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Molecular cloning, developmental expression, promoter analysis and functional characterization of the mouse *CNBP* gene☆

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Abstract

Striking conservation in various organisms suggests that cellular nucleic acid-binding protein (CNBP) plays a fundamental biological role across different species. However, the regulated expression and physiological properties of the CNBP gene are unknown. In this study, we report the molecular cloning, promoter characterization, developmental expression and functional analysis of the mouse *CNBP* gene. The gene contains five exons and is localized to chromosome 6 in the region corresponding to band 6 D1-D2. Primer extension assay indicates that the transcription start site is located 230 bp upstream of the initiator Met codon. Our promoter analysis indicates that strong transcription enhancer and silencer regions lie within the 1.6 kb proximal region of the promoter and the upstream −3.0 to −1.6 kb region, respectively. The promoter activity is 10 fold higher in embryonic carcinoma cells than that in fibroblast, as determined by CAT assay. Consistent with its function as a transcription factor, *CNBP* protein is located in the nucleus of cells. During mouse embryogenesis, *CNBP* is expressed in the anterior region of the early embryo and in the limb, tail and craniofacial region. Overexpression of CNBP strongly stimulates cell proliferation and increases *c-myc* promoter activity. Our finds suggest that CNBP may play an important role in cell proliferation and tissue patterning during anterior-posterior axis, craniofacial and limb development by targeting c-Myc.

Keywords: Transcriptional regulation; Chromosomal localization; Zinc-finger protein; Proliferation; c-Myc

1. Introduction

CNBP encodes a 19 kDa protein containing seven tandem zinc finger repeats of 14 amino acid residues (Cys-X_2-Cys-X_4-His-X_4-Cys) (Covey, 1986). CNBP cDNAs have also been cloned from mouse (Warden et al., 1994), rat (Yasuda et al., 1995), chicken (Ruble and Foster, 1998; van Heumen et al., 1997), *Xenopus* (De Dominicis et al., 2000; Flink and Morkin, 1995) and *Bufo arenarum* (Armas et al., 2001). The amino acid sequence of *CNBP* is highly conserved; human *CNBP* is 94.1% identical to that of Xenopus laevis (Flink et al., 1998), 93.8% identical to that of *B. arenarum* (Aruffo and Seed, 1987), 99% identical to that of chicken (van Heumen et al., 1997), 100% identical to that of rat (Yasuda et al., 1995) and 100% identical to the mouse homolog (Warden et al., 1994). This striking conservation, together with the fact that homologous genes have been found in various organisms, suggests that CNBP plays an essential biological role across different species.

The human CNBP cDNA was originally isolated by screening with a promoter fragment containing a sterol regulatory element (SRE) from the gene encoding for the hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase enzyme (Rajavashisth et al., 1989). CNBP was postulated to function as a negative transcriptional factor in the coordinate control of cholesterol metabolism, but this has not been confirmed (Ayala-Torres et al., 1994; Warden et al., 1994).

Abbreviations: ADE, anterior definitive endoderm; AVE, anterior visceral endoderm; CNBP, cellular nucleic acid-binding protein; FISH, fluorescence in situ hybridization; HMG-CoA, hydroxymethylglutaryl-coenzyme A; M-CSF, macrophage colony-stimulating factor; SRE, sterol regulatory element.

☆ The GenBank accession number of the *CNBP* gene is AY176064.

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Through direct binding to their respective promoter regions, CNBP can negatively control the expression of the early promoter-enhancer of the JC virus, a polyoma virus that commonly infects humans (Liu et al., 1998), and the β-myosin heavy chain (Flink and Morkin, 1995), but upregulates the expression of macrophage colony-stimulating factor (CSF-1) in fibroblasts (Konicek et al., 1998) and a reporter gene via the CT promoter element from c-Myc promoter (Michelotti et al., 1995). An association with c-Myc suggests that CNBP might potentially have a role in normal and abnormal cell proliferation and differentiation. However, this biochemical property has not been substantiated with physiological studies.

A possible role for CNBP during embryogenesis has also been suggested. *Xenopus CNBP* is detected in the unfertilized egg, strongly decreases after fertilization, starts accumulating again at gastrulation, gradually increases and is widely expressed throughout the neurula and tailbud stages (Flink et al., 1998). At the early gastrula stage, *Xenopus CNBP* is expressed in ectodermal, endodermal, and mesodermal germ layers (Flink et al., 1998). The *Xenopus* posterior gene, a homolog of CNBP, is also expressed in early development starting after the midblastula transition (Sato and Sargent, 1991). In *B. arenarum*, *CNBP* is also expressed during oogenesis and early embryogenesis (Armas et al., 2001). The expression pattern in *Xenopus* and *B. arenarum* embryos suggests a potentially important role for *CNBP* during early embryonic development.

A comparative analysis of the CNBP gene between organisms has not been conducted since only the human gene has been isolated and characterized (Flink and Morkin, 1995). A single copy of the CNBP gene is present in the human genome that consists of five exons, four of which contain coding information for two alternatively spliced products, CNBPα and CNBPβ. Localization studies have mapped the human CNBP gene to the long arm of chromosome 3 (Lusis et al., 1990). In the mouse genome, two genetic loci on chromosome 5 and 6 have been suggested (Warden et al., 1994) that encode multiple cDNAs homologous to human CNBPα and CNBPβ (Flink and Morkin, 1995). However, other reports indicate that there is only a single copy of the mouse CNBP gene (Lusis et al., 1990; Ruble and Foster, 1998). Only single gene copies have been identified in chickens (Ruble and Foster, 1998) and Xenopus (De Dominicis et al., 2000).

Here we report the molecular cloning, promoter characterization, developmental expression and functional analysis of the mouse *CNBP* gene. The mouse genome contains a single copy of the CNBP gene, which is located on chromosome 6. We analyzed CNBP expression during early to late embryo development by Northern blotting, whole-mount in situ hybridization and immunohistochemical studies. We also characterize CNBP gene regulation by promoter analysis. A potentially significant role in cell proliferation and expression regulation of c-Myc is identified. These findings provide direction for more definitive functional studies.

2. Materials and methods

2.1. Isolation of mouse CNBP cDNA and gene

A full-length CNBP cDNA was obtained by screening an E17.5 mouse cDNA library (Clontech). A mouse 129/SVJ genomic library, constructed on lambda FIX II (Stratagene), was screened using a 1650 bp radiolabeled mouse *CNBP* cDNA (Li et al., 1996). About 1×10^6 plaques were screened with a ^{32}P-random primer labeled probe derived from the mouse full-length *CNBP* cDNA. The positive clones were isolated and subcloned into pBluescript vector (Stratagene). The structure of the isolated genes was determined by restriction endonuclease mapping and Southern blot analysis. Restriction endonuclease digested DNA was fractionated on 0.8% agarose gel and transferred to nylon membranes. The DNA fragments containing portions of the *CNBP* gene exons were identified by hybridization with nick-translated DNA probes from different portions of the mouse *CNBP* cDNA. Standard methods were used for library screening, purification of phage DNA inserts, mapping of restriction enzyme digestion sites and Southern blotting (Sambrook et al., 1989).

2.2. DNA sequencing

A 20 kb fragment (clone C8) containing the 5′-end of the mouse *CNBP* gene and all five exons was isolated from a bacteriophage clone and subcloned into pBluescript vector (Stratagene). The subclone was sequenced as double-stranded templates with Sequenase in combination with dideoxynucleotide chain terminators according to the supplier's recommendation (U.S. Biochemical Corp.) or with an ABI 377 sequencer. Initial DNA sequencing was performed with T7 and T3 primers. Subsequent reverse orientation sequencing was performed using insert-specific primers. The DNA sequence was assembled and analyzed for the location of exons and introns by comparison to the sequence of mouse *CNBP* cDNA using the DNAsis II program (Hitachi).

