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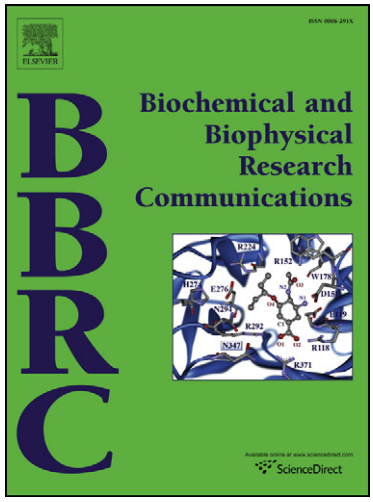

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Multiple coding and non-coding RNAs in the *Hoxb3* locus and their spatial expression patterns during mouse embryogenesis

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ABSTRACT

Hoxb3 plays important roles in embryogenesis and it has a complex transcription profile of mRNAs, non-coding RNAs and anti-sense RNAs. Characterization of the spatial expression patterns of these RNAs is important to understand their functions. We investigated the regulation and spatial expression patterns of multiple RNA transcripts derived from the *Hoxb3* gene locus. By 5' RACE we identified four novel transcription initiation sites and initiating exons, by luciferase activity assay we identified a new promoter region. Expression pattern analysis of the alternative transcripts containing specific initiation exons in mouse embryos suggests that there are co-operations between the initiation exons, their adjacent promoters and enhancer elements to orchestrate overlapping neural tube specific transcription profiles for *Hoxb3*. Furthermore, we showed that anti-sense transcripts derived from the *Hoxb3* locus were expressed in the hindbrain with distinct rhombomere boundaries, in a pattern complementary to the sense coding mRNA transcripts. Our results suggest that the multiple non-coding RNAs could be involved in the regulation of *Hoxb3*.

Keywords:

Hoxb3, initiation exon, promoter, non-coding RNA, anti-sense RNA, expression patterns

Introduction

Hox genes encode transcription factors that play important roles in anteroposterior axis patterning and regional identity during embryogenesis. In mammals there are four *Hox* clusters (A to D) containing 39 genes organized into 13 paralogous subgroups [1-3]. As *Hox* genes have a specific colinear characteristic between organization of the genes on the cluster and their expression domains, expression of *Hox* genes requires multiple levels of complex control [4-6]. Previous studies on identification and characterization of cis-acting regulatory elements and trans-acting factors focused on the regulation of protein-coding mRNA transcripts, and sharing of cis-acting elements by neighboring *Hox* genes [7-9]. We have previously reported multiple regulatory elements within *Hoxb3*, including two initiation exons, two promoters and multiple enhancer elements that interact with each other to maintain a complex transcriptional control throughout embryogenesis [7]. However, the involvement of other RNA-based regulatory mechanisms has not been investigated.

It has been shown that 31% of mouse genes have alternative initiation exons and 25% have alternative terminal exons. In the *Hox* clusters, the unannotated transcripts can represent up to 60% of the total transcriptional output [10,11]. The discovery of multiple forms of RNA molecules, including non-coding RNA (ncRNA),

micro-RNA (miRNA) and anti-sense RNA, have been shown to have important impact on gene regulation [3,11,12]. The miRNAs are short 21-23 nucleotide function in gene silencing [13,14]. The *Hox* clusters contain several miRNA genes, including miR-196 and miR-10 that are transcribed in the same orientation as the mRNA [3,14,15]. The presumed targets of the *Hox* miRNAs are predominantly located 3' to the miRNA locus, suggesting that the miRNAs may have cis-acting function in controlling genes of different paralogous groups [16,17]. Anti-sense transcript is a type of ncRNA complementary to sense mRNA with suggested regulatory function. For example, the anti-sense *IGF2* transcripts play an important role in muscle development [18] and expression of anti-sense *ENC-1* regulate *ENC-1* level in leukemai cell leukemia [19]. The complementary expression patterns of sense and anti-sense *Msx1* transcripts strongly suggest a role of the anti-sense transcript in *Msx1* regulation in tooth development [20]. In addition to duplex formation, anti-sense transcripts may also exert an epigenetic regulatory mechanism, such as demethylation of CpG sites in *Kpsla* [21]. Computational analysis estimated that up to 20% of human and mouse genes form sense-anti-sense transcript pair [22].

