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Zebrafish *cnbp* Intron1 Plays a Fundamental Role in Controlling Spatiotemporal Gene Expression During Embryonic Development

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ABSTRACT

Cellular nucleic acid binding protein (CNBP) is a strikingly conserved zinc-finger nucleic acid chaperone required for forebrain development. Its depletion causes forebrain truncation mainly as a consequence of a reduction in size of craniofacial structures and neural crest derivatives. The CNBP expression pattern is complex and highly dynamic, but little is known of the underlying mechanisms regulating its spatiotemporal pattern. CNBP expression is highly conserved between all vertebrates characterized. In this study we have combined comparative sequence analysis and in vivo testing of DNA fragments in zebrafish to identify evolutionarily constrained regulatory motifs that likely control expression of the *cnbp* gene in embryos. We found a novel exon sequence located 5′ upstream of the Exon1-sequence reported in most databases, and two transcription start sites that generate two primary-transcripts that differ in their 5′UTRs and expression profile during zebrafish embryonic development. Furthermore, we found a region inside the intron1 sequence that controls the *cnbp* developmental-specific transcriptional activation. Conserved binding sites for neural crest transcription factors were identified in this region. Mutagenesis analysis of the regulatory region revealed that Pax6/FoxD3 binding sites are required for proper zygotic *cnbp* expression. This is the first study that identifies, in vivo, *cis*-regulatory sequences inside intron sequences and typical neural crest transcription factors involved in *cnbp* spatiotemporal specific transcriptional control during vertebrate embryonic development. J. Cell. Biochem. 108: 1364–1375, 2009.

KEY WORDS: TRANSCRIPTIONAL REGULATION; *CNBP*; EMBRYONIC DEVELOPMENT; ZEBRAFISH; NEURAL CREST

Cellular nucleic acid binding protein (CNBP) is a nucleic acid chaperone [Armas et al., 2008b] required for forebrain development in mouse [Chen et al., 2003], chicken [Abe et al., 2006], and zebrafish [Weiner et al., 2007]. Its depletion causes forebrain truncation mainly as a consequence of a reduction in size of craniofacial structures and neural crest (NC) derivatives. This loss apparently occurs via cell death of the precursor population rather than via a cell fate switch [Weiner et al., 2007; Armas et al., 2008a]. CNBP is expressed in a temporal and spatial fashion that correlates with its biological function. In mouse and chick embryos, *cnbp* is expressed in the forebrain, midbrain, craniofacial structures, limb buds, and somites [Shimizu et al., 2003; Abe et al., 2006]. In zebrafish, *cnbp* is mainly expressed at the border between the midbrain and hindbrain, and in the retina. During the hatching period, it is expressed in the prospective craniofacial structures, the lateral fins and the liver [Weiner et al., 2007].

The structural and biochemical properties of CNBP have been extensively described. Studies have analyzed the structural organization and expression of the gene and protein [Warden et al., 1994; Flink and Morkin, 1995; Michelotti et al., 1995; Yasuda

Abbreviations used: CNBP, cellular nucleic acid binding protein; EGFP, enhanced green fluorescent protein; hpf, hours post-fertilization; NC, neural crest; MBT, mid-blastula transition.

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et al., 1995; van Heumen et al., 1997; De Dominicis et al., 2000; Armas et al., 2001, 2004; Shimizu et al., 2003], biochemical activity [Armas et al., 2008b], and developmentally regulated phosphorylation that modifies the nucleic acid chaperone activity [Lombardo et al., 2007]. Moreover, it was recently reported that a natural proteolytic NH_2-terminal CNBP form may act as dominant negative during NC development [Armas et al., 2008a]. However, the mechanisms controlling CNBP embryonic developmental or tissue-specific expression are still poorly understood.

A preliminary characterization of human, mouse, and zebrafish putative promoter regions has been done. The human *cnbp* locus is comprised of 6,453 bp from the transcription start point to the polyadenylation signal, plus additional 5′ (201 bp) and 3′ (259 bp) flanking sequences [Flink and Morkin, 1995]; however, no functional analysis has been performed to examine developmental or tissue specific expression of CNBP. In mouse, a 1.6 kb region upstream of the putative transcriptional start site was shown to be sufficient to confer major promoter activity in P19 embryonic carcinoma cells [Shimizu et al., 2003]. There is neither a TATA box nor a CAAT box in the region near the transcription start site. Promoter constructs greater than 1.6 kb had significantly lower transcriptional activation abilities, suggesting the presence of negative regulatory elements upstream of that region [Shimizu et al., 2003]. Finally, in silico analysis of zebrafish gene sequence also showed the absence of a canonical promoter region [Armas et al., 2004].

Defining promoter and regulatory regions and predicting the biological function of such sequences require sophisticated systems for empiric testing. In vitro approaches fail to represent the complete repertoire of transcriptional programs occurring in vivo. Currently, a systematic strategy combining in silico identification with biological validation in intact organisms is required to record functional *cis*-acting sequences. The zebrafish is not only a system that has a divergent genome—useful for comparative analysis—but also it is an extremely tractable experimental system. Fluorescent reporter gene expression in both stable and transiently transgenic zebrafish embryos is usually employed to explore *cis*-regulation of gene expression on a gene-by-gene basis and to study the spatiotemporal impact of these regions on the expression of particular genes during embryonic development [Allende et al., 2006; Islam et al., 2006; Jin et al., 2006].

In this study, stable and transiently transgenic zebrafish embryos were used to perform an exhaustive analysis of putative zebrafish *cnbp* promoter regions. Data permitted the identification of transcription start sites, the elucidation of the actual 5′UTR sequences and the recognition of *cis*-acting specific regulatory elements responsible for regulation of *cnbp* developmental expression. We found a not reported exon located 5′ upstream of the previously reported exon1, and two transcription start sites that generate two transcripts that differ in their 5′UTRs and display differential expression during zebrafish embryonic development. We also found putative TATA-less promoter sequences and conserved transcription binding sites for Pax6 and FoxD3 in intron1, which are required for *cnbp* transcriptional regulation during zebrafish embryonic development. This is the first study that identifies, in vivo, *cis*-regulatory sequences inside intron sequences and transcription factors responsible for *cnbp* transcriptional control during embryonic development.

RESULTS

MAPPING THE *cnbp*–mRNA 5′UNTRANSLATED REGION

Previous works reported a CNBP gene general organization of five exons and four introns [Flink and Morkin, 1995; Shimizu et al., 2003; Armas et al., 2004]. Current genomic databases localize the zebrafish *cnbp* gene in the chromosome 23, between positions 4,495,466 and 4,502,064, in the negative strand. In these databases, *cnbp* contains six exons and five introns (Fig. 1A). *cnbp* coding sequence spans from exon2 to exon5, which are represented with broader boxes in Figure 1A. Zebrafish exons have similar size to those observed in mammal genes [Warden et al., 1994; Flink and Morkin, 1995; Michelotti et al., 1995; Yasuda et al., 1995; Shimizu et al., 2003]. The zebrafish *cnbp* 3′UTR was previously amplified and sequenced [Armas et al., 2004], whereas the 5′UTR has not been thoroughly analyzed yet. From the current databases, it was difficult to accurately establish the transcription start site or the length of *cnbp* 5′UTR, both data critically important for studying the promoter region. Hence, we performed 5′RACE using total RNA extracts obtained from ovary of adult zebrafish females. We chose ovary since we had previously detected high expression levels of *cnbp* mRNA in this tissue [Armas et al., 2004]. A specific reverse primer named 5′RACE (indicated in Fig. 1A) was used together with a commercial oligodT forward oligonucleotide. Products were re-amplified with a set of specific nested primers. A single band of approximately 400 bp was obtained, cloned and eventually sequenced (Fig. 1B). Sequences were in silico analyzed by BLAT. The 5′RACE product (shown as ''5′RACE product'' in Fig. 1A) matched to a genomic region that spans from exon4 to a region located approximately 400 bases 5′ upstream of exon1. The 5′RACE product comprised exons 2, 3, and 4 sequences but did not contain the Exon1-sequence. This finding allowed us to speculate about the existence of a novel exon, which was called Exon1′. It is important to note that in the present report we kept the name of ''exon1'' and ''intron1'' for the sequences informed as *cnbp* exon1 and intron1 in the Ensembl database.