2.3. Identification of the transcription start site of the mouse CNBP gene

The initiation site for the transcription of the mouse *CNBP* gene was analyzed by primer extension in a commercially available primer extension kit (Promega) according to the manufacturer's instructions. Four synthetic 30 base oligomers antisense to the transcribed strand were used to prime mRNA. They are located at −174 to −203 (P1: 5′-TCGCCCACGGATCCAATCAGCGCAGGAAGC-3′), −302 to −331 (P2: 5′-AGCGCTCGCGGCCTTCCC-

GCCCGTACCTGG-3′), −429 to −458 (P3: 5′-AGTGG-TCGCAGCTTGCGGGAGCCAATCTCC-3′), and −562 to −591 (P4: 5′-AGCGGCTGTGGCTACCGTTACGGTC-CATGA-3′) upstream of the initiator Met codon (as +1) of the mouse *CNBP* gene. The primers were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Life Technologies, Inc). The ^{32}P-labeled primers were hybridized with 10 μg total RNA from mouse osteoblast-like cells, prepared as described previously (Li and Chen, 1999), and extended using AMV reverse transcriptase. The extended cDNA product was analyzed on a sequencing gel with DNA sequence reactions and dephosphorylated ϕ X174 Hinf I DNA fragment size markers (Promega). As a control, a similar experiment was performed in parallel using the mouse Cathepsin K gene promoter, which is described in Li and Chen (1999).

2.4. Isolation of the mouse CNBP gene and promoter

We have cloned eight unique and overlapping λ clones. In order to characterize the promoter and 5′-flanking region of the mouse *CNBP* gene, sequence analysis and Southern blot using the primer encoding the exon 1 portion of mouse *CNBP* cDNA were performed. The cloned DNA were digested using *Not* I and were blotted onto a nylon filter. We designed an oligo probe P1: 5′-CGGACGAAGAACCAA-GAAGGCGAC-3′ that was located in exon 1, which was end-labeled with [γ-^{32}P]ATP. The membrane was prehybridized in BLOTTO solution and then hybridized to ^{32}P-labeled oligo probe P1 at 48 °C for 24 h. The membrane was washed at 50 °C in 6 × SSC and 0.1% SDS, and exposed to X-ray film (Fuji; Tokyo, Japan) for 96 h.

2.5. Cell culture

Rat2 and P19 cell lines were purchased from ATCC. The Rat2 line was derived from a 5-bromo-2′-deoxyuridine resistant strain of the Fischer rat fibroblast 3T3 like cell line. The P19 line was derived from an embryonic carcinoma induced in a C3H/He mouse. Rat2 and P19 cells were grown in DMEM medium containing 10% FBS (Gibco BRL; Gaithersburg, MD) and 0.3 mg/ml glutamine at 37 °C in an atmosphere of humidified air and 5% CO_2.

2.6. DNA transfection and CAT assay analysis

Rat2 or P19 cells were co-transfected with 5 μg of a reporter plasmid, 5 μg of expression plasmid and 2 μg of pSV-βgal using lipofectAMINE reagent (BRL) as described previously (Chen and Li, 1998). DNA co-transfections were performed in triplicate and repeated at least four times. *c-Myc* promoter-luciferase reporter plasmid was a gift from Dr Kenneth W. Kinzler. The pCMV-mCNBP-sense and -antisense were constructed by inserting the CNBP cDNA in a sense or antisense orientation, respectively, under transcriptional control of the CMV promoter region of the pCDNA3.1 vector (Invitrogen; San Diego, CA). Stably transfected P19 cells were selected in medium containing 300 μg/ml G418 (Gibco BRL). Transfection efficiency was normalized to β-galactosidase and CAT assays were performed on lysates as described previously (Neumann et al., 1987). Luciferase activities were measured using a kit (PACKARD) following the manufacturer's instructions. Galactosidase activities were measured as described previously (Chen and Li, 1998).

2.7. Chromosomal localization of mouse CNBP by fluorescence in situ hybridization

DNA from mouse *CNBP* gene clone C8 was labeled with digoxigenin dUTP by nick translation. Labeled probe was combined with sheared mouse DNA and hybridized to normal metaphase chromosomes. Chromosomes were derived from mouse embryo fibroblast cells in a solution containing 50% formamide, 10% dextran sulfate and 2 × SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated anti-digoxigenin antibodies followed by counterstaining with DAPI (Shi et al., 1997). The C8 (CNBP) probe was co-hybridized with the control probe (Genome Systems, Inc.) which is specific for the centromeric region of chromosome 6.

2.8. Northern blot analysis

Total RNA was isolated from mouse embryos at different stages of development. Whole cell RNA from human tissues was purchased from Clontech (Palo Alto, CA). Human cell lines were purchased from ATCC. Northern blot analysis of mouse *CNBP* was performed as described previously (Li et al., 1995).

2.9. In situ hybridization

Whole-mount RNA in situ hybridization was done as described previously (Wilkinson, 1993) using a digoxigenin-labeled RNA probe made from the entire 1.65 kb *CNBP* cDNA. In situ hybridization in tissue sections was done on freshly isolated E6.0 through E10.5 mouse embryos using procedures described previously (Li et al., 1996).

2.10. Tissue section CNBP immunostaining

Craniofacial regions of mouse embryos (form E 6.0 to E10.5) were processed, embedded in paraffin and sectioned. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 20 min. Nonspecific background staining was blocked by incubating sections in 10% goat serum in phosphate-buffered saline (PBS) for 20 min. Primary antibody (anti-*CNBP*, 1:100) was applied for 1 h at room temperature. The horseradish peroxidase avidin-biotin complex system (Rabbit Elite ABC Kit, Vector

Laboratories; Burlingame, CA) and diaminobenzidine were used to visualize bound antibody.

2.11. Cell proliferation assay and statistical analysis

To study cell growth, the P19 cells were stably transfected with the sense and antisense expression constructs and selected with G418 for 14 days. The stably transfected P19 clones were re-plated in six-well multiwell plates at 10^4 and grown in medium containing 10% FBS (Feng et al., 1989). On days 1, 3, or 4, medium was removed. Cells were rinsed twice with PBS, trypsinized, dispersed, resuspended in PBS and counted using a Coulter counter. Experiments were performed in triplicate and repeated at least four times. Data are presented as mean ± SEM. All statistical analyses were performed by Student's *t*-test using GraphPad (San Diego, CA) software. $P < 0.05$ was considered significant for establishing differences.

3. Results

3.1. Organization of the mouse CNBP gene

A description of the human *CNBP* gene structure was reported in 1995 (Flink and Morkin, 1995), but the mouse *CNBP* gene remains to be characterized. In order to clone and characterize the mouse CNBP gene, a 129/Sv strain mouse genomic library was screened for segments of the *CNBP* gene using a full-length mouse *CNBP* cDNA. Using this approach, eight overlapping lambda bacteriophage clones were identified and isolated (C1–C8). DNA from the eight-lambda clones was hybridized with different portions of mouse cDNA by Southern blot analysis. One clone, designated C8, was approximately 20 kb in length and hybridized to all portions of the *CNBP* cDNA indicating that it contained all the coding exons. *Not* I digestion of C8 generated two 10 kb fragments that were subcloned into pBluescript (Stratagene) for sequencing. A primer walking strategy was employed to obtain contiguous sequences that encompassed all exons, intervening introns, a 3 kb putative promoter region upstream of the transcription start site and 3 kb of 3′ sequence that extended to the polyadenylation signal (Fig. 1). The intron–exon boundaries were identified by sequence comparison between the *CNBP* cDNA and genome, as shown in Table 1. The intron–exon boundary sequences were generally consistent with the 5′ and 3′ splice consensus sequences and conformed to the GT/AG rule with the exception of GT/TG at the beginning and end of intron 3. We determined that the *CNBP* genome is composed of five exons ranging from 93 to 981 nucleotides (Table 1). The ATG translation initiation site is located in exon 2. Exon 5 contains the TAA stop codon and putative consensus polyadenylation signals (AATAAA) 856 bp downstream from the stop codon. The four introns range in size from 98 bp to 4.2 kb (Table 1). The entire CNBP gene is approximately 7 kb in length.