Among the *Hox* gene cluster there are 14 anti-sense non-coding transcriptional units that are polyadenylated and alternatively spliced, and most of them have similar position compared to the human clusters, suggesting functional importance [10]. For

example, the *Hoxa11* anti-sense transcript was involved in *Hoxa11* regulation in limb development [23]. The presence of anti-sense transcripts and alternative initiation exons would contribute to the complexity of *Hoxb3* regulation. This study reveals several novel sense *Hoxb3* mRNA transcripts with different initiation exons, and an abundantly expressed *Hoxb3* anti-sense transcript in mouse embryo that provide invaluable information for understanding the precise control of *Hoxb3* expression.

Materials and methods

In silico analysis of Hoxb3 conserved region

Mouse *Hoxb3* genomic sequence on chromosome 17 (Build 37.1, **NT_165773.2**, from 7720370 to 7758370) was compared to the sequence of human, pig, cow and zedrafish to search for conserved region using the Vista program with default setting (http://genome.lbl.gov/vista/) [24].

5'-Rapid amplification of cDNA ends (5'-RACE)

Total RNA was extracted from 12.5dpc mouse embryo with Trizol (Invitrogen) and first-strand cDNAs were synthesized using a *Hoxb3* primer (GCTGTT GCTAGTGGTACTGGTA). The *Hoxb3* cDNAs were subjected to 5' RACE reaction using the SMART RACE cDNA Amplification Kit (Clontach) with another *Hoxb3* primer (TGGCCTCATGCAGCTGCCATTGAGC) and a universal primer mix. A secondary PCR was performed using a nested universal primer A (NUP) and a series of nested *Hoxb3* primers (AGAACACGCTGAGCGCTGGTAGT, CGTAGTAGGTGGCTTTCTGCATCGCT, GGGCCTCGAATCCATCTTAGGAAC

and CGGCAGGCGAGAAATCTCCCCTC). The PCR products were sequenced.

RT-PCR

Total RNAs extracted from 12.5dpc mouse embryo and F9 teratocarcinoma cells were reverse transcribed using Superscript II (Invitrogen) and oligo-dT primer, and subjected to a first round PCR with an exon4 primer (CCACCACCACAACCTTCTG) and primers specific to exon1a (CCCAAGCTTCTCCTTTGTCATTATTCAACAGCA), exon3a (CCCAAGCTTGCAATGGTCCACGAAGCAGT) and exon1b to 1e shown in Figure 1. The products were used for a second round PCR with an exon3b primer (GGCCTCATGCAGCTGCCATT) and other exon specific primers described above. For detection of the anti-sense transcript, first strand cDNA was subjected to a PCR with primers (AGCGGAGAGCTGGAACTGGA) and (CTCTACGGCCTCAATCACCT). Finally the PCR products were sequenced.

Whole-mount in situ hybridization (ISH)

Mouse embryos were fixed and subjected to whole-mount ISH with short anti-sense

probes (~130 to 300 base) complementary to the initiation exon (1a, 1b, 1c and 3a) as previously described [7],. The short anti-sense probes were generated by *in vitro* transcription from T3 promoter plasmid containing unique sequences for the four initiation exons. For comparison of sense and anti-sense transcript expression, anti-sense and sense probes derived from exon4 were used (Fig 1A).

Luciferase assay

A full length (2.2kb) *Hoxb3* BamH1-HindIII exon2 genomic fragment and its deleted versions were cloned into a luciferase reporter plasmid pGL3-enhancer. The reporter plasmids were co-transfected with the *Renilla* luciferase control plasmid into F9 tertecarcinoma cells using the LipofectAMINE 2000 kit (Invitrogen). The cells were cultured with or without retinoic acid, luciferase activities were assayed in triplicate using dual-luciferase reporter assay system (Promega) and the results shown as relative activity.

