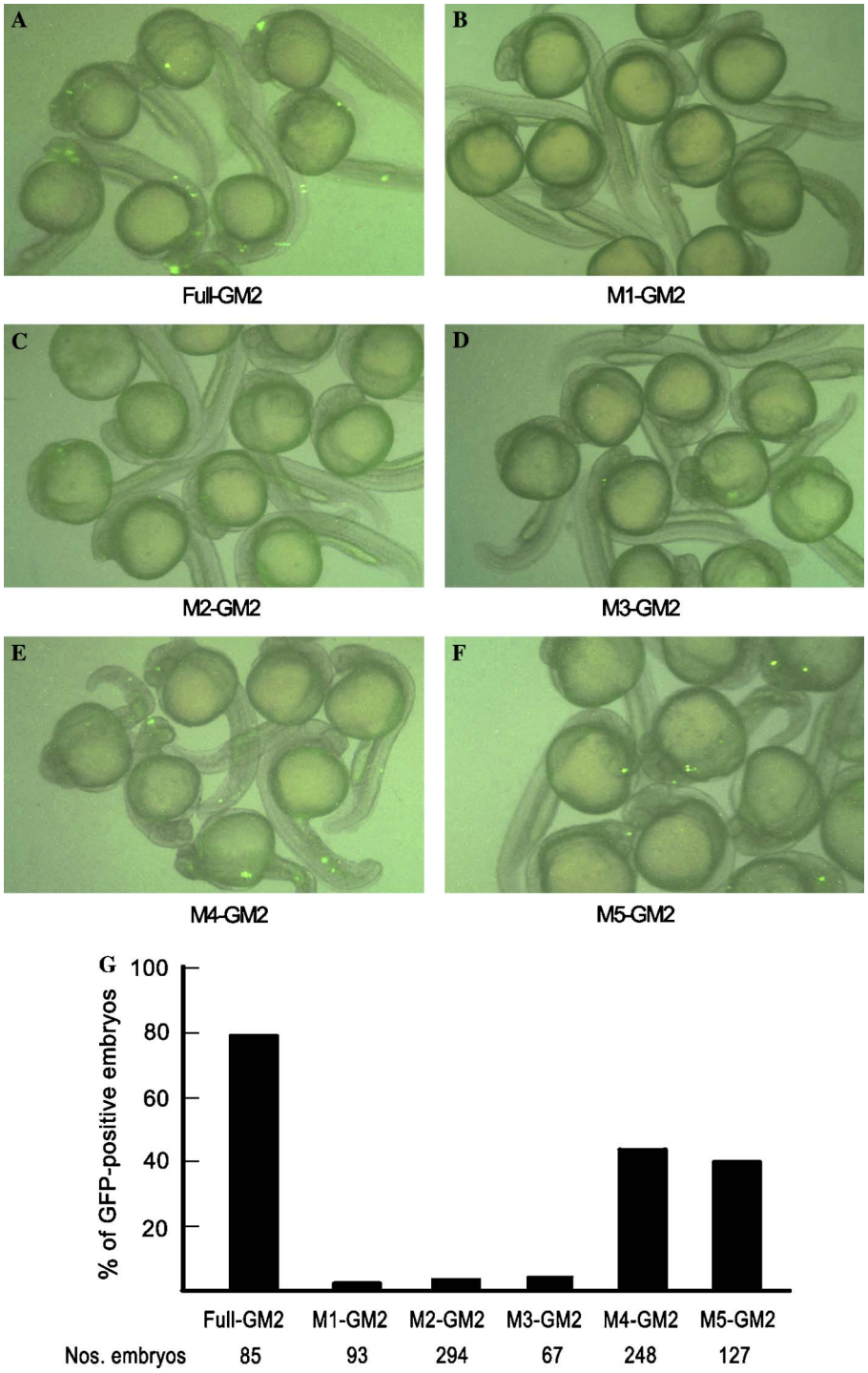

Fig. 2. Transient expression of GFP in injected zebrafish embryos. (A–F) Live embryos at 24 hpf. The embryos were injected at one-cell stage with different expression transgene constructs and observed at 24 hpf under a fluorescent microscope. (G) Percentage of embryos with GFP expression in trunk neurons. The number of observed embryos was indicated.