3.2. Identification of the transcription start site of the mouse CNBP gene

The CNBP gene promoter has not been reported from any species. To determine the mouse *CNBP* transcription initiation site, primer extension analysis was performed using mouse *CNBP* antisense oligonucleotide primers. The 5′-UTR of the mouse *CNBP* gene was predicted by homology with the human cDNA. Based on this potential exon 1 sequence, we made the antisense primer for the primer extension experiment. Four end-labeled antisense primers P1, P2, P3 and P4 that were homologous to the 5′-flanking region of *CNBP* cDNA or promoter region were annealed to total RNA isolated from mouse E10.5 mouse embryos. The primers were extended with AMV reverse transcriptase and analyzed by polyacrylamide-urea gel electrophoresis. The end-labeled P1 primer used to extend to the start of transcription yielded a product of 57 bp (Fig. 2) indicating that there is a transcription start site at 230 bp upstream of the initiator Met codon. No other bands were seen in the rest of the gel and no product was observed with the upstream primers P2, P3 and P4 (data not shown). The transcript initiation at 230 bp upstream of the initiator Met codon is, therefore, the major transcription initiation site.

3.3. Structural analysis of the putative promoter region of the mouse CNBP gene

In order to identify clones that may contain segments of the CNBP promoter, Southern blot of DNA isolated from lambda bacteriophage clones was hybridized to a primer that is homologous to a portion of exon 1. Genomic clones were digested using *Not* I endonuclease (which cuts just downstream of exon 1), blotted onto a nylon filter and

Table 1
Exon–intron junction of the mouse CNBP gene

Exon number and size (nt)	cDNA position of exon	5′ splicing site	Intron number and size (kb)	3′ splicing site
1 (216)	1–216	ACGCCGAAG gtgagaggc	1 (5.025)	attccaaag CTCTAACTG
2 (138)	217–354	CGGATAGAG gtatttttg	2 (0.098)	tcaaaatag GGTTCCAGT
3 (93)	355–447	AGGAGGATG gtaagtatt	3 (0.141)	ttattgttg AAGCCTGCT
4 (202)	448–649	GTGCTATAG gtgagtcat	4 (0.978)	ttttttcag GTGTGGTGA
5 (981)	650–1630			

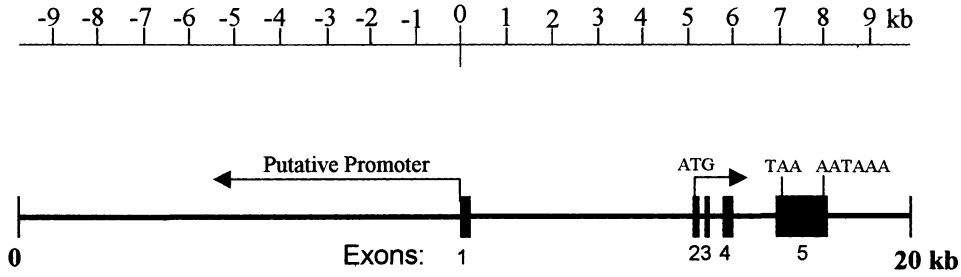

Fig. 1. Genomic organization and restriction map of the mouse *CNBP* gene. Schematic diagram of the mouse *CNBP* gene with flanking regions. The entire gene is composed of five exons and four introns distributed over approximately 7 kb. Solid bars represent exons. The locations of the initiator MET code (ATG) and stop code (TAA) are indicated.

hybridized to an exon 1-specific oligo probe, P1: 5′-CGGACGAAGAACCAAGAAGGCGAC-3′. The Southern blot result indicated that varying fragments (from 0.5 to 12 kb in length) of the *CNBP* promoter were obtained (Fig. 3). All clones were sequenced and overlapping sequences were completely identical in all clones (data not shown).

To identify basic promoter elements and consensus sequences for transcription factor binding sites, the basepair sequence of a 3 kb fragment of the *CNBP* gene promoter was analyzed by database search. Interestingly, the TATA box is located at position −718 bp, which is uncommonly far from the transcriptional start site, and there is no CAAT motif in this region. At positions −27 and −2161, we identified two GC boxes for Sp1 binding, a component of the basic transcription initiation complex, that function like a CAAT box. A number of AML-1a, E2F, MyoD, AP-1 and GATA binding sites were also present, indicating that these transcription factor families may play an important role in regulating *CNBP* gene expression. Other putative regulatory elements in the CNBP promoter include Oct-1, Ets1, SP1, and C/EBP (Table 2).

3.4. Chromosomal mapping of the mouse CNBP gene

Localization of the CNBP gene on the mouse chromosome was performed by fluorescence in situ hybridization (FISH) using the mouse CNBP cDNA as a probe. This experiment resulted in the specific labeling of the centromere (control probe) and the middle portion of chromosome 6 (*CNBP* probe) (Fig. 4A). Measurements of ten specifically labeled chromosomes demonstrated that *CNBP* is located at a position that is 59% of the distance from the heterochromatic-euchromatic boundary to the telomere of chromosome 6, an area that corresponds to band

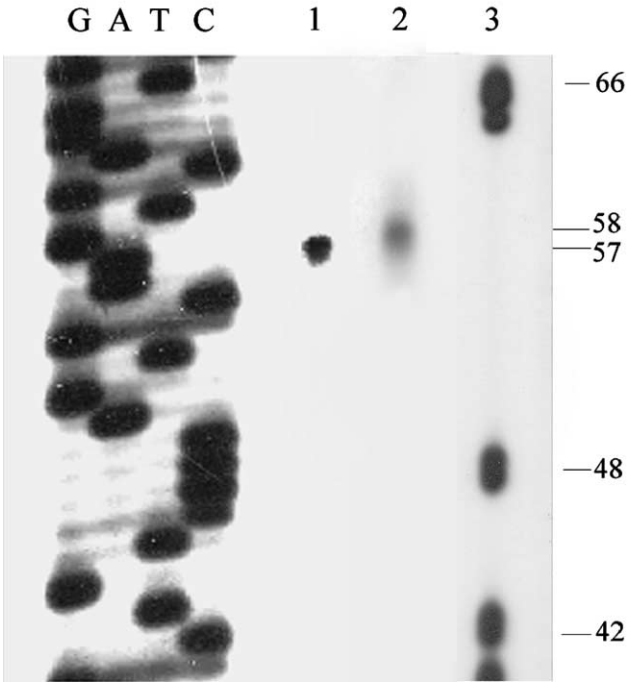

Fig. 2. Identification of the mouse *CNBP* transcription initiation site by primer extension. The primer extension product was determined on denaturing polyacrylamide gel electrophoresis using the adjacent DNA sequencing reaction (GATC) as a size marker. Line 1, *CNBP* primer extension 57 bp product; line 2, cathepsin K primer extension 58 bp product; line 3, ^{32}P-phosphorylated ϕ X174 Hinf I markers.