Results

Expression profile and differential splicing of Hoxb3 transcripts

According to the Vertebrate Genome Annotation (Vega, http://vega.sanger.ac.uk/), there are two coding transcripts of *Hoxb3* (Hoxb3-001 and Hoxb3-002) that are either initiated from exon3a or exon1a [25] and two sense non-coding transcripts (Hoxb3-003 and Hoxb3-004) with differential splicing patterns (Fig. 1A). To reveal additional *Hoxb3* coding transcripts, we performed 5' RACE reaction and identified four novel alternative initiation exons, designated exon1b to 1e. To confirm the presence of these novel exons and to investigate their splicing patterns, RT-PCR experiments were performed. All four novel exon1s were expressed in 12.5dpc mouse embryos. However, in F9 teratocarcinoma cells only exon1d and exon1e containing transcripts were amplified (Fig. 1A). Therefore, based on the RT-PCR results, we identified four novel *Hoxb3* transcripts named Hoxb3-N1 to N4, each containing a novel alternative initiating exon1 (Fig. 1A). Sequence analysis indicated that all four novel exon1s had no open reading frame (ORF), ended before a GT splice donor site, and spliced onto the same AG splice acceptor site on exon2 (Fig. 1D). Interestingly, the novel transcript Hoxb3-N2 shares an identical splicing pattern

as the non-coding transcript Hoxb3-003 reported in the database, except that the third exon of Hoxb3-003 is prematurely terminated. Similar phenomenon was found in the novel transcript Hoxb3-N4 and the non-coding transcript Hoxb3-004, in which an alternative third exon is used by Hoxb3-004 before termination (Fig. 1A). Our results show that the transcription and differential splicing profiles of mouse embryonic *Hoxb3* mRNA transcripts are highly complex, involving the generation of multiple coding and non-coding RNAs.

A comparison between the human and mouse *Hoxb3* transcript expression profiles shows that the human *HOXB3* coding transcript (HOXB3-001) has similar splicing pattern to the mouse *Hoxb3* coding transcript, they are initiated from a 5' non-coding exon, spliced to exon2, followed by exon3 and exon4 (Fig. 1 A and C). Genomic sequence comparison between human and mouse demonstrates high sequence homology among the *Hoxb3* initiation exons (exon1a: 89%; exon1b: 89%; exon1c: 87%; exon1d: 93%; exon1e: 100%; exon3a 91% in the last 100bp). Such a high level of sequence conservation strongly suggests that the multiple initiation exons are under evolutionary selective pressure, and could be functionally important (Fig. 1B).

A novel promoter (P3) in the mouse Hoxb3 locus

Two *Hoxb3* promoters, P1 and P2, upstream of exon3a and exon1a, respectively, have been previously described [7]. With our identification of novel initiation exons we hypothesized that there should be additional promoter regions. Here we examined a 2.2 kb DNA fragment P3, which covers exon1d, exon1e and exon2, in a luciferase activity assay. The P3 fragment exhibited promoter activity which could be further enhanced by retinoic acid (RA) (Fig. 2). To define the P3 promoter region, luciferase assay was performed using different 3' and 5' deletions and the promoter activity was identified within a 1.58kb 3' fragment. Further analysis of this fragment showed that there were blocks of sequences highly conserved in mammals, and a ~150bp sequence also conserved zebrafish (Fig. 2). The addition of a 620bp upstream fragment (P3-5'), which itself had no promoter activity, reduced the activity of the 1.58kb fragment. The presence of at least 3 promoters in the *Hoxb3* locus suggests that there are multiple regulatory units within this gene, with differential usage of enhancer elements, promoters and exons for transcriptional initiation.

Embryonic expression patterns of multiple sense Hoxb3 transcripts in CNS

During embryonic central nervous system (CNS) development, the hindbrain is transiently segmented into a series of rhombomeres (r) with distinct cell lineage commitments. The identity of rhombomeres and their anterior boundaries are precisely defined by combinational expression of *Hox* genes [9,26]. Having shown that there is alternative utilization of initiation exons among *Hoxb3* transcripts, we further addressed the differential expression patterns of these transcripts in the developing CNS of mouse embryos. Whole-mount ISH was preformed using riboprobes derived from specific initiation exon1s, except exon1d and exon1e which are too short to be used. In the *Hoxb3* locus, exon1a is located between the P2 promoter and the enhancer element Ia (Fig. 1A) which direct anterior neural tube expression up to the boundary between r6/7. Using an exon1a probe, we found that the Hoxb3-002 transcript was expressed in the neural tube from posterior to the rhombomere r7/8 boundary (Fig. 3), that is similar to the expression pattern of *Hoxb4.* The transcripts initiated from exon1b and exon1c are downstream to the P2 promoter and the two enhancer elements Ia and Ib (Fig.1A). Exon1b- and exon1c-containing transcripts displayed a neural tube expression boundary at r6/7 (Fig. 3) that reflected the activity of enhancer element Ia. The 3' most initiation exon (exon3a) is immediately downstream of the promoter P1 and the neural tube specific enhancer element IVa that drives r5 expression, and the mesoderm specific elements in region

IVb and Va (Fig. 1A). Expression of the exon3a transcript extended to r5 at 9.5dpc and 10.5dpc (Fig. 3) and was also found in the mesodermal region. In summary, the six initiation exons represent differential *Hoxb3* transcript expression patterns which correspond to the activity of their immediate upstream enhancers.