Sp1-like transcription factors. The proximal GC-rich box, designated as p-GC box, is located from −172 to −149 and has a sequence of GGTGGGGGCTGGAG GGATGGGGGG; and the distal one, designated as d-GC box, is located from −218 to −208 and has a sequence of GGGGGGGGCAG (Fig. 1B). A CCAAT box lies between −64 and −60. This CCAAT box has been previously found to be an essential element for *huC* transcription [20], but the regulatory roles of the two GC boxes are not known. Thus, we investigated the potential roles of the d-GC and p-GC boxes in regulation of *huC* transcription using transgenic approach.

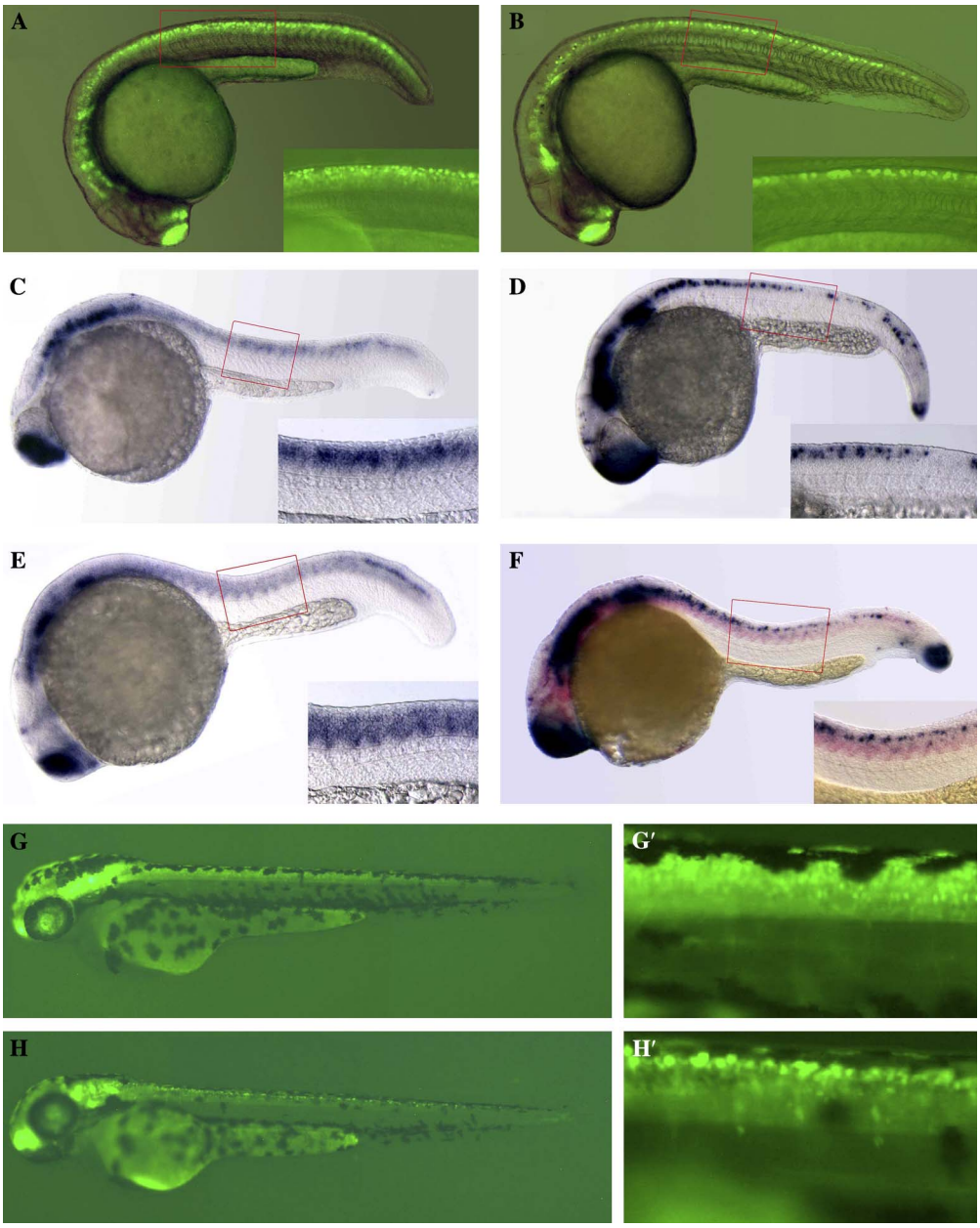

Fig. 3. Expression patterns of GFP in transgenic embryos and of endogenous *huC*. (A) GFP expression in a live Full-GM2 transgenic embryo at day 1. (B) GFP expression in a live M4-GM2 transgenic embryo at day 1. Note that GFP is absent in the middle and ventral region of the spinal cord. (C) Whole-mount in situ hybridization detected *gfp* mRNA distribution in a Full-GM2 transgenic embryo at day 1. (D) Whole-mount in situ hybridization detected *gfp* mRNA distribution in an M4-GM2 transgenic embryo at day 1. (E) Endogenous *huC* expression in a 1-day-old FULL-GM2 transgenic fish, detected by whole-mount in situ hybridization using digoxigenin-labeled *huC* antisense RNA probe. (F) Double staining with fluorescein-labeled *huC* antisense RNA probe and digoxigenin-labeled *gfp* RNA probe revealed expression of endogenous *huC* (red) throughout the spinal cord and expression of *gfp* (blue) in the dorsal RB neurons in an M4-GM2 transgenic embryo. The insets in (A–F) showed the boxed region at a higher magnification. (G) GFP expression in a 2-day-old Full-GM2 transgenic embryo. A trunk region of this embryo was shown in (G′) at a higher magnification. (H) GFP expression in a 2-day-old M4-GM2 transgenic embryo. The higher magnification (H′) of a trunk region of this embryo indicates that GFP-positive cells are larger RB neurons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

P-GC box is absolutely required for the huC promoter activity