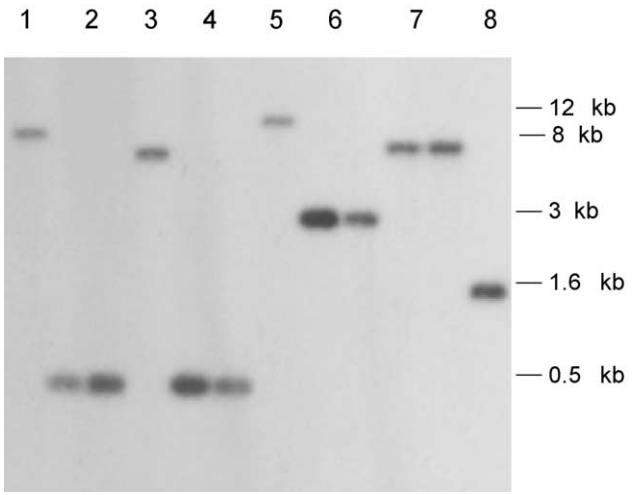

Fig. 3. Isolation of the mouse CNBP gene promoter. Southern blot analysis of *CNBP* gene clones digested by *Not* I using exon 1 oligo probe (P1) 5′-CGGACGAAGAACCAAGAAGGCGAC-3′ as a probe. The result indicates that the varied length 5′-flanking region fragments of CNBP promoter were obtained. The relative lengths of fragments shown on the Southern blot are depicted on the right.

Table 2
The potential recognition sequences for transcription factors in the mouse CNBP promoter region

Name of Cis-element	Position	Sequence
AML-1a	−2773, −701	TGTGGT
	−453	TGAGGT
	−199, −119	AGTGGT
AP-1	−2488	TGAGTCAT
	−2480	TGACTCAG
	−1839	TGACTCTT
	−984	TGACCAAGA
	−892	TGACTTAG
AP-4	−2658, −2484	CAGCAG
	−2569, −2560	CAGCTG
	−2044	CAGCTT
C/EBP	−2208	TTTGGAAA
	−2039	TTTGGTAA
	−1164	TTTGCTTA
	−703	TGTGGTAA
C/EBPβ	−2209	ATATTTGGAAAT
c-Ets-1(p54)	−2628	AAAGGAAAA
	−1934	AATGGAAGT
	−854	AGCGGAAAC
	−670	ACAGGAAAA
	−66	CCGGAAGA
CHOP-C/EBPα	−422	TTGCAATAGCC
c-Myc/Max	−2570	TAGCACATGCCT
	−479	AATCACCTGTCA
CRE-BP1/c-Jun	−2657	TGACTTTA
	−1684	TGATGTCA
	−1677	TGACATCA
E2F	−1862	TTGGGCGC
	−1851	CTTGGCGC
	−439	TTTGGGGC
GATA-1	−2334	GAGGAT
	−2121, −791	CCAGAT
	−1911, −1746	TGTGAT
	−1640	GCAGAT
	−1493, −460	GAAGAT
	−1435, −187	GCTGAT
	−1412	CAGGAT
	−473	GGTGAT
	−359	TGAGAT
	−228	GGAGAT
	−1460	GCAGATAAAG
	−2403	TAGAGGATAACTGA
	−1882	ATAGTGATAATTAG
GATA-2	−2401, −2187, −1911, −1640, −1460, −1263	GATA
	−2334, −1412, −460	GATG
	−228	GATT
GATA-3	−1912	GATAGTT
	−1745, −616	GATTGTG
	−1461	GATAAAG
	−1056	GATATGG
	−461	GATGGTG
	−227	GATTGGC
	−1765	AAAGATCAAA
	−1049	AAAGGTCTTA
	−976	AGAGATCCTA
GATA-X	−1462	GATAAAGC
	−652	GATAAATT
MyoD	−2783	CACCTGTA
	−2571	CACATGCC
	−478	CACCTGTC
	−1219	CAGCATGTGTTG

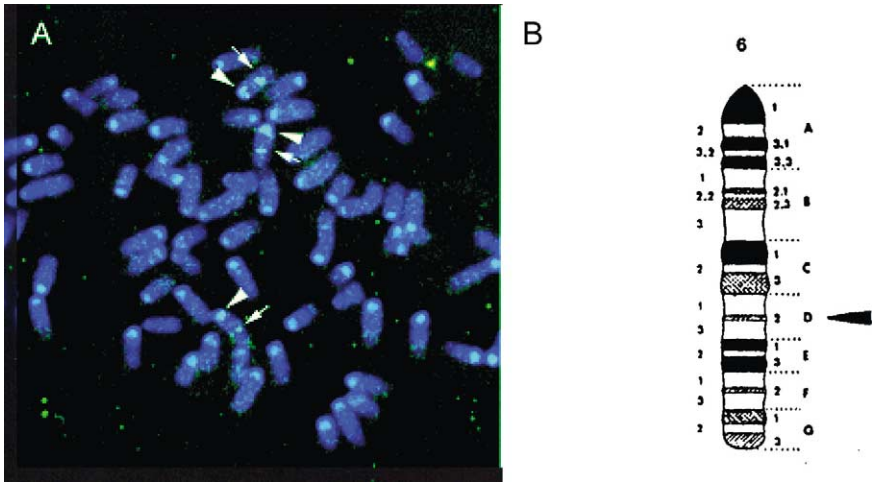

Fig. 4. Chromosomal localization of mouse *CNBP* by fluorescence in situ hybridization (FISH). FISH of the mouse *CNBP* gene on normal metaphase chromosomes derived from mouse embryo fibroblast cells. A probe, which is specific for the centromeric region of chromosome 6, was co-hybridized with the *CNBP* gene as control (A, short arrow). C8 (*CNBP*) probe hybridized at the middle portion of chromosome 6 D1-D2 (A, arrow; B, short arrow).

6 D1-D2 (Fig. 4B). A total of 80 metaphase cells were analyzed with 71 exhibiting specific labeling.

3.5. Northern blot analysis of CNBP gene expression during embryogenesis and CNBP tissue distribution

To determine the time course of *CNBP* gene expression during mouse embryogenesis, Northern blot analysis was performed using total cellular RNA extracted from whole embryos at different days during development. Blots were hybridized with a 1.65 kb ^{32}P-labeled mouse *CNBP* cDNA probe. Relative to *GDPH* levels, a high level of *CNBP* expression is maintained at all stages of mouse embryonic development (Fig. 5A). Adult tissues and several cell lines were also examined, which showed that CNBP is expressed in a wide range of cells at varying levels (Fig. 5B). For example, CNBP is highly expressed in brain and kidney but weakly expressed in T lymphocyte (HSB-2).