Expression of Hoxb3 anti-sense non-coding transcripts

In the *Hoxb3* locus, there are seven Vega annotated anti-sense transcripts (Fig. 1A). To obtain a full picture of the *Hoxb3* transcription profile we searched for additional anti-sense transcript from EST libraries. Although no additional anti-sense transcript was identified, sequence analysis of the EST **BY742432** indicates that the initiation site of Hoxb3-s004 is 69bp more upstream than that described in Vega. Interestingly, we found that the anti-sense transcripts had distinct splicing patterns and some exons had sequences complementary to exon4 of the *Hoxb3* sense transcripts with considerable sequence homology (Fig. 4B). The presence of complementary sequence suggests a potential regulatory function. The anti-sense transcripts are identified in various tissue sources, ranging from embryonic to adult, and present in both normal and tumor tissues (s001: infiltrating ductal carcinoma, s002: mouse embryo, s003: thymus, s004: retina, s005: mammary gland, s006: tumor,

Rp23-9G13.9-001: spinal cord). Expression of the anti-sense transcript Hoxb3-s002 was experimentally confirmed by RT-PCR in mouse embryo and F9 cells (Fig. 4A) To assess the importance of the anti-sense transcripts during neural development, we further investigated their expression patterns by ISH. The anti-sense *Hoxb3* transcript was expressed from 8.5dpc in r4 and posterior hindbrain and in the spinal cord, by 9.0dpc expression was also detected in r6. At 9.5dpc strong expression was found in the hindbrain extending anteriorly up to the r2/3 boundary, with a diminishing level of expression in the posterior end, this expression pattern persists till 10.5dpc. The anti-sense *Hoxb3* transcript was also expressed in the anterior half of the third and fourth branchial arches and the neural crest cells migrated from r4 (Fig. 4C). Interestingly, the expression patterns of the anti-sense RNAs in the hindbrain was complementary to the sense coding RNAs which were found abundantly in r5 at 9.0-9.5dpc, and much reduced in r5 at 10.5dpc. The embryonic expression patterns of these anti-sense transcripts suggest that multiple forms of *Hoxb3* RNAs are involved in normal development of the hindbrain and spinal cord during embryogenesis.

Discussion

Utilization of multiple alternative initiation sites

The *Hox* transcription factors are evolutionarily conserved and required for axial patterning during embryogenesis. In mouse there are four *Hox* clusters (a to d) each consisting of 9 to 11 individual genes [2,3,5]. The *Hoxb3* locus occupies the largest genomic interval of ~32kb that harbors complex regulatory regions. Here we characterized four novel coding transcripts (Hoxb3-N1 to Hoxb3-N4) with distinct initiation exons (exon1b to exon1e). There is no evidence of polycistronism, which is found in *Hoxc4*, *Hoxc5* and *Hoxc6* [10]. The RNA transcription profile of the *Hoxb3* gene is highly complex and could be regulated at multiple levels.

Coordinated regulation by promoter and cis-acting regulatory elements

We examined the differential expression patterns of the multiple forms of *Hoxb3* transcripts and found that they represented the activities of their upstream cis-acting regulatory elements. In addition to the promoter regions previously described [7], we identified a novel promoter region P3, located adjacent to exon1d

and exon1e. Our results suggest that in the *Hoxb3* locus, several transcript initiation units, each containing its own cis-regulatory elements, initiation exon and promoter, have evolved to control *Hoxb3* transcription in a coordinate manner. The sequence of these initiation exons are highly conserved, they could serve as targets of gene regulation mediated by sequence recognition. Over a third of mammalian protein-coding genes have been under selective pressure to maintain pairing to miRNA [22] and some miRNAs have been shown to regulate *Hox* genes through base-pairing. The 3' UTR of *Hoxa7*, *Hoxb8*, *Hoxc8* and *Hoxd8* have been shown experimentally to be the target of mi-R196 [14]. The conserved *Hoxb3* initiation exons may also provide docking sites for miRNAs or other ncRNAs that mediate post-transcriptional regulation, and therefore provide an additional level of gene regulation.