Alignment of zebrafish, tetraodon, fugu, *Xenopus tropicalis*, mouse and human *cnbp* gene sequences revealed exon1′ and exon1 are not conserved (not shown). Lack of conservation might be due to imprecise sequence information on the genomic region or to scant information about the real *cnbp* 5′ region. To further explore this, zebrafish ESTs that matched to the *cnbp* 5′ were compared with the Exon1′-transcript (5′RACE product) and Exon1-transcript sequences (Fig. 1B). An important group of ESTs corresponded to short products starting from exon2 (146 ESTs). These ESTs were probably obtained as a consequence of incomplete 5′-end synthesis of cDNA. Among the remaining ESTs, 93% of them contained the Exon1′-sequence. This finding reinforces the hypothesis about the existence of a not reported exon sequence of 43 bp located 394 bases upstream of the previously informed exon1.

The existence of a novel Exon1′-sequence may be explained if, (1) there are two *cnbp* genes, each one coding for a different

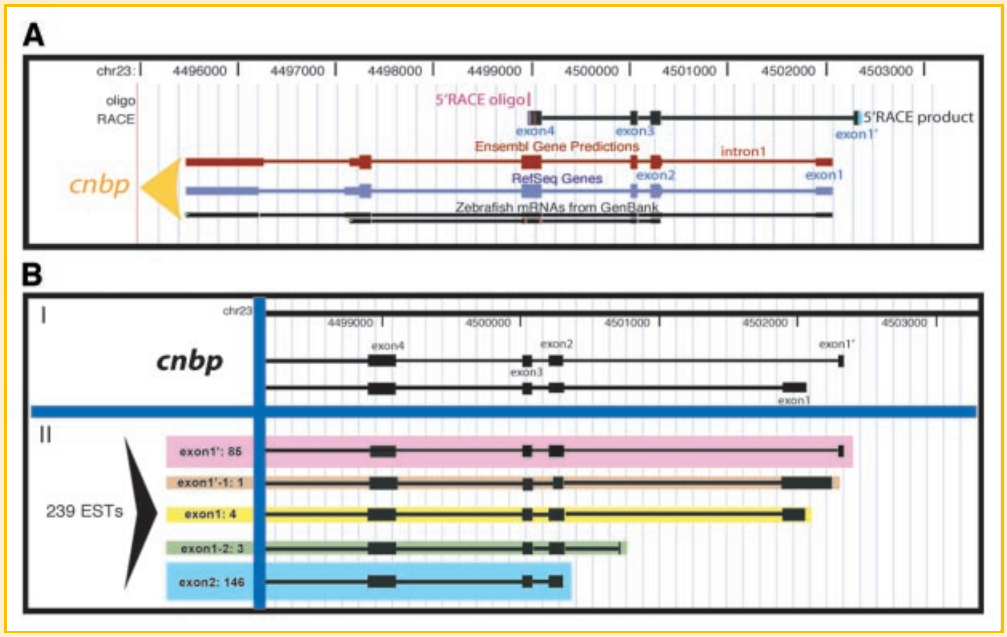

Fig. 1. In silico analysis of *cnbp* gene and ESTs sequences. A: Zebrafish chromosome 23 and *cnbp* gene general organization representation. Exons are represented with boxes and introns with lines. Different colors indicate different genomic databases for annotated *cnbp* genes. The 5′RACE oligonucleotide is shown in purple and the 5′RACE product is represented with black boxes. All these sequences are shown as matches for that genomic window. B: Diagram showing *cnbp* reported 5′-ends. Panel I shows results obtained by BLAT of the 5′product against current *cnbp* genomic sequence, and Panel II depicts the 239 ESTs found that extend over exon1′ to exon4. Among ESTs, 85 contain exon1′ but do not contain exon1 (highlighted in pink); 1 contains exon1 and an extra 5′ sequence but excludes exon1′ (highlighted in orange); 4 contain exon1 but not exon1′ (highlighted in yellow); 3 start from a small region located between exon1 and exon2 (highlighted in green); and 146 begin in exon2 (highlighted in blue). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

transcript; (2) Exon1 does not exist and intron1 comprises 1,974 bp instead of 1,580 bp; (3) the *cnbp* gene has 7 exons and 6 introns, and Exon1′ and Exon1 are alternatively spliced; and (4) there are two alternative transcription start sites, which generate two different *cnbp* transcripts. In silico analysis of the current zebrafish genome sequence yielded a single putative *cnbp* gene sequence, in agreement with previous data [Armas et al., 2004]. This fact allowed us to rule out the first possibility. The second possibility could be ruled out since 4 ESTs actually contain Exon1-sequence (Fig. 1B). The experiments that follow were performed to explore the other two possibilities.

DETERMINATION OF *cnbp* TRANSCRIPTION INITIATION SITE AND PRE-mRNA SPLICING PATTERN

To evaluate the existence of a not reported exon in the *cnbp* gene, the first issue was to establish the transcription start site. For this purpose, primer extension reactions were performed using total RNA prepared from 24 hpf-embryos and specific Exon1 and Exon1′ primers. A band of 51 bases was obtained for Exon1′-oligonucleotide (Fig. 2A, red arrow), confirming that Exon1′ was expressed. The transcript obtained has 46 bases, that is, three bases longer that the product obtained by 5′RACE. The sequence is written at the bottom of Figure 2A. This result confirmed that the Exon1′-sequence amplified by 5′RACE actually represent an exon of the zebrafish *cnbp* gene. Conversely, no band was detected in primer extension using Exon1-oligonucleotide, even when higher amount of total RNA was used (Fig. 2A, black arrow indicates the oligonucleotide used). Of the ESTs reported thus far, only four EST contain the Exon1-sequence and, thus, the transcription start site from Exon1 might not be detected due to the low representation of Exon1-transcripts in addition to the relative low sensibility of the primer extension assay. Taken together, our results indicate the existence of two different transcription start sites in the zebrafish *cnbp* gene.

To further analyze the nature of both transcripts, we cloned, sequenced and compared the *cnbp*-transcripts. RT-PCRs were done using a 3′oligonucleotide that match on exon4 and specific 5′oligonucleotides that matched on Exon1 or Exon1′-sequences. The use of Exon4 and Exon1′-oligonucleotides yielded a 350 bp product conformed by exons 1′, 2, 3, and 4 while the combination of Exon1 and Exon4-oligonucleotides gave rise to a product of 450 bp conformed by exons 1, 2, 3, and 4 (Fig. 2B). Therefore, it seems that the pre-mRNA synthesized from the Exon1′-start site loses splicing signals necessary to preserve the Exon1-sequence in the mature mRNA. Pre-mRNA synthesized from Exon1-start site follows a canonical splicing processing.

Exon1 and Exon1′-sequences were analyzed using the mfold 3.0 program for possible secondary structure formation. Exon1-sequence is able to form six different secondary structures (not shown) with ΔG between -29.76 and -35.30 kcal/mol. Conversely, Exon1′-sequence can form only 2 structures (not shown) with ΔG of -10 and -11 kcal/mol. Free energy data suggest that Exon1 putative secondary structures are more stable that the ones formed by Exon1′. Thus, it is tempting to speculate about a differential mechanism of *cnbp* expression regulation depending on the 5′UTR features.