3.6. Whole-mount in situ hybridization of CNBP during mouse embryogenesis

Whole-mount in situ hybridization and immunohistochemistry were performed in order to obtain a more detailed understanding of the precise tissue regions that express CNBP. We examined *CNBP* expression from pre-gastrulation to organogenesis stages. Before gastrulation, at E5.5, *CNBP* expression is initially symmetric and uniform in the

Table 2 (*continued*)

Name of Cis-element	Position	Sequence
	−468	TGACAGGTGATT
NF-E2	−2490	TGCTGAGTCAT
Oct-1	−1879	ATAATTA
	−753	TTAATGA
	−502	ATAATGA
	−446	CTAATGA
	−1605	CATAGTGAGATCCT
	−606	CACCATGAAATGCA
P53	−2572	GCACATGCCT
	−1709	TGACTTGTTC
	−1607	AGTCTTGCCT
P300	−574	AGGAGTGACCG
Sp1	−1817	TGGGCGTGGT
	−1379	GAGGCAGGGT
	−1343	GTGGCTGGGT
	−2159	GGGGGCGGAGG
	−29	AGGGGCGGGGC
STATx	−662	TTCCTGTAA
	−308	TTCCCCCAA
v-Myb	−2398	GATAACTGAA
	−353	GTAACGG
YY1	−472	CCATCTTCAAC

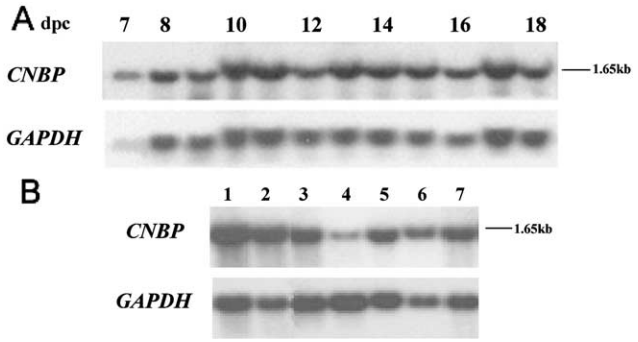

Fig. 5. The time course of CNBP expression during embryonic development and its tissue distribution. (A) Northern blot analysis was performed using total cellular RNA extracted from different days during embryogenesis, which indicated that a 1.6 kb *CNBP* transcript is expressed throughout embryonic development. (B) Northern hybridization of CNBP cDNA to total RNA from human tissues and cell lines. Lane 1, adult brain; lane 2, adult kidney; lane 3, T lymphocyte (HSB-2); lane 4, laryngeal carcinoma (Hep-2); lane 5, myelomonocytic (U-937); lane 6, pancreatic adenocarcinoma (AsPC-1); lane 7, neuroblastoma SK-N-MC. GAPDH probe was used to control for differences in loading. d.p.c., days post coitum.

epiblast and in the extra-embryonic visceral endoderm (Fig. 6A,B). At E7.5, *CNBP* expression was confirmed at the protein level by immunostaining using anti-*CNBP* polyclonal antibodies, shown in brown stain (Fig. 6C). At this early-primitive streak stage, *CNBP* expression becomes asymmetrical and localizes to all three germ layer regions of the anterior conceptus, suggesting a possible role in anterior-posterior axis patterning. Later *CNBP* is expressed in the forebrain (E9.0 and E10) and in the midbrain (E11.5). From E9.0 to E11.5, *CNBP* is also expressed in the early craniofacial structures, limb buds and somites (Fig. 6D–F). Regions of highest expression in the face include the cranial and caudal regions of the mandibular prominences, the budding maxillary prominences and the roof of the stomodeum (Fig. 6E). In the distal limb region, at E13.0, *CNBP* protein lines the outer regions of the developing phalanges (Fig. 6G) within the nucleus of the cells (Fig. 6H), which is consistent with the function of *CNBP* as a transcription factor.

3.7. Analysis of the mouse CNBP gene promoter

CNBP is abundantly expressed in *Xenopus* (Flink et al., 1998) and *B. arenarum* embryos (Armas et al., 2001). In order to identify the regions of the *CNBP* promoter that are directly involved in transcription regulation, DNA transfection and CAT assay were performed. Three different length fragments of the *CNBP* promoter were subcloned upstream of a CAT reporter gene (1.6-CAT, 3.0-CAT and 7.0-CAT) and transiently transfected into both rat fibroblast-like cells (Rat2) and mouse embryonic carcinoma cell lines (P19). CAT activity was normalized to β-galactosidase activity, which was co-transfected as a control for transfection

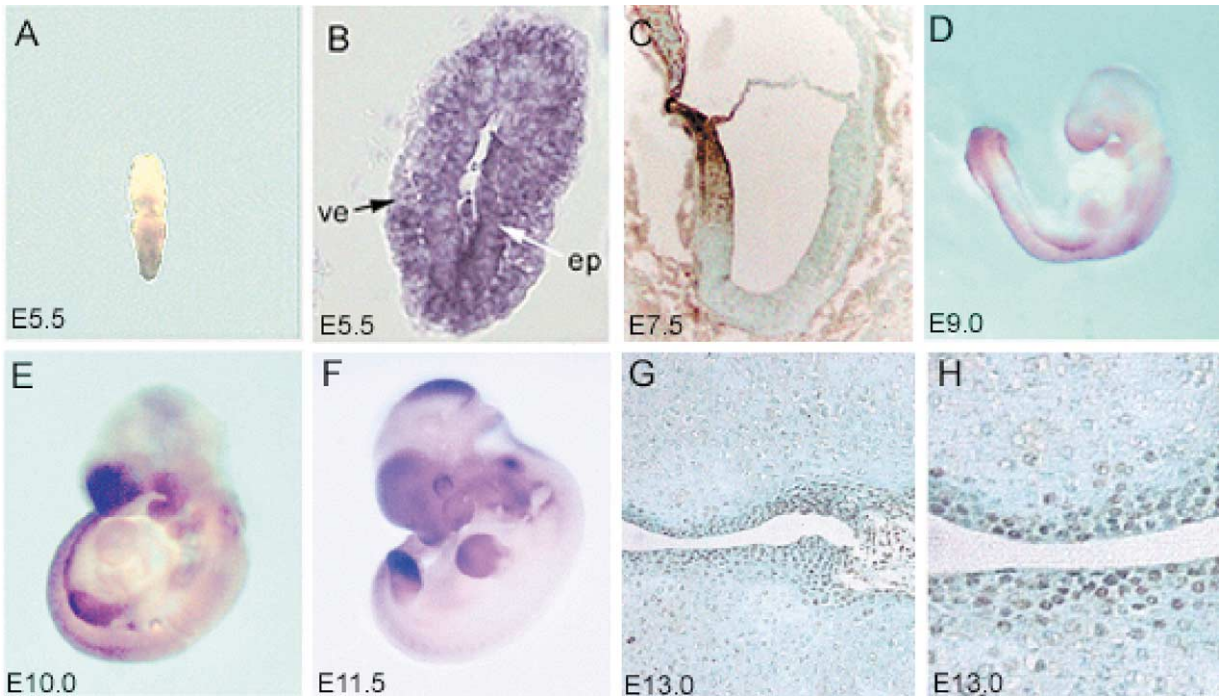

Fig. 6. Expression of *CNBP* at pre-gastrulation to organogenesis stages. At pre-gastrulation stage embryos, E5.5, *CNBP* expression is initially symmetric and uniform (A). A cross-section shows expression in the epiblast (ep) and in the extra-embryonic visceral endoderm (ve; B). CNBP immunostain of E7.5 embryo in tissue section shows that CNBP protein is restricted to the anterior end of the embryo in all three germ layers, anterior ectoderm, anterior visceral endoderm and axial mesendoderm (C). By E9.0, early organogenesis stage, *CNBP* was expressed in craniofacial structures (D), which persists at E10 (E) and 11.5 (F) stages. As organogenesis proceeds, CNBP becomes more abundant in the limbs and midbrain (E,F). CNBP protein is also localized to the phalanges (G,H). A higher magnification of the boxed region in (G) is shown in (H), which indicates that CNBP is localized to the nucleus (arrows).

efficiency. Transfection of either of the CNBP promoter constructs resulted in 13–17 fold greater CAT activity in P19 cells as compared to Rat2 cells. The patterns of CAT activity between the three different length promoter fragments were similar in both cell types. The greatest CAT activity was observed with the 1.6 kb fragment, particularly in P19 cells. Significantly less activity was detected with the 3 kb promoter and the 7 kb fragment had even slightly less activity (Fig. 7). These results indicated that within the 1.6 kb region upstream of the putative transcriptional start site is sufficient to confer major promoter activity in cells. Promoter constructs greater than 1.6 kb had significantly lower transcriptional activation abilities, suggesting the presence of negative regulatory elements upstream of the −1.6 kb region.