Expression of Hoxb3 anti-sense transcripts

One interesting feature of the mouse *Hoxb3* transcripts is a long 3' UTR (~1,500 bp) that is not conserved among other vertebrate species. Unlike the initiation exons, only some stretches of the 3'UTR are conserved that may serve as targets for regulation by other RNA molecules. In Zebrafish *HoxB3a* gene, there are miR-10

target sites in the mRNA 3'UTR and overexpression of miR-10 leads to its downregulation [17]. Although there is no known miRNA complementary to the 3' UTR of the *Hoxb3* coding transcripts identified, *Hoxb3* anti-sense transcripts do present. It has been estimated that anti-sense transcription represents up to 38% of the spliced transcript in the *Hox* clusters and there is an anti-sense transcription unit located between *Hoxb2* and *Hoxb3* [10]. In the present study, we found that anti-sense transcripts derived from the *Hoxb3* locus, Hoxb3-s001 to s005, utilize a common anti-sense exons complementary to the 3'UTR of exon4 (Fig. 4B), suggesting potential post-transcriptional regulatory function. According to the Vega database, anti-sense transcripts are also found in human *HOXB3* at comparable position and with similar splicing pattern of mouse *Hoxb3* (Fig. 1), although the sequences are not conserved.

Functional implication of the Hoxb3 ncRNAs

Previous studies showed a limb specific expression pattern of anti-sense *Hoxa11* and a trend of sense transcripts disappearing as the anti-sense transcript appear [23,28]. A probable function of ncRNAs is to negatively regulate its respective sense transcripts. Micro-RNA miR-124 is the most abundant brain specific ncRNA

that regulate a hundred mRNA [29], miR-196 mediates a limb-specific inhibition of *Hoxb8* [14,30] and miR-181 mediated suppression of *Hoxa11* [31]. Although most of the known ncRNA process a negative regulatory function, previous study of Evf-2 indicated that it can increase Dlx6 transcription [32]. Thus, there is no consistent regulatory function of ncRNAs and their expression patterns may provide a cue. We have conducted ISH to reveal the expression pattern of Hoxb3-s002, and an interesting complementary pattern was seen between Hoxb3-s002 and *Hoxb3* sense transcripts, suggesting a potential suppressive function. The complementary sequence of the anti-sense transcripts Hoxb3-s002 and Hoxb3-s006 extend to the *Hoxb3* coding region, suggesting that they may also directly affect protein translation. Moreover, Hoxb3-s003 and Hoxb3-s004 comprise complementary sequence of the *Hoxb3* region Va enhancer. The multiple features of *Hoxb3* anti-sense transcripts suggest that they may regulate *Hoxb3* expression at multiple levels.

Specific expression of the anti-sense transcript Hoxb3-s002

The anti-sense transcript Hoxb3-s002 has a sharply defined anterior boundary, suggesting that it is highly regulated. Although most of the *Hoxb3* anti-sense transcripts share common exon2 and exon3, their initial exons are distinct. For

Hox-s001 and Hox-s002, their common initial exon is adjacent to a *Hoxb2* enhancer [27,33,34], and the embryonic expression pattern of Hoxb3-s002 represents the *Hoxb2* enhancer activity. Therefore, the *Hoxb2* enhancer may work in a bidirectional manner to drive the expression of both the *Hoxb2* sense and the *Hoxb3* anti-sense transcripts. The *Hoxb3* anti-sense transcripts Hoxb3-s003, s004 and s005 show complementary sequence not only to *Hoxb3*, but also to exon2 of *Hoxb2*. Therefore, these anti-sense transcripts could regulate both *Hoxb2* and *Hoxb3*. However, the anti-sense transcripts of human *HOXB3* do not share any complementary sequence homology to *HOXB2*. In terms of genome conservation, these different features between the human and mouse anti-sense transcription profiles may represent an evolutionary divergence.

In this study, multiple novel coding transcripts were identified and their physical arrangements in the *Hoxb3* locus as well as their expression patterns suggest that they may cooperate with specific cis-acting enhancer elements for gene regulation. Moreover, the hindbrain rhombomere-specific expression patterns of the *Hoxb3* anti-sense ncRNAs suggest that they could be involved in the post-transcriptional regulation of *Hoxb3* expression during embryogenesis.