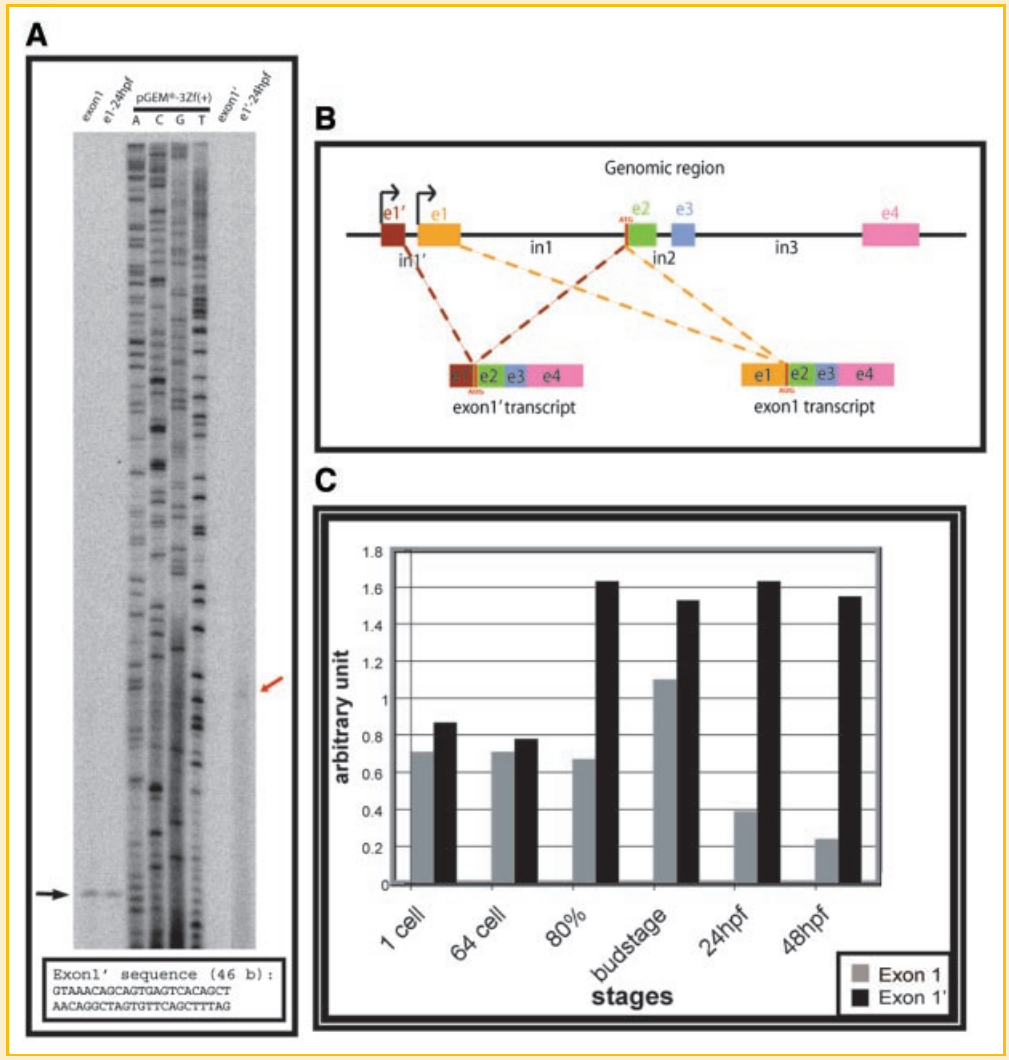

Fig. 2. *cnbp* mRNAs transcription expression analysis. A: Identification of zebrafish *cnbp* transcription start sites by primer extension. Exon1 and Exon1′-oligonucleotides were used as primers. Products were run on denaturant polyacrylamide gels. Lines at the extreme-left correspond to reactions without RNA (exon1) and with RNA prepared from 24 hpf embryos (e1-24 hpf). Lines at the extreme-right correspond to reactions without RNA (exon1′) and with RNA prepared from 24 hpf embryos (e1′-24 hpf). The pGEM® 3Zf(+) DNA sequencing reaction (lines in the middle, ACGT) was used as a size marker. The red arrow indicates extended product from Exon1′-oligonucleotide and black arrow indicates Exon1-oligonucleotide that was not elongated. B: Representation of *cnbp* transcripts detected by RT-PCR. The color boxes represent exons and the black lines the introns. The *cnbp* gene arrangement is shown within the genomic region. Below, Exon1′ and Exon1-transcripts are represented by joining the different exons. Exon1′-transcript is formed by exons (e) 1′, 2, 3, and 4 whereas Exon1-transcript is formed by exons 1, 2, 3, and 4. The translation start codon (ATG or AUG) is indicated in red in both genomic as well as transcript drawings. Discontinued lines indicate splicing between exon1 or exon1′ and exon2. C: Differential expression of *cnbp*-transcripts. Bar graphic compares Exon1- (gray bars) and Exon1′- (black bars) transcript expression profiles during embryonic development. β-Actin RT-PCR results were utilized to normalize product amounts (not shown). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cnbp-mRNA DIFFERENTIAL EXPRESSION DURING EMBRYONIC DEVELOPMENT

We wondered if Exon1′ and Exon1-transcripts display similar developmental behavior. Therefore, RT-PCRs were performed using total RNAs prepared from embryos at different developmental stages and the set of oligonucleotides described above. A set of specific oligonucleotides was used to amplify *β-actin* mRNA as control for sample integrity and quantification. Both transcripts were detected at similar levels at early developmental stages (Fig. 2C). This finding revealed the maternal origin of Exon1′ and Exon1-transcripts. Significant differences were detected beyond the mid-blastula transition (MBT). Exon1-transcript decreased reaching the lowest level at 48-hpf while Exon1′-transcript increased and remained constant from 80%-epiboly stage onwards (Fig. 2C). The low presence of Exon1-transcript may explain the higher number of EST containing the Exon1′-sequence and the failure to detect Exon1-transcription start site by primer extension assays.

IN VIVO ANALYSIS OF DIFFERENT DNA FRAGMENTS AS PUTATIVE PROMOTERS OF *cnbp* EXPRESSION

The mechanism by which *cnbp* is differentially expressed is poorly understood. Results using mouse cultured-cells showed that *cnbp* enhancer and silencer *cis*-regions lie within the 3 kb proximal region

of the promoter [Shimizu et al., 2003]. These data may represent a partial vision of the elements governing *cnbp* expression and not necessarily be responsible for the differential expression pattern observed during vertebrate embryonic development. Therefore, we decided to isolate and characterize the putative promoter and enhancer regions that control the *cnbp* expression during zebrafish development.