3.8. CNBP increases c-Myc promoter activity

CNBP has been shown to regulate the CT element of the human c-Myc protooncogene in a heterologous promoter (Michelotti et al., 1995). However, whether CNBP participates directly in the regulation of *c-myc* promoter has not been addressed. We examined the regulation of c-Myc promoter activity by co-transfection of *c-Myc* promoter-luciferase reporter plasmid (He et al., 1998) and mouse *CNBP* expression plasmid (CMV-mCNBP) constructs into P19 cells. *c-Myc* promoter was stimulated 2.5 fold by the CNBP expression vector (Fig. 8). We therefore conclude that *CNBP* behaves as a transactivator of the *c-Myc* promoter.

3.9. CNBP enhances cell proliferation

The c-Myc gene is a critical cell cycle regulator that is also associated with carcinogenesis. To investigate the potential function of CNBP on cell growth, P19 embryonic carcinoma cells were stably transfected with the full-length CNBP cDNA in a sense or antisense orientation to alter

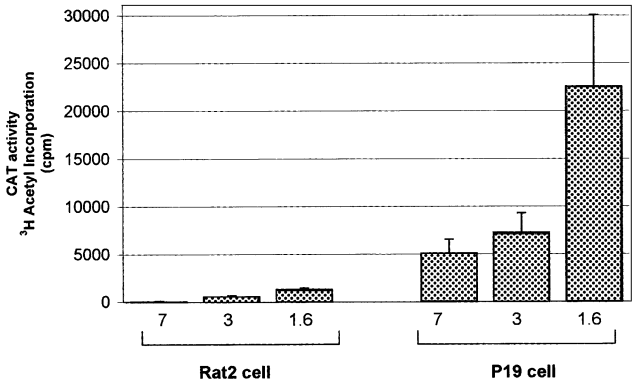

Fig. 7. CAT activity of Rat2 and P19 cells transfected with varying sizes of CNBP promoter. CAT activities were much higher in P19 than Rat2 cells with every size of DNA that was transfected. The 1.6 kb construct showed significantly higher CAT activity than 3 or 7 kb transfected constructs. CNBP promoter activity is much higher in P19 cells than that in Rat cells.

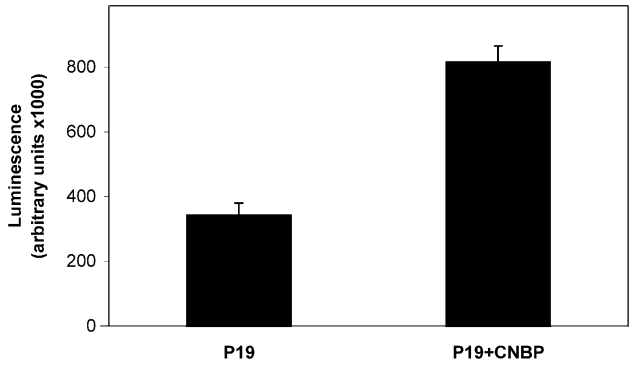

Fig. 8. CNBP increases c-Myc promoter activity. Overexpression of mouse *CNBP* expression in P19 cells stimulated c-*Myc* promoter activity by 2.5 fold, as determined by luciferase activity.

CNBP production. Compared with control cells transfected with vector alone, *CNBP* overexpression (sense orientation of CNBP) cells showed a 2.5 fold increase in the rate of cell proliferation 3 days after plating (Fig. 9). By day 4, *CNBP* overexpressing cells showed a 200% increase in cell number compared with vector controls. Cells transfected with antisense CNBP cDNA showed a 50% decrease in growth compared with empty vector. Collectively, our data suggest a mechanism whereby CNBP regulates cell proliferation perhaps through the specific targeting and upregulation of *c-Myc* expression. The fact that CNBP regulates *c-Myc* expression and cell proliferation may have intriguing implications for normal and neoplastic cell growth.

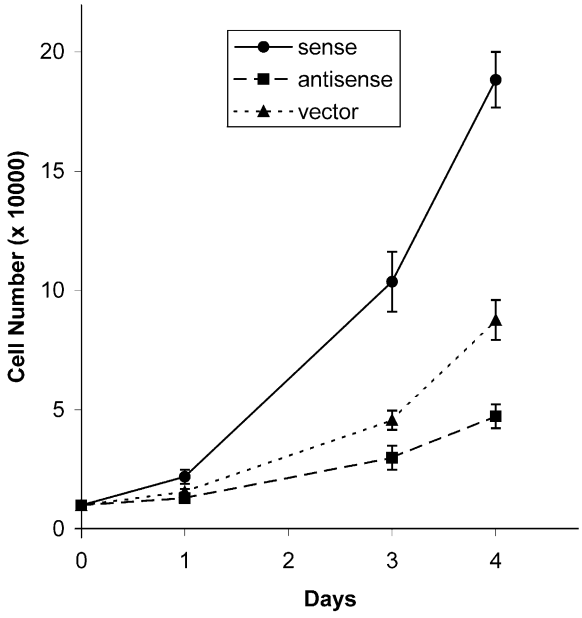

Fig. 9. CNBP enhances cell proliferation. P19 cells transfected with vectors containing CNBP in sense and antisense orientations resulted in a 200% increased and 50% decreased cell proliferation, respectively, relative to cells transfected with vector alone. Experiments were performed in triplicate and repeated at least four times.

4. Discussion

The CNBP cDNA was cloned over a decade ago but the function of CNBP is still not known. CNBP homologs are widespread throughout the animal kingdom (van Heumen et al., 1997) and its amino acid sequence is highly conserved, suggesting a fundamental function in different species throughout evolution. Although, the role of *CNBP* in mammals remains unclear, studies in yeast have shown that Byr3, a protein highly homologous to *CNBP*, can suppress sporulation defects in cells that lack ras1 (Xu et al., 1992). Our expression analysis, promoter characterization and functional studies should begin to shed some light on the potential role of CNBP during embryogenesis.

Our results indicate that the mouse *CNBP* gene is located at a single locus on chromosome 6. Others have reported that genetic mapping of the *CNBP* gene in interspecific and intersubspecific mouse backcrosses identified two loci that hybridize to *CNBP* cDNA and are located on chromosomes 5 and 6: *CNBPl*1, located in chromosome 5 at 58 cM, and *CNBPl*2, located in chromosome 6 at 39 cM (Warden et al., 1994). We further examined our data in order to address this discrepancy. Two other lines of evidence also indicate that there is only a single copy of the *CNBP* gene in the mouse genome. First, sequence analyses of all our mouse CNBP gene clones were found to be identical. Our original screening for the mouse *CNBP* gene resulted in the isolation of eight overlapping λ clones. These clones were digested with *Not* I, generating eight varied length (from 0.5 to 12 kb) 5′-flanking promoter region fragments of the *CNBP* gene. These non-coding regions are much more variable than coding regions and would be of different sequence if more than one CNBP gene was present in the mouse. Second, analysis of mouse genomic DNA using several restriction enzymes resulted in a single band on Southern blots (data not shown). If two copies of CNBP, on chromosome 5 and 6, exist in the mouse we would predict a minimum of two bands. It is very unlikely that digestion of both chromosomes results in similar length CNBP gene containing fragments that are indistinguishable by Southern analysis. We also do not observe any conflict between predicted restriction sites and banding patterns using restriction enzymes that cut multiple times within the first and last exons. We would expect discrepancies if there were more than one copy of *CNBP*. The presence of a single CNBP gene in the mouse genome is consistent with other organisms, including chickens (Ruble and Foster, 1998), *Xenopus* (De Dominicis et al., 2000) and humans (Lusis et al., 1990), which all have a single copy of CNBP.