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Figure legends

Fig. 1: Analysis of coding and non-coding RNAs of the *Hoxb3* locus. (A) Mouse *Hoxb3* RNA transcription profiles. Genomic organization, cis-acting elements (Ia to Va), promoter regions (P1 to P3), positions of the novel exon1s (*) are shown in the middle. Sense transcripts are shown above the gene structure in blue, anti-sense transcripts are shown below in red. Exons are indicated in boxes, *Hoxb4* and *Hoxb2* transcripts are indicated as open boxes. Expression of novel exon1 containing transcripts in mouse embryos and F9 cells was analysed by RT-PCR and shown on the left. The short line above exon4 indicates the sequence used for RNA probes for ISH. (B) Homology between human and mouse *Hoxb3* genomic sequence (Build 37.1, **NT_165773.2**). Conserved exon (blue) and intron (red) sequences are highlighted. (C) Transcription profile of human *HOXB3*. The genome structure is shown in the middle, sense exons and transcripts HOXB3-001 and HOXB3-002 are shown above the gene structure and in blue, anti-sense transcripts shown in red below. (D) DNA sequence of novel *Hoxb3* exon1s. The primer sequences used for RT-PCR are underlined, the gt/ag splicing sites and exon2 sequence are in blue.

Fig 2. A novel *Hoxb3* P3 promoter region. The P3 promoter region covers exons 1d,

1e and 2 and shares sequence homology with other mammalian species (Build 37.1, **NT_165773.2**) as shown (conserved intron: light gray, exon: dark gray). Full length (P3) and deleted (P3-5', P3-3' and P3-3'd) *Hoxb3*-luciferase reporter constructs were transfected into F9 cells with or without retinoic acid (RA) treatment. The relative luciferase activities for each construct are shown.

Fig 3: Expression patterns of alternatively spliced *Hoxb3* mRNA transcripts in the developing central nervous system. Exon-specific anti-sense probes for exons 1a, 1b, 1c and 3a were used in whole-mount ISH to identify specific form of alternatively spliced transcript. Arrowheads indicate the position of hindbrain r5.

Fig. 4: (A) Expression of *Hoxb3* anti-sense transcripts in mouse embryo and F9 cells analysed by RT-PCR. (B) Comparison between *Hoxb3* sense transcript exon4 (homeobox and 3'UTR) and anti-sense transcripts 001-006 (exons shown as red boxes) showing regions of complementary sequence. Anti-sense exons conserved between human and mouse are highlighted in dark gray. (C) Expression patterns of sense and anti-sense *Hoxb3* transcripts during mouse embryogenesis. Sense and anti-sense probes derived from the same exon4 sequence (indicated in Fig. 1A) were used for whole mount ISH.