Once the correct *cnbp* 5′UTR was established, different sets of forward and reverse primers were designed to amplify genomic DNA sequences containing putative *cnbp cis*-acting elements located from −2,500 to +2,000 bp (Fig. 3). The promoting activity of each of the 12 amplified sequences was analyzed using the Tol2 transposon system [Kawakami et al., 2004]. DNA constructs were injected in four independent experiments, which comprised approximately 200 embryos each. Higher amounts of DNA, until reaching evident signs of toxicity, were microinjected for those constructs that did not promote EGFP expression. The fluorescence observed by the injection of the pT2KXIG plasmid was used as a positive control. A summary of results is shown in Figure 3. In the column on the right side are indicated the constructs that promoted (''Yes'') or did not (''No'') promoted EGFP expression. From the 12 constructs analyzed, only two constructs, p1A and p4E, were able to promote EGFP expression during zebrafish development (Fig. 3, on the right). The p1A construct, located 1,000 bp upstream of Exon1 or 750 bp upstream of Exon1′, promoted EGFP expression from 10 hpf onwards. At early stages, EGFP expression was ubiquitous, being higher in the cephalic region (Fig. S1A). At 24 hpf, embryos ubiquitously expressed the reporter gene, with higher expression levels in the optic tectum and forebrain and midbrain regions (Fig. S1B). High expression in muscle cells was also observed. In later developmental stages (Fig. S1C), strong EGFP expression was observed in muscle cells, epithelial cells and optic tissue. We noticed strong fluorescence in epithelial cells of 20 hpf embryos onwards (shown as ''ec'' in Fig. S1B,C). Hence, while p1A was able to promote the expression of EGFP in injected embryos, the observed fluorescence pattern did not exactly match with the *cnbp* expression pattern observed by whole-mount in situ hybridization [Weiner et al., 2007]. The other construct, p4E, contains exon1′, intron1′, exon1, and intron1 sequences. Embryos injected with p4E expressed EGFP from 10 hpf onwards (Fig. S2A,B). The expression at 10 hpf was ubiquitous and not as strong as the one observed for p1A. At 24 hpf, EGFP expression was highly detected in brain regions and in the presumptive retina (Fig. S2C–E). EGFP expression was observed over the anterior neural tissue, inside the cells of the midbrain hindbrain border and in the hindbrain territory. In addition, cells that will constitute the retina also expressed the reporter gene at this embryonic stage. At 48 hpf, EGFP expression was reduced, being expressed in muscle cells, brain tissue and retina (Fig. S2F,G). The EGFP expression governed by p4E closely matched the *cnbp* mRNA expression revealed by whole-mount in situ hybridization experiments [Weiner et al., 2007], suggesting that elements within this genomic region could be responsible for the spatiotemporal transcriptional control of *cnbp* expression during embryonic development. To confirm this, zebrafish embryos injected with the p4E construct were kept in our facility to establish a stable transgenic fish line. Sexually mature fish were in-crossed to obtain F1 stable transgenic lines and the EGFP expression pattern was analyzed during embryonic development (Fig. 4). EGFP expression pattern in transgenic fish (Fig. 4B,C,E,G) recapitulated the expression of *cnbp* observed in whole-mount in situ hybridization (Fig. 4A,D,F). These results allow us to conclude that the *cis*-acting elements responsible for *cnbp* differential expression during embryonic development are located within intron1.

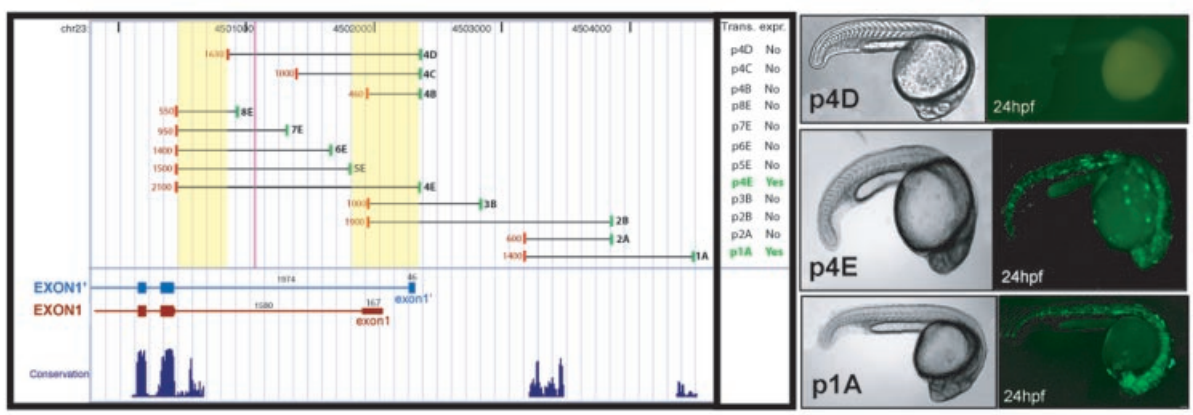

Fig. 3. Scheme showing *cnbp* genomic window and amplified putative promoters. In the upper part of the diagram are represented the forward (from 1 to 8 in green boxes) and reverse (from A to E in red boxes) primers used for identifying putative *cnbp* promoter regions. The 12 different amplified sequences are labeled with black lines between the respective forward and reverse oligonucleotides used. The name of each product is indicated on the right side while their approximate length (bp) on the left side. Fragments obtained were 1A (1,700 bp), 2A (600 bp), 2B (2,000 bp), 3B (800 bp), 4B (450 bp), 4C (985 bp), 4D (1,630 bp), 4E (2,000 bp), 5E (1,500 bp), 6E (1,400 bp), 7E (950 bp), 8E (550 bp). Below, Exon1 and Exon1′-transcripts are represented in red and blue, respectively. At the bottom of the figure it is shown the conservation between zebrafish and other vertebrate genomes (UCSC Genome Browser; where included genomes are: Tetraodon, *X. tropicalis*, mouse and human). The column on the right shows the EGFP expression observed for each construction in zebrafish embryos, where "Yes" indicates promoted expression and "No" not promoted expression. Bright-field and fluorescent images are shown on the right of 24hpf transiently transgenics embryos for p4D, p4E and p1A constructions (lateral views). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