The CNBP promoter has not been characterized from any species. In order to characterize the promoter and 5′-flanking region of the mouse *CNBP* gene, primer extension, DNA transfection and CAT assay analysis were performed. The potential regulatory elements in the *CNBP* gene promoter were identified by database search. There is neither a TATA box nor a CAAT box in the region near the transcription start site. These features are characteristic of genes that are specifically regulated during development, rather than being constitutively active, and initiate transcription at only one or a few clustered sites (Smale and Baltimore, 1989). Two GC boxes for Sp1 binding, which are components of the basic transcription initiation complex that function like a CAAT box, were identified. A number of AML-1a, E2F, and MyoD response elements were found. Multiple potential AP-1 and GATA binding sites located in the 5′-flanking regulatory region of the mouse *CNBP* gene indicate that AP-1 and GATA transcription factor families may also play important transcriptional regulatory roles. Other putative regulatory elements found included Oct-1, Ets1, SP1 and C/EBP (Table 2).

CNBP expression has been reported in different tissues of amphibia (Calcaterra et al., 1999), chickens (Ruble and Foster, 1998; van Heumen et al., 1997) and mammals (Flink and Morkin, 1995). Very little is known about *CNBP* expression during embryonic development. In *Xenopus*, *CNBP* is expressed in the unfertilized egg, and is widely expressed throughout early development at the gastrula, neurula, and tailbud stages. At the early gastrula stage, *Xenopus CNBP* is expressed in ectodermal, endodermal, and mesodermal germ layers (Flink et al., 1998). CNBP expression has also been reported in oocytes to late-gastrula stages in *B. arenarum* (Armas et al., 2001). In oocytes, *CNBP* is ubiquitously expressed and as development proceeds, the localization of CNBP protein moves from the cytoplasm to the nucleus (Armas et al., 2001). Here we report a dynamic tissue specific pattern of CNBP expression in the developing mouse embryo. We showed by Northern hybridization that *CNBP* is expressed throughout development, suggesting that it may have an important role during all stages of embryogenesis. A more specific localization of CNBP was examined by in situ hybridization and immunohistochemistry. We found that the early ubiquitous *CNBP* expression became anterior specific during pre-gastrulation and late-gastrulation stages. In the mouse embryo, separate cell populations organize the development of head and trunk structures. The head organizer is located in the anterior visceral endoderm (AVE) but the node, node derivatives and the anterior epiblast are also involved in defining anterior structures (Lu et al., 2001). During the mid-primitive streak to the late-primitive streak, the proximodistal (or embryonic/abembryonic) axis of the blastocyst gives rise to the anterior-posterior axis of the egg cylinder. *CNBP* expression becomes restricted to the anterior region and is abundant in the anterior neural ectoderm, anterior definitive endoderm (ADE) and axial mesendoderm tissues (Fig. 7C). CNBP appears to play a role in defining anterior tissue during anterior-posterior axis formation. A similar expression pattern has been observed for Otx2 (Simeone et al., 1993; Ang et al., 1994) and Hesx1 (Thomas and Beddington, 1996), genes that are necessary for anterior patterning in the mouse (Acampora et al., 1995). CNBP may also be important for face and limb morpho-

genesis, two structures that frequently express the same genes (Schneider et al., 1999), since *CNBP* is expressed in the developing facial prominences and limb buds at E9.0, E10 and E11.5 (Fig. 7D–F).

We found that CNBP protein is localized in the nuclei of expressing cells, which is concurrent with its proposed role as a transcription factor. CNBP has been purified from nuclear extracts (Michelotti et al., 1995) and can bind to the promoter region of a number of genes (LDL receptor (Rajavashisth et al., 1989); c-Myc (Michelotti et al., 1995); β-MHC (Flink and Morkin, 1995)). Others have shown that CNBP protein is also present in the cytoplasm of cells, functioning in the translational control of ribosomal protein mRNAs in *Xenopus* (Pellizzoni et al., 1997). It is not known whether CNBP modulates translation of mRNA in all animal species. In particular, this feature has not been substantiated in the mouse model although there has been a report that CNBP is primarily localized in the cytoplasm and endoplasmic reticulum in the mouse liver and is undetectable in the nucleus (Warden et al., 1994). We found that consistent with its proposed function as a transcription factor, CNBP is indeed located in the nucleus of mouse cells and also show that CNBP can alter gene transcription. Future studies should clarify the multifunctional actions of CNBP in the nucleus and cytoplasm of cells.

Functioning as a transcriptional regulator, CNBP regulates transcription from the CT element of the c-Myc protooncogene (Michelotti et al., 1995). CNBP also regulates transcription of macrophage colony-stimulating factor (M-CSF; CSF-1), a cytokine that acts on monocytic cells (Konicek et al., 1998). Together these findings have intriguing implications for CNBP as a regulator of cell proliferation. We have shown that CNBP regulates c-Myc expression and may act through c-Myc to regulate cell proliferation, thereby identifying a potentially very significant gene upstream of c-Myc in the cell cycle progression of cells. Our studies are the first to show that overexpression of CNBP can directly stimulate cell proliferation. This finding was particularly striking because the P19 embryonic carcinoma cells that we used in our study already have a very high rate of turnover. The 2.5 fold increase in proliferation we observed in response to *CNBP* overexpression indicates that CNBP is a potent stimulator of cell proliferation. Whether this finding and its association with c-Myc translate into an involvement in neoplasia should be resolved in the future.

The interaction between CNBP and both c-Myc and CSF-1 also suggests a potential role for CNBP in the differentiation of cells, particularly during early A-P axis development when *CNBP* is expressed. Interestingly, c-Myc knockout mice die early in development with defects in anterior development, including the failure of neural tube closure (Davis et al., 1993). *c-Myc* expression during pre-gastrulation and gastrulation stages overlaps with *CNBP* expression (Downs et al., 1989), although *c-Myc* is less tissue specific than *CNBP*. Presumably, *c-Myc* expression is CNBP dependent in some tissues but CNBP independent in others. Several exciting unanswered questions remain to be addressed from our studies.

Here we are the first to report the characterizations of the mouse CNBP gene and gene promoter. We are also the first to analyze the developmental expression of CNBP during mouse embryogenesis, which suggests a role for CNBP in axis development, head development and organogenesis, perhaps as a regulator of cell proliferation and differentiation through c-Myc. Future investigations that address the functional role of CNBP should tie together and clarify these intriguing observations.

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References

Acampora, D., Mazan, S., Lallemand, Y., Avantaggiato, V., Maury, M., Simeone, A., Brulet, P., 1995. Forebrain and midbrain regions are deleted in Otx2 − /− mutants due to a defective anterior neuroectoderm specification during gastrulation. Development 121, 3279–3290.

Ang, S.L., Conlon, R.A., Jin, O., Rossant, J., 1994. Positive and negative signals from mesoderm regulate the expression of mouse Otx2 in ectoderm explants. Development 120, 2979–2989.

Armas, P., Cabada, M.O., Calcaterra, N.B., 2001. Primary structure and developmental expression of Bufo arenarum cellular nucleic acid-binding protein: changes in subcellular localization during early embryogenesis. Dev. Growth Differ. 43, 13–23.