We first generated an expression construct Full-GM2, in which the GM2 cDNA was placed downstream of the 2.8-kb *huC* promoter [20]. Based on this construct, a series of deletional constructs were made, which in the promoter region lack either the d-GC box or the p-GC box or both. DNAs of various constructs were microinjected into one-cell stage embryos, followed by observing GFP expression at 24 h postfertilization (hpf) and counting the number of GFP-positive embryos.

The 2.8-kb *huC* promoter drove efficient expression of the reporter GFP in the injected embryos at 24 hpf (Fig. 2A), which was similar to observation made by Park et al. [20]. We found that 78.8% (67/85) of injected embryos had GFP expression in trunk neurons (Fig. 2G). Injection with M5-GM2, which lacks the sequence upstream of the d-GC box in the promoter, allowed 40.1% (51/127) of embryos to express GFP in neurons (Figs. 2F and G). In contrast, only 3.2% (3/93) of embryos injected with M1-GM2 that lacks d-GC and p-GC boxes showed GFP expression (Figs. 2B and G). This suggests that those two GC-rich boxes are necessary for *huC* expression in neurons.

Next we asked whether both d-GC and p-GC boxes are indispensable for *huC* promoter activity. When injected with M2-GM2 or M3-GM2, both of which lack the p-GC box, only 3.4% (10/294) or 4.7% (3/67) of embryos had GFP-positive neurons (Figs. 2C and D), suggesting absolute requirement of the p-GC box for *huC* transcription. On the other hand, injection with M4-GM2 that lacks the d-GC box only still permitted 44.4% (110/248) of embryos to express GFP in neurons (Fig. 2E). This observation by transient transgenic analysis raises a possibility that the d-GC box only exerts an additive effect on or is dispensable for *huC* transcription.

d-GC box is necessary to drive GFP expression in interneurons and motoneurons

In the transient transgenic assays, we were unable to precisely determine the locations of GFP-positive neurons. Thus, we screened germline transgenic fish by mating injected adult fish to wild-type fish and observing GFP expression in their progeny. Because embryos injected with M1-GM2, M2-GM2 or M3-GM2 rarely gave rise to GFP expression in neurons, we focused on screening germline transgenic fish for Full-GM2 and M4-GM2 only. Subsequently, we identified one germline transgenic male among Full-GM2 founder fish and two germline transgenic male among M4-GM2 founder fish. Progeny of the two M4-GM2 transgenic fish produced the same GFP expression pattern.