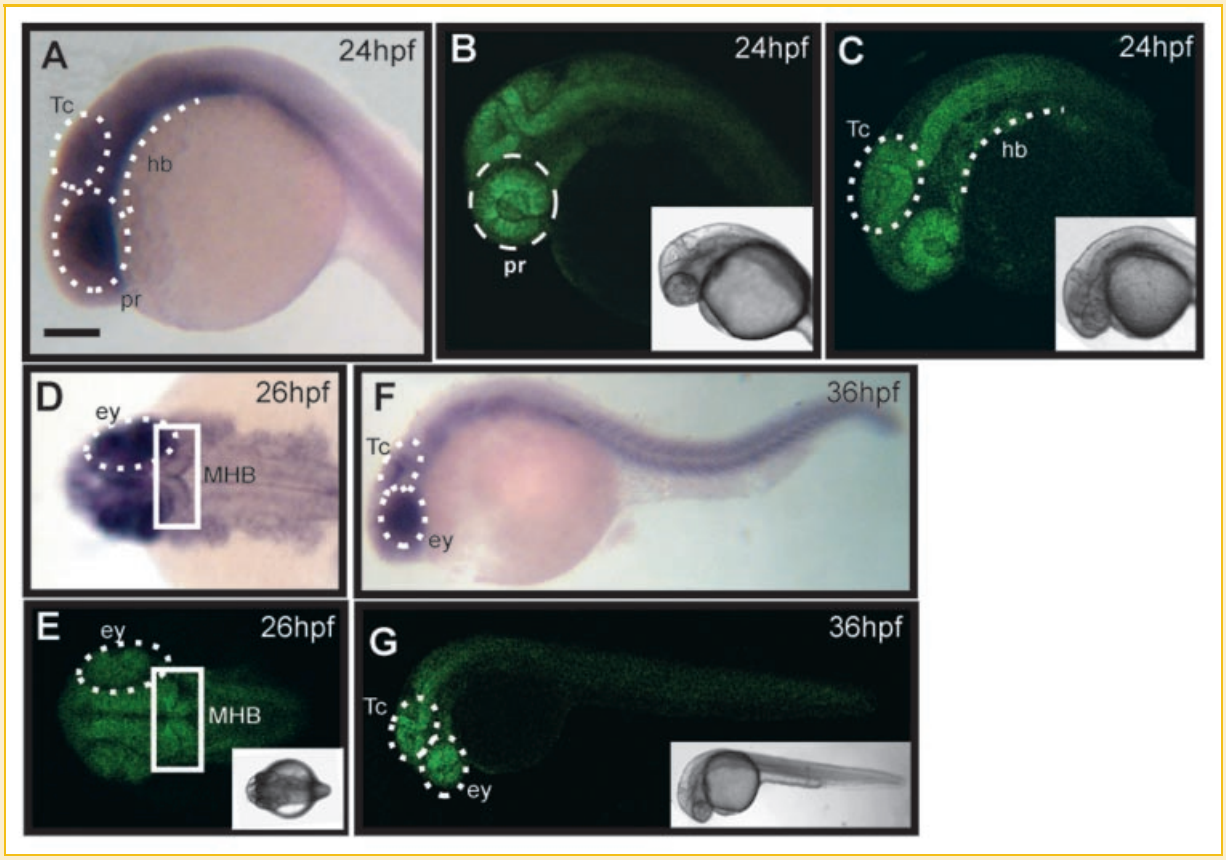

Fig. 4. Comparison of p4E EGFP expression and *cnbp* gene expression pattern. Bright-field and fluorescent images of zebrafish stable transgenic lines (B,C,E,G) as well as *cnbp* in situ hybridization expression pattern (A,D,F). In B and C, EGFP expression promoted by p4E construct at 24 hpf is shown. The reporter expression is high in the cephalic region, especially in the presumptive retina (pr) and brain domains, such as tectum (Tc) and hindbrain (hb). This expression is best detected in dorsal views (E), where the eye (ey) and the midbrain–hindbrain border (MHB) regions express EGFP. This expression can be compared with *cnbp* in situ hybridization at 26 hpf (D), where the gene is highly detected at eye (ey), and the midbrain–hindbrain border (MHB). At 36 hpf (G) expression is maintained in the same cephalic regions as in the previous stage analyzed (in the eye (ey) as well as the tectum (Tc) region). Lateral views on A, B, C, F and G; dorsal views on D and E. Scale bar in A: 0.17 for A, B and C; 0.23 for D and E; and 0.28 for F and G. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

PROMOTER AND TRANSCRIPTION FACTOR BINDING SITES SEQUENCE ANALYSIS IN SILICO AND IN VIVO

The next goal was to identify specific *cnbp* promoter and *cis*-acting sequences inside the p4E sequence. We searched in silico for promoter sequences by using the Promoter 2.0 Prediction Server [Knudsen, 1999] and the Neural Network Promoter Prediction Server [Reese and Eeckman, 1995]. Four sequences were found, one located at the 5′end of Exon1-sequence and three in the intron1 sequence (Fig. 5A,B). None of these sequences are conserved among vertebrates (not shown). The carefully dissection of the in vivo expression results (see Fig. 3, regions highlighted with yellow boxes) revealed that the four putative promoter sequences found were required for proper *cnbp* expression since deletion of any of them impaired EGFP expression. To find conserved transcription factor binding sites, we compared the zebrafish 4E fragment sequence with sequences from the human, mouse, rat, and chicken *cnbp* introns 1. Numerous conserved transcription factor sites were found (Table I) being the most conserved Pax6 and FoxD3 binding sites (Fig. 6A). This finding led us to explore the relevance of Pax6 and FoxD3 transcription binding sites on *cnbp* embryonic developmental expression. For this purpose, a deletion mutant of the p4E construct ($p4E_{\Delta pax6\text{-}foxd3}$) was generated and tested in vivo by using the Tol2 transposon system as described above. It is important to note that both transcription factor binding sites are separated by only 14 nucleotides, thus, the deletion mutation removed a 58 nucleotide region that comprises the two putative sites as well as a non-conserved site for HNF-3beta homolog *axial* in *Danio rerio*. Interestingly, embryos injected with $p4E_{\Delta pax6\text{-}foxd3}$ developed as controls but did not express EGFP (compared Fig. 6B with C), even when 100-fold more DNA was injected compared to p4E, or when evident signs of toxicity were observed. Five independent experiments were done using approximately 200 embryos each. These results indicate that the deleted region is required for activating *cnbp* expression during development. However, it is still unknown whether the presence of either Pax6 or FoxD3 or both *cis*-acting sequences are required for suitable *cnbp* expression. Since EGFP expression was completely prevented by the deletion of this region, even in regions where Pax6 and FoxD3 were not detected [Thisse and Thisse, 1995], the sequence deleted should support the expression outside the above mentioned transcription factors

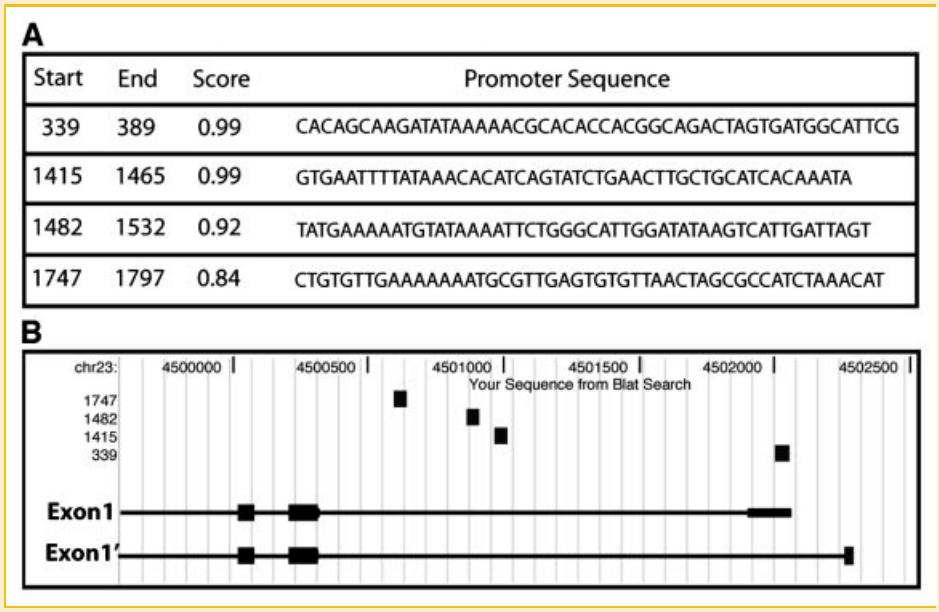

Fig. 5. In silico analysis of putative promoter sequences. A: Summary of the TATA-less putative promoter regions found in the p4E sequence. The position –relative to the first nucleotide of the (Exon1′-transcript sequence), score (number from zero to one that indicates the proximity in the neural network between the analyzed sequence to the formed ones; higher the score, more similar the sequences are), and nucleotide sequences are indicated. B: Diagram of chromosome 23 showing the position where the four putative promoters align. Promoters were named with the number of each origin (339, 1,415, 1,482, and 1,747), considering that Exon1′ first nucleotide is the initial point of the analyzed sequence. Exon1 and Exon1′-transcripts are also represented.

boundaries. The *axial* transcription factor is expressed in zebrafish 24 hpf-embryos in the forebrain/midbrain boundary and in the ventral midbrain and hindbrain region [Strahle et al., 1996]. Together, Pax6-FoxD3-Axial gene expression patterns resembles p4E construct EGFP expression. In conclusion, the deleted region in $p4E_{\Delta pax6\text{-}foxd3}$ construct is necessary for expressing *cnbp* during embryo development, and Pax6, FoxD3 as well as *axial* transcription factors seem to be involved in its expression.