Figure-1

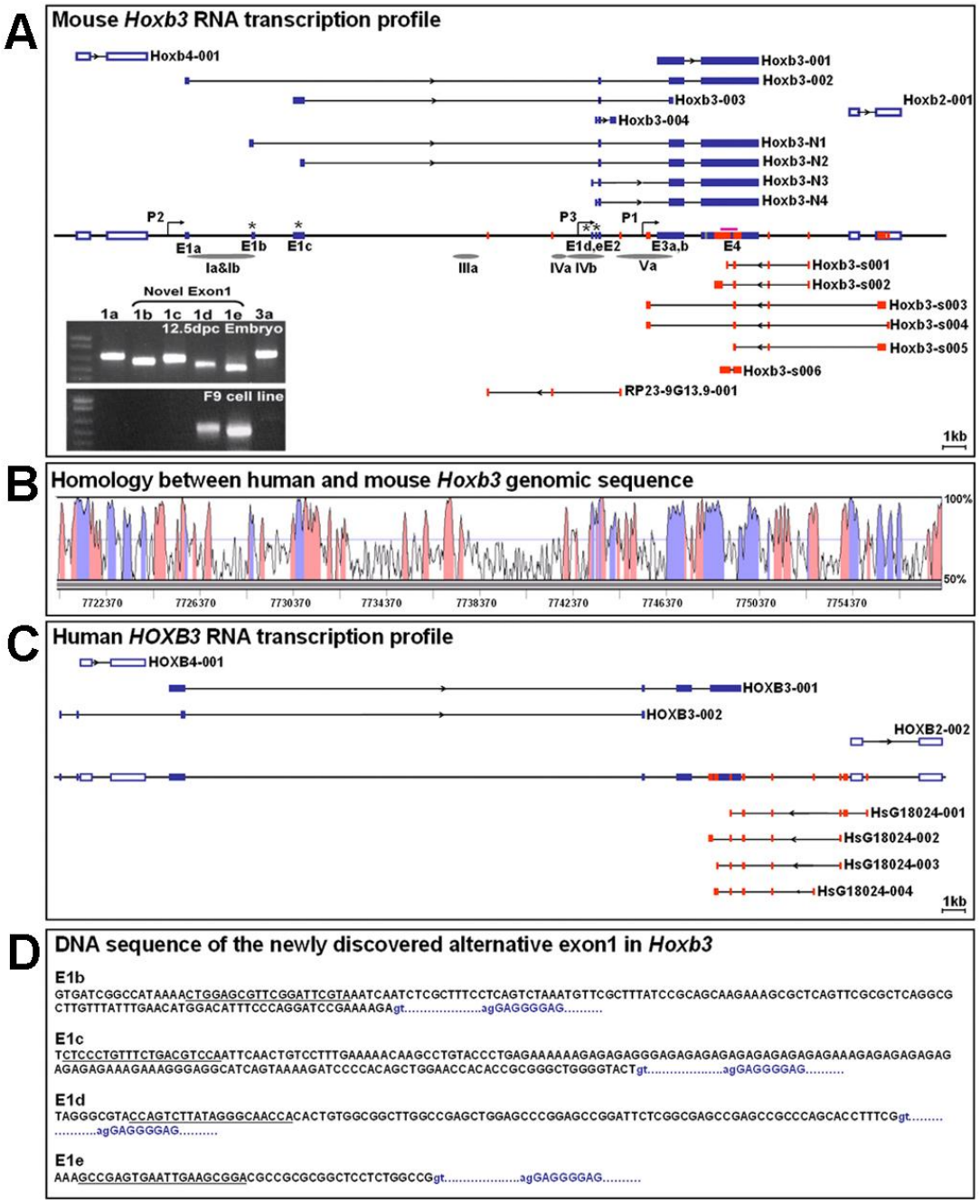

Figure-2