We then observed GFP expression in the transgenic embryos by fluorescence microscopy. GFP expression was seen in telencephalon, diencephalon, hindbrain, and the whole spinal cord along the anterioposterior and dorsoventral axes in 24-hpf Full-GM2 transgenic embryos (Figs. 3A and C), which mimicked endogenous *huC* expression pattern (Fig. 3E) and shared the same pattern as in *huC:GFP* transgenic fish generated by Park et al. [20]. In 24-hpf M4-GFP transgenic embryos, GFP was also detected in telencephalon, diencephalon, and hindbrain (Figs. 3B and D), which were similar to those in Full-GM2 transgenic embryos. Unlike in Full-GM2 transgenic embryos, however, GFP in the trunk region of M4-GM2 transgenic embryos was only expressed in larger, scattered dorsal neurons but absent in interneurons and motoneurons derived from the spinal cord. A double staining with *GFP* and *huC* antisense probes confirmed that M4-GM2 was unable to drive GFP expression in interneurons and ventral neurons (Fig. 3F). The location and shape of GFP-positive neurons in the trunk region of M4-GM2 transgenic embryos suggest that they are primary RB sensory neurons. Even at 48 hpf, the difference in GFP expression between Full-GM2 and M4-GM2 transgenic embryos remained (Figs. 3G and H). Taken together, these results suggest that the p-GC box alone confers the transcriptional activity of the *huC* promoter in dorsal neurons of the spinal cord while the d-GC box is required for *huC* expression in the full spectrum of neurons in the spinal cord.

Sp1-like transcription factors may be involved in regulation of huC transcription

Using transgenic approach, we demonstrate that in the *huC* promoter two GC-rich boxes, d-GC box, and p-GC

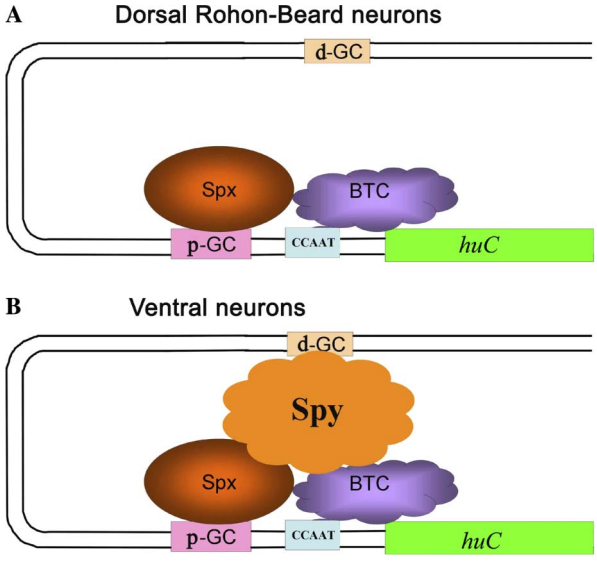

Fig. 4. Models for regulation of *huC* transcription by GC-rich boxes. Regulatory model of the D-GC and P-GC elements in the transcription of zebrafish *huC* gene. (A) In the dorsal RB sensory neurons, an unknown Sp1-like factor (Spx) binds to the p-GC box and interacts with the basic transcriptional complex (BTC) to activate *huC* transcription. The d-GC box is not required in this case. (B) In interneurons and motoneurons, transcription of *huC* is activated only when another unknown Sp1-like transcription factor (Spy) binds to the d-GC box and forms a transcription complex with the Spx/BTC complex.

box, play distinct roles in controlling neuronal expression of *huC* in zebrafish embryos. The GC box regulatory element present in the promoter of many genes is bound and activated by Sp1-like transcription factor family members [21]. We propose a model for regulation of *huC* transcription in subtypes of neurons in zebrafish embryos (Fig. 4). In the dorsal Rohon-Beard neurons, an unknown Sp1-like factor (Spx) binds to the p-GC box and interacts with the basic transcriptional complex (BTC) to activate *huC* transcription. In these neurons, the d-GC box is not necessary for *huC* transcription. In neurons generated from the middle and ventral domains of the spinal cord, however, efficient transcription of *huC* requires another Sp1-like transcription factor (Spy), which binds to the d-GC and forms a transcriptional complex with Spx and the BTC.

Several members of the Sp1-like transcription factor family, including Sp1, Sp2, and Sp3, have been found to be ubiquitously expressed in vertebrate embryos [21]. On the other hand, some members of this family are specifically expressed in the brain and the spinal cord. In zebrafish, *sp4* (http://zfin.org), *sp5/bts1* [25], *spr2/sp5-like* [26], *sp8* (http://zfin.org), *sp8-like* [27,28], and *sp9* [28] all are expressed in the central nervous system. These genes may play a role in neurogenesis of zebrafish embryos. Further studies are needed to elucidate the roles of individual or collective Sp1-like proteins in regulating *huC* transcription.

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