DISCUSSION

In this study we report the cloning and functional characterization of zebrafish *cnbp* putative promoter and regulatory regions, located in an intron sequence of the single *cnbp* gene. Furthermore, we report the identification of a region required for *cnbp* transcriptional activation located inside intron1 sequence. This region contains conserved specific neural crest Pax6/FoxD3 binding elements and a non-conserved site for HNF-3beta homolog *axial* in *D. rerio*. While further work will be required to show that these transcription factors regulate *cnbp* expression, our findings suggest a novel mechanism of regulation for this gene in zebrafish and raise the possibility that *cnbp* transcription may also be more complex than previously described [Shimizu et al., 2003].

THE ZEBRAFISH *cnbp* INTRON1 CONTAINS FUNCTIONAL TATA-LESS PROMOTER SEQUENCES

The 5′-flanking sequence of the zebrafish *cnbp* gene is not a typical RNA polymerase II-transcribed gene. Immediately upstream from the transcription initiation sites found in this work, there is neither TATA-like nor CAAT-like motifs, in agreement with previous experimental and in silico results [Shimizu et al., 2003; Armas et al., 2004]. The sequence located 1,000 bp upstream of Exon1′, represented in the p1A construct in this work, contains two regions of high conservation (Fig. 3, bottom graph). This finding led us to

TABLE I. Conserved Transcription Factor Binding Sites Found in *cnbp* Intron1 Sequences

Transcription factors	*D. rerio*	*H. sapiens*	*M. musculus*	*R. novergicus*	*G. gallus*
Pax-6	1[a]	1	1	1	1
FoxD3	1	5	2	1	
AP-1		5	2	2	1
Nkx2-5		2	1	3	2
Pax-4		12	1	3	3
v-Maf		1	1	1	1
HNF-4	1	1			2
COMP1	1	2			1
v-Myb	1				
HNF-3beta	1				

[a]Numbers indicate how many times each site is in the corresponding sequence.

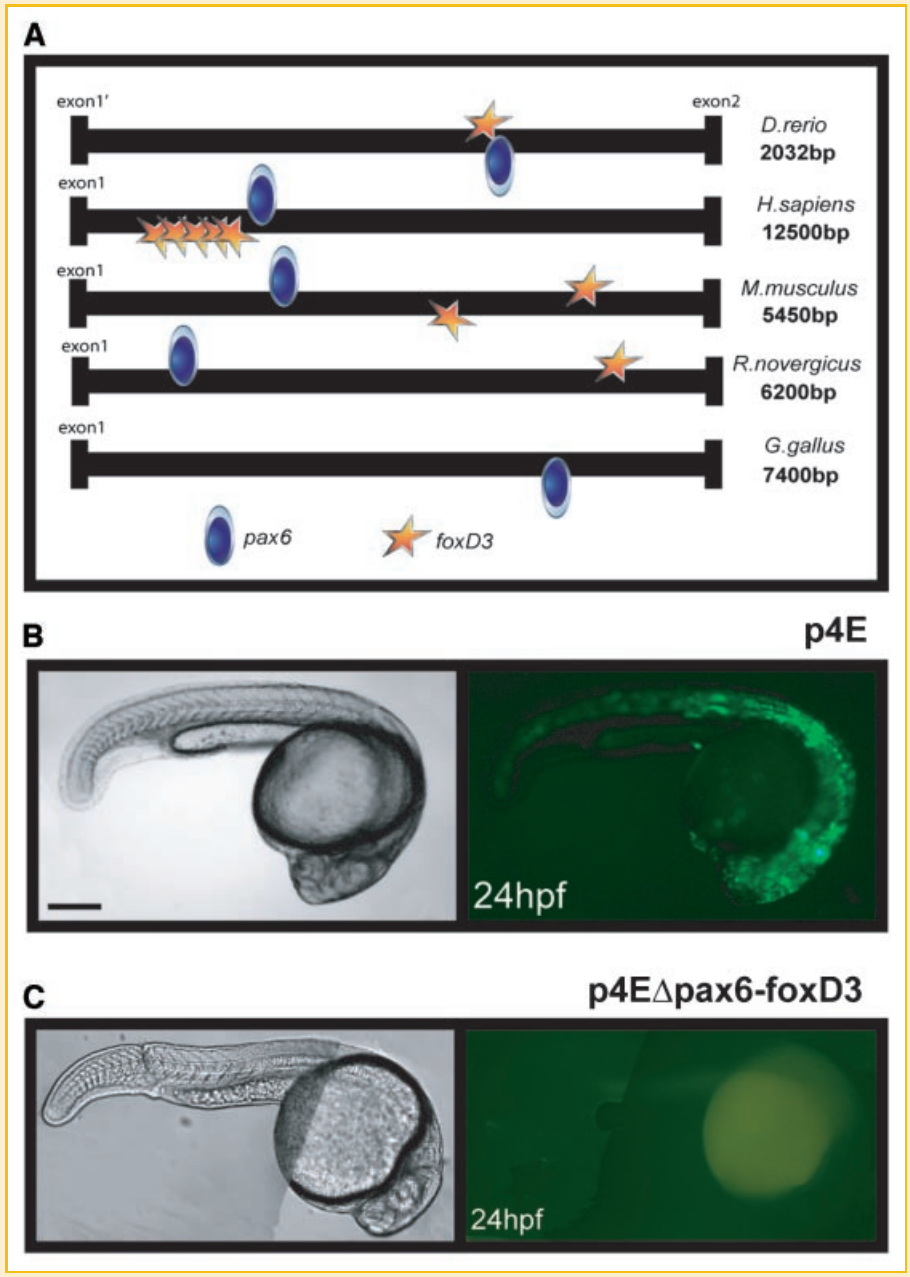

Fig. 6. In silico and in vivo analysis of putative transcription factor binding domains. A: Representation of *D. rerio*, *H. sapiens*, *M. musculus*, *R. novergicus*, and *G. gallus* intron1 indicating for each species the transcription factor binding sites for Pax6 (blue oval) and FoxD3 (orange star). B: Bright-field and fluorescent pictures of a 24 hpf embryo injected with the p4E construct. C: Bright-field and fluorescent images of a 24 hpf embryo injected with $p4E_{\Delta pax6\text{-}foxD3}$ construct. Notice that the contrast of the fluorescent image was enhanced in order to detect autofluorescence on the yolk, since the embryo did not show EGFP expression. Scale bar in C: 0.25 for C and B. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

speculate about the existence of a TATA-less promoter in the *cnbp* genomic sequence. Although the EGFP expression was actually promoted by p1A, the fluorescence pattern did not match with the one observed for *cnbp* in situ hybridization. Consequently, p1A does not appear to be the genomic region responsible for the regulation of spatiotemporal expression of *cnbp* during embryonic development. More than 700 bp separates these conserved regions from the Exon1′-transcript start site. Given the high synteny among zebrafish and other vertebrate genomes, these conserved sequences might correspond to regions involved in the control of another conserved gene. It is worth mentioning that upstream *cnbp* gene is localized v-raf-1 murine leukemia viral oncogene homolog 1 gene (*raf1*). However, the possibility that 1A fragment contributes quantitatively and/or temporally to *cnbp* regulation could not be ruled out since different genomic regions outside 1A sequence may repress ectopic expression during zebrafish development.

We emphasize the advantage of using zebrafish embryos to assess gene regulatory regions. Indeed, we might erroneously conclude

that the p1A construct contained the *cnbp* promoter region if an in vitro cell system were used instead of intact embryos. By using in vivo expression analysis we could accurately conclude that the genomic region represented by the p4E construct is the region that promotes the specific spatiotemporal *cnbp* embryonic expression profile. Furthermore, by dissecting the 4E sequence, we further found that putative promoter sequences are organized as modules in this region, and that the deletion of any of these elements impairs the expression of the gene. We generated zebrafish transgenic lines that contain the p4E genomic sequence linked to the EGFP reporter gene and demonstrated that this genomic sequence is able to recapitulate the expression pattern of endogenous *cnbp*. The transgenic lines not only provide excellent materials for studying the regulatory mechanism of *cnbp*, but they also will facilitate screens to identify novel mechanisms that regulate NC cell development and more detailed studies on the morphogenesis of the rostral head.