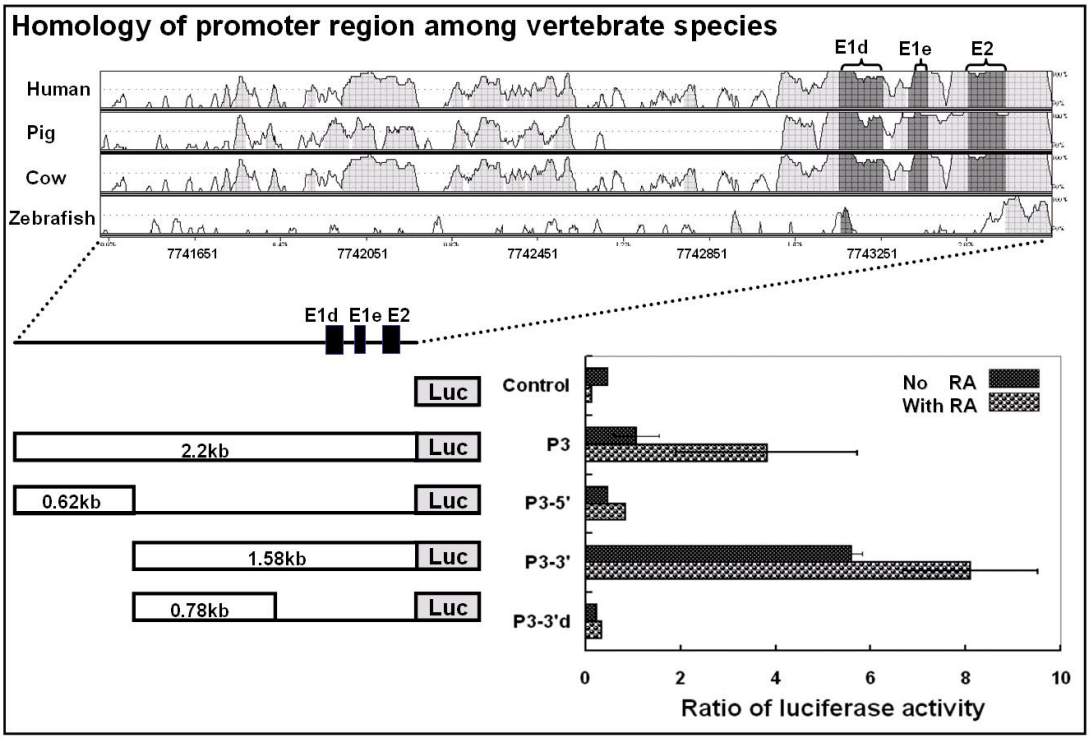

Figure-3

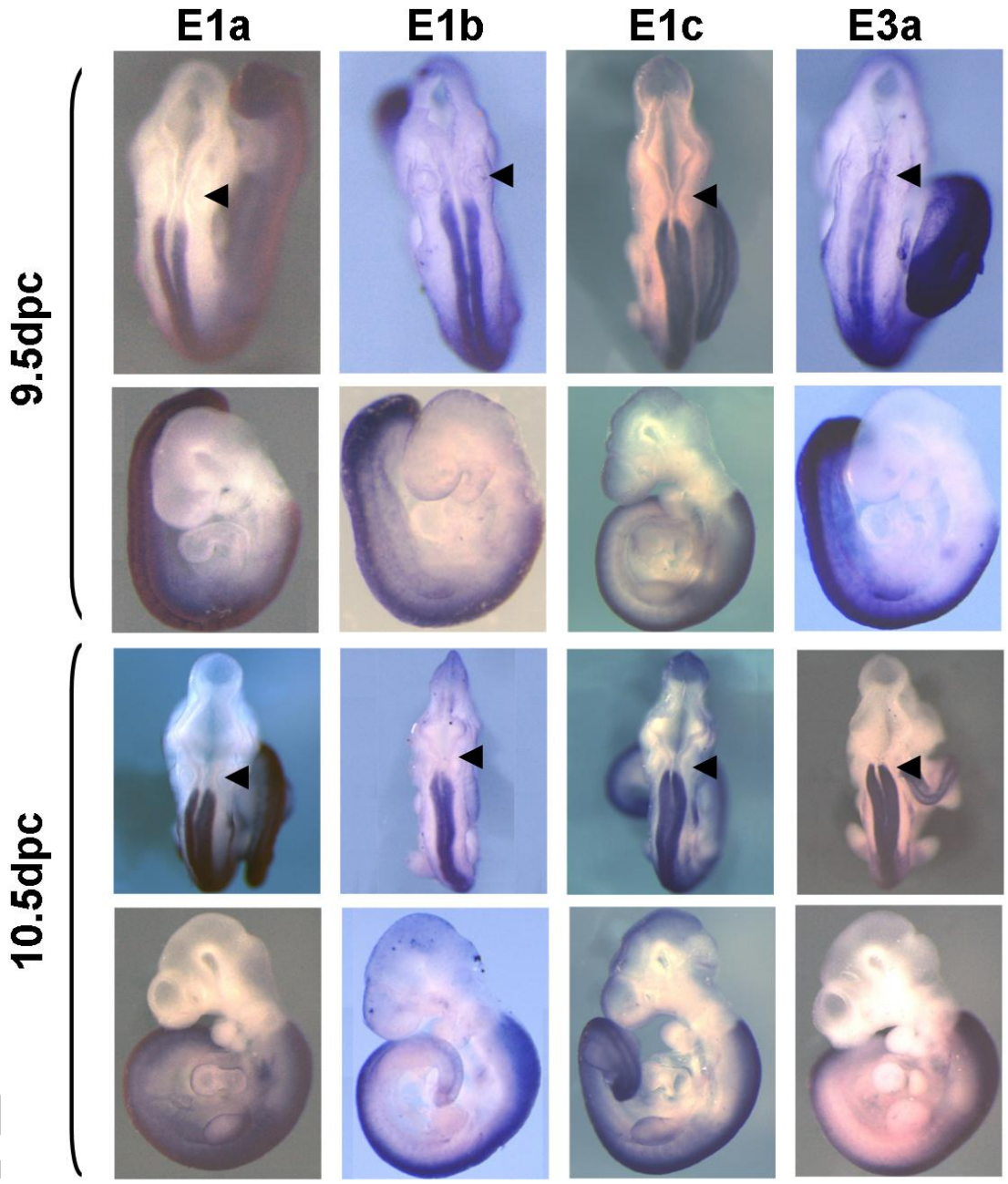

Figure-4