Aruffo, A., Seed, B., 1987. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. Proc. Natl. Acad. Sci. USA 84, 8573–8577.

Ayala-Torres, S., Johnson, B.H., Thompson, E.B., 1994. Oxysterol sensitive and resistant lymphoid cells: correlation with regulation of cellular nucleic acid binding protein mRNA. J. Steroid Biochem. Mol. Biol. 48, 307–315.

Calcaterra, N.B., Palatnik, J.F., Bustos, D.M., Arranz, S.E., Cabada, M.O., 1999. Identification of mRNA-binding proteins during development: characterization of Bufo arenarum cellular nucleic acid binding protein. Dev. Growth Differ. 41, 183–191.

Chen, W., Li, Y.P., 1998. Generation of mouse osteoclastogenic cell lines immortalized with SV40 large T antigen. J. Bone Miner. Res. 13, 1112–1123.

Covey, S.N., 1986. Amino acid sequence homology in gag region of reverse transcribing elements and the coat protein gene of cauliflower mosaic virus. Nucleic Acids Res. 14, 623–633.

Davis, A.C., Wims, M., Spotts, G.D., Hann, S.R., Bradley, A., 1993. A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. Genes Dev. 7, 671–682.

De Dominicis, A., Lotti, F., Pierandrei-Amaldi, P., Cardinali, B., 2000. cDNA cloning and developmental expression of cellular nucleic acid-

binding protein (CNBP) gene in Xenopus laevis (in process citation). Gene 241, 35–43.

Downs, K.M., Martin, G.R., Bishop, J.M., 1989. Contrasting patterns of myc and N-myc expression during gastrulation of the mouse embryo. Genes Dev. 3, 860–869.

Feng, Z.M., Li, Y.P., Chen, C.L., 1989. Analysis of the 5′-flanking regions of rat inhibin alpha- and beta-B-subunit genes suggests two different regulatory mechanisms. Mol. Endocrinol. 3, 1914–1925.

Flink, I.L., Morkin, E., 1995. Alternatively processed isoforms of cellular nucleic acid-binding protein interact with a suppressor region of the human beta-myosin heavy chain gene. J. Biol. Chem. 270, 6959–6965.

Flink, I.L., Blitz, I., Morkin, E., 1998. Characterization of cellular nucleic acid binding protein from Xenopus laevis: expression in all three germ layers during early development. Dev. Dyn. 211, 123–130.

He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B., Kinzler, K.W., 1998. Identification of c-MYC as a target of the APC pathway. Science 281, 1509–1512.

Konicek, B.W., Xia, X., Rajavashisth, T., Harrington, M.A., 1998. Regulation of mouse colony-stimulating factor-1 gene promoter activity by AP1 and cellular nucleic acid-binding protein. DNA Cell Biol. 17, 799–809.

Li, Y.P., Chen, W., 1999. Characterization of mouse cathepsin K gene, the gene promoter, and the gene expression. J. Bone Miner. Res. 14, 487–499.

Li, Y.P., Chen, W., Stashenko, P., 1995. Characterization of a silencer element in the first exon of the human osteocalcin gene. Nucleic Acids Res. 23, 5064–5072.

Li, Y.P., Chen, W., Stashenko, P., 1996. Molecular cloning and characterization of a putative novel human osteoclast-specific 116-kDa vacuolar proton pump subunit. Biochem. Biophys. Res. Commun. 218, 813–821.

Liu, M., Kumar, K.U., Pater, M.M., Pater, A., 1998. Identification and characterization of a JC virus pentanucleotide repeat element binding protein: cellular nucleic acid binding protein. Virus Res. 58, 73–82.

Lu, C.C., Brennan, J., Robertson, E.J., 2001. From fertilization to gastrulation: axis formation in the mouse embryo. Curr. Opin. Genet. Dev. 11, 384–392.

Lusis, A.J., Rajavashisth, T.B., Klisak, I., Heinzmann, C., Mohandas, T., Sparkes, R.S., 1990. Mapping of the gene for CNBP, a finger protein, to human chromosome 3q13.3-q24 (published erratum appears in Genomics 1991;9(3):564). Genomics 8, 411–414.

Michelotti, E.F., Tomonaga, T., Krutzsch, H., Levens, D., 1995. Cellular nucleic acid binding protein regulates the CT element of the human c-myc protooncogene. J. Biol. Chem. 270, 9494–9499.

Neumann, J.R., Morency, C.A., Russian, K.O., 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. Biotechniques 5, 444–447.

Pellizzoni, L., Lotti, F., Maras, B., Pierandrei-Amaldi, P., 1997. Cellular nucleic acid binding protein binds a conserved region of the 5′ UTR of Xenopus laevis ribosomal protein mRNAs. J. Mol. Biol. 267, 264–275.

Rajavashisth, T.B., Taylor, A.K., Andalibi, A., Svenson, K.L., Lusis, A.J., 1989. Identification of a zinc finger protein that binds to the sterol regulatory element. Science 245, 640–643.

Ruble, D.M., Foster, D.N., 1998. Molecular cloning and characterization of a highly conserved chicken cellular nucleic acid binding protein cDNA. Gene 218, 95–101.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sato, S.M., Sargent, T.D., 1991. Localized and inducible expression of Xenopus-posterior (Xpo), a novel gene active in early frog embryos, encoding a protein with a 'CCHC' finger domain. Development 112, 747–753.

Schneider, R.A., Hu, D., Helms, J.A., 1999. From head to toe: conservation of molecular signals regulating limb and craniofacial morphogenesis. Cell Tissue Res. 296, 103–109.

Shi, Y.P., Mohapatra, G., Miller, J., Hanahan, D., Lander, E., Gold, P., Pinkel, D., Gray, J., 1997. FISH probes for mouse chromosome identification. Genomics 45, 42–47.

Simeone, A., Acampora, D., Mallamaci, A., Stornaiuolo, A., D'Apice, M.R., Nigro, V., Boncinelli, E., 1993. A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo. EMBO J. 12, 2735–2747.

Smale, S.T., Baltimore, D., 1989. The "initiator" as a transcription control element. Cell 57, 103–113.

Thomas, P., Beddington, R., 1996. Anterior primitive endoderm may be responsible for patterning the anterior neural plate in the mouse embryo. Curr. Biol. 6, 1487–1496.

van Heumen, W.R., Claxton, C., Pickles, J.O., 1997. Sequence and tissue distribution of chicken cellular nucleic acid binding protein cDNA. Comp. Biochem. Physiol. B Biochem. 118, 659–665.

Warden, C.H., Krisans, S.K., Purcell-Huynh, D., Leete, L.M., Daluiski, A., Diep, A., Taylor, B.A., Lusis, A.J., 1994. Mouse cellular nucleic acid binding proteins: a highly conserved family identified by genetic mapping and sequencing. Genomics 24, 14–19.

Wilkinson, D.G., 1993. *In situ* hybridization. In: Stern, C.D., Holland, P.W.H. (Eds.), Essential Developmental Biology: A Practical Approach, IRL Press, Oxford, pp. 257–274.

Xu, H.P., Rajavashisth, T., Grewal, N., Jung, V., Riggs, M., Rodgers, L., Wigler, M., 1992. A gene encoding a protein with seven zinc finger domains acts on the sexual differentiation pathways of Schizosaccharomyces pombe. Mol. Biol. Cell 3, 721–734.

Yasuda, J., Mashiyama, S., Makino, R., Ohyama, S., Sekiya, T., Hayashi, K., 1995. Cloning and characterization of rat cellular nucleic acid binding protein (CNBP) cDNA. DNA Res. 2, 45–49.