EVIDENCE FOR DEVELOPMENTALLY REGULATED ALTERNATIVE TRANSCRIPTION START SITE USAGE IN THE ZEBRAFISH *cnbp* GENE

Results presented here indicate that *cnbp* has a TATA-less promoter able to yield two possible transcripts, which differ in their 5′UTRs. The variation in 5′UTRs may provide alternative regulatory mechanisms for gene expression without changing the protein sequence, a process that could be used to mediate the complex expression pattern of *cnbp*. Exon1′ and Exon1-transcripts were found at developmental stages earlier than the MBT, and in similar amounts. This finding suggests a maternally inherited origin of both transcripts, but reveals differential developmental behavior for both. Exon1′-transcript level increased after MBT and did not change during embryonic development whereas Exon1-transcript level was constant before MBT but decreased as embryonic development progressed. Data suggest that the Exon1′-transcript is synthesized by the embryo while Exon1-transcript is not. The decrease of Exon1-transcript may therefore be due to transcription repression or mRNA degradation during embryonic development. Thus, Exon1′-transcript may be important for embryonic developmental stages beyond MBT, whereas Exon1-transcript is likely to play a role during earlier embryonic stages.

Sequence analysis of both transcripts rule out the possibility of an alternative splicing of a unique pre-mRNA. The absence of alternative splicing in addition to the existence of a unique *cnbp* gene in the zebrafish genome point out differential transcription start site usage in vivo. It is worth mentioning that a number of TATA-less promoters are known to start transcription from more than one site [Bender and Kuehl, 1986; Stauffer et al., 1990]. Transcription initiation complexes that involve different sets of proteins may regulate the expression of both transcripts. Beyond MBT, Exon1′-transcript synthesis may be started by transcription initiation complexes mostly composed by proteins synthesized from developmentally regulated genes. Differences in transcriptional initiation complex composition may explain differences in transcription products, as it was reported for other genes [Wessely and De Robertis, 2000]. Computer analysis of p4E identified conserved putative binding sites for various nuclear factors. Interestingly, the most conserved were binding sites for transcription factors involved in NC development, such as Pax6 and FoxD3. Pax6 regulates diverse developmental processes in craniofacial and ocular morphogenesis. As for the migration of NC cells, it was reported that homozygous Pax6 mutant rats show craniofacial defects due to impaired migration of midbrain NC into the nasal region [Matsuo et al., 1993]. *FoxD3* is one of the earliest NC genes to be expressed in mice, zebrafish, *Xenopus* and chick embryos [Steventon et al., 2005]. Pax6 and FoxD3 expression start after MBT and display an anterior-most pattern expression that match with the *cnbp* spatiotemporal expression profile. Our results suggest that Exon1 and Exon1′-transcript synthesis is regulated during oogenesis through a transcriptional complex composed of maternal proteins. Another regulatory complex mainly containing transcription factors encoded by the zygotic genome and in an specific spatiotemporal expression pattern, for example, Pax6 and FoxD3, may interact with the transcriptional initiation complex and promote the synthesis of Exon1′-transcript after MBT. It is important to note that experimental approach used in this work restricted the analysis only to transcripts synthesized from zygotic genome.

Results from the mutant construction $p4E_{\Delta pax6\text{-}foxd3}$ suggest that Pax6, Foxd3, and Axial transcription factors are involved in *cnbp* expression control during zebrafish embryonic development. It is important to realize that Pax6 factor is expressed in the diencephalon, hindbrain and presumptive eye during somitogenesis [Thisse and Thisse, 1995]. Later in the development, Pax6 expression pattern is focused on cephalic regions that will constitute the fish brain and retina. FoxD3 is widely expressed by the NC progenitors at the neural plate border and maintained in the premigratory NC cells, but rapidly downregulated as NC cells differentiate and migrate [Stewart et al., 2006]. Axial factor is expressed along the ventral midline of the neural tube, where anterior expression terminates at the mid-diencephalic boundary being broader in the midbrain region [Strahle et al., 1996]. This factor is also expressed all along the ventral spinal cord. Since *cnbp* expression is detected in a narrower domain, our results lead to propose that *cnbp* gene regulation requires the presence of Pax6, FoxD3 and Axial transcription factors to demarcate its spatiotemporal gene expression pattern. Nevertheless, other regions present in p4E fragment (shaded in yellow in Fig. 3) are involved in *cnbp* expression control since their loss also prevented the EGFP expression.

Thus, in summary, results presented here established the existence of a TATA-less promoter in the first intron of zebrafish *cnbp*, which initiates synthesis of two transcripts from two different transcription start sites. Different enhancers and repressors interacting with the initiation complex may modulate the efficiency of transcription from the two alternative transcription start sites. One of the transcripts, which contains a novel Exon1′-sequence, is maternally inherited and also synthesized from the zygotic genome once specific transcription factors have been translated. The other transcript, which contains the Exon1 sequence, is also maternally inherited but is not transcribed beyond MBT. Further mutational analysis might reveal the relative importance of the identified sites, but our data indicates that regulation is likely to depend on multiple sites within the critical region identified in intron1, with these acting at least partially to drive *cnbp* expression during embryonic zebrafish development.

EXPERIMENTAL PROCEDURES

ANIMALS

Adult zebrafish were maintained at 28°C in a 14/10 h light/dark cycle as previously described [Westerfield, 1995]. Embryos were obtained by natural mating and raised in E3 medium at 28.5°C until appropriate stages. Morphological features were used to stage embryos [Kimmel et al., 1995]. When necessary, 0.1 mM of 1-phenyl-2-thiourea was added to the E3 medium to prevent pigment formation [Karlsson et al., 2001].

mRNA EXPRESSION ANALYSIS

Total RNA from different embryonic stages was obtained using TRIZOL® Reagent (Invitrogen) following the manufacturer's instructions. Purified RNA was treated with RQ1 DNAse (Promega) and used to perform RT-PCR. Total RNA was retro-transcribed with SuperScript II enzyme (Invitrogen) and oligodT (5′-TTTTTT-TTTTTTTTTTTCGAACTCGAGCTCAGGAG-3′). Specific *cnbp* forward primers for Exon1′ (5′-CACAGCTAACAGGCTAGTGTTCA-3′) and Exon1 (5′-ACACCACGGCAGACTAGTGA-3′) were used with specific 5′RACE reverse oligonucleotide (5′-GTAGCACTTCTGCTCGTTGG-3′) that hybridized on *cnbp* exon4. Products were resolved in 1.2% (p/v) agarose gels stained with ethidium bromide. β-Actin RT-PCR was performed as positive control as well as to calculate relative amounts of *cnbp* during development.

5′RAPID AMPLIFICATION OF cDNAs ENDS (5′RACE) AND PRIMER EXTENSION ASSAYS

For 5′RACE, total RNA prepared from ovary was used to obtain single stranded cDNAs as described above. The RNA was removed by incubating for 20 min at 37°C using RNAse H (Promega), and then the cDNA was heated at 65°C during 5 min. For the tailing, the cDNA was mixed with dATP and Terminal Deoxynucleotidyl Transferase enzyme (Promega) in reaction buffer and incubated at 37°C during 1 h. The obtained product was subjected to a PCR reaction with oligodT and 5′RACE oligo to amplify 5′UTR sequences. PCR reaction conditions were one step of 5 min at 95°C, 40 cycles of three steps of 1 min at 95°C, 1 min at 53°C and 2 min at 72°C, and a final elongation step of 10 min at 72°C. The use of 5′RACE oligonucleotide, which hybridizes to exon4, allowed us to evaluate contaminant amplification from genomic DNA. Products were cloned into pGEM-T Easy Vector System (Promega) and sequenced. For primer extension reactions, oligonucleotides that match Exon1′ (5′-CGCggatccGCCTGAACACTAGCCTGTTAGCTGTG-3′) and Exon1 (5′-CGCggatccGCCGCTCCAGACCTCAAACCTCA-3′) were used. Both primers were end-labeled using [γ-^{32}P] ATP (NEB) and T4 polynucleotide kinase (Fermentas). Labeled primers were purified using a Sephadex G-25 column and 1 pmol of each of them was annealed with 25–50 μg of total RNA purified from embryos at 50°C during 45 min. Extensions were carried out by adding 20 U of Mu-MLV reverse transcriptase (Promega) and incubating during 1 h at 37°C. Samples were denatured at 90°C and separated on 6% (p/v) polyacrylamide gel electrophoresis alongside di-deoxynucleotide sequencing ladders derived from the *fmol*® DNA Cycle Sequencing System (Promega). Specific product length was assigned by comparing product length with pGEM®-3Zf(+) plasmid sequence.

CLONING OF *cnbp* PUTATIVE PROMOTER FRAGMENTS

Zebrafish genomic DNA was prepared from adult fish as described [Westerfield, 1995]. Specific primers were designed to amplify DNA fragments corresponding to the *cnbp* putative promoter. Forward primers have *Xho*I restriction site tails (indicated in lower case) for posterior sub-cloning into pT2KXIG vector [Kawakami et al., 2004]. Names and sequences were as follow: For1, CCGgtcgacAAGTCA-TATTGGATGCTATAGAGGTACCGTTGG (42 bp); For2, CCGgtcgac-GCCGAAGTATAGTACAAGCAATGCAAAAA (38 bp); For3, CCG-gtcgacGCCGGGCTTACAAGGCTGCTGTA (32 bp); For4, CCGgtcgac-GCCGCCCCACTGTTTCTGTCGCTCAG (35 bp); For5, CCGgtcgac-GCCTGAAACGTCACGTTATG (29 bp); For6, CCGgtcgacGTAGCTG-TAGCCGTGGTGCTA (30 bp); For7, CCGgtcgacGTGACTGCCGCTC-TACCAAGTG (31 bp); For8, CCGgtcgacGCCATTGCAATCGTGAT-TTTCG (31 bp); Reverse primers have *Bam*HI restriction site tails (indicated in lower case) for posterior sub-cloning in pT2KXIG vector. Names and sequences are as follow: RevA, CGCggatccGCC-TAGGGATGTTGCTTGGACCT (32 bp); RevB, CGCggatccGCCGCTC-CAGACCTCAAACCTCA (32 bp); RevC, CGCggatccGCCAAGACACT-GAAAAGTCTGCCCTA (35 bp); RevD, CGCggatccGCCCACATCCA-AGCGCTCTATCA (32 bp); RevE, CGCggatccGCCAAGCAACAAA-CTTTGACTTC (32 bp). Several forward and reverse oligonucleotide combinations were done to perform PCR on genomic DNA. PCRs were carried out using 4.8 ng/μl of genomic DNA, 0.1–0.2 μM of primers, 0.02 U/μl of Taq DNA polymerase per reaction tube (Invitrogen), 2.5 mM of $MgCl_2$, 200 μM of dNTPs, and 1× of reaction buffer. Products were named by combining the primer letter and number used in each case. Fragments were cloned in pGEM-T Easy (Promega) Vector System and sequenced. Sequences were analyzed by BLAT [Kent, 2002]. Positive clones were sub-cloned in pT2KXIG vector by using *Xho*I and *BamHI* restriction sites. To obtain the p4E deletion ($p4E_{\Delta pax6\text{-}foxd3}$), two oligonucleotides containing a MluI restriction site each designed. One oligonucleotide matched the 3′-end of the Pax6 transcription factor site (deletePax6, CGacgcgt-GACTCAAATGAGTAGCGGCTTG) and the other the 5′-end of the FoxD3 site (deleteFoxD3, CGacgcgtTATAAACACATCAGTATCT-GAACTTGC). Two PCRs were performed with different pairs of oligonucleotides, one with For4-deletePax6 and the other with deleteFoxD3-RevE. Both products were digested with *Mlu*I restriction enzyme, purified and ligated. Then, foxD3-pax6 deleted fragment was cloned in pT2KXIG vector by using *Xho*I and *BamHI* restriction sites, and eventually the obtained clones were sequenced.

MICROINJECTIONS AND ZEBRAFISH HANDLING

Plasmids with different DNA genomic fragments were purified and diluted for microinjection. The mRNA coding for Tol2 transposase was in vitro transcribed from pCS2+TP plasmid [Kawakami et al., 2004] by using SP6 polymerase (Fermentas), treated with RQ1 DNAse (Promega), purified by ethanol precipitation and its concentration was measured at 260 nm. For all experiments, our wild type zebrafish stocks were used. Five nanoliter of 5/25 ng/μl DNA/mRNA mixture was injected into one-cell stage embryos using glass capillaries made with a Sutter Instruments needle puller. Injected fish were observed at 10, 24, and 48 hpf for EGFP expression and then raised to sexual maturity. Injected adult fish were outcrossed with wild type individuals and at least 50 F1

embryos were screened with a fluorescent microscope (Olympus inverted microscope) equipped with 4×, 10×, and 20× lenses, and a 500/20 nm excitation filter and a 515 nm BP emission filter (Chroma) for EGFP. Photographs of live positive embryos were taken using a Nikon digital sight camera and associated software (NIS Elements). p4E construct was tested in at least two independent transgenic lines. Specimens were mounted and imaged using a Nikon confocal microscope. Images were processed in Adobe Photoshop by adjusting levels.

IN SILICO ANALYSIS OF DNA AND RNA SEQUENCES

Genomic sequences were obtained from the University of California at Santa Cruz (UCSC) Genome Browser (genome.ucsc.edu). Sequence alignments were carried out using the BLAT software program [Kent, 2002]. Zebrafish *cnbp* Exon1′ and Exon1-sequences were analyzed for secondary structures with mfold predictor using default settings [Zuker, 2003]. Exon1 sequences for *D. rerio*, *Homo sapiens*, *Rattus novergicus*, *Mus musculus*, *Xenopus laevis*, and *Gallus gallus* were subjected to MUSCLE for creating a multiple alignment and then subjected to BOXSHADE to obtain printouts from the multiple-aligned sequences (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=boxshade). The alignment was analyzed using "Ident and Sim" from the Sequence Manipulation Suite webpage (http://www.ualberta.ca/~stothard/javascript/ident_sim.html). Human (chr3: 130,371,122–130,385,389, March 2006 UCSC assembly), mouse (chr6: 87,793,074–87,800,989, July 2007 UCSC assembly), rat (chr4: 122,033,038–122,041,891, November 2004 UCSC assembly), chicken (chr12: 5,253,629–5,262,898, May 2006 UCSC assembly), and zebrafish (chr23: 4,495,466–4,502,064, March 2006 UCSC assembly) intron sequences were obtained and submitted to TRANSFAC [Matys et al., 2003] version 8.1 using the Matrix Search for Transcription Factor Binding Sites (MATCH). MATCH parameters were set to identify TRANSFAC entries using the "minimize false negatives" setting. In silico promoter analysis was performed using Promoter 2.0 Prediction Server [Knudsen, 1999] and the Neural Network Promoter Prediction Server [Reese and Eeckman, 1995].

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