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Characterisation and developmental regulation of the *Xenopus laevis* CCAAT-enhancer binding protein β gene

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Abstract

We report here the cloning, characterisation and developmental expression profile of the *Xenopus laevis* CCAAT-enhancer binding protein β (xC/EBPβ) gene. The protein synthesised from the xC/EBPβ gene interacts specifically with a C/EBP-recognition sequence and acts as a transcriptional activator. Several conserved regions are present in the xC/EBPβ sequence, including the basic region, leucine zipper, activation domains, three in-frame AUG codons, and a consensus site for mitogen activated protein kinase. The corresponding mRNA is present at high levels in the kidney, liver, lung, muscle and adipose tissue, and at low levels in the ovary, brain and heart. Although the xC/EBPβ mRNA and protein are present throughout embryogenesis, there is a biphasic increase in their expression levels during development. Whole-mount in situ hybridisation shows a restricted spatial expression profile of the xC/EBPβ gene during early embryogenesis, with transcripts present around the blastopore lip and in the endodermal cells at the mid-gastrula stage, and, the whole dorsal side at the neurula and early tailbud stage. The expression domain becomes almost ubiquitous during later embryonic development, and includes the brain, spinal cord, somites and regions that give rise to the liver and the heart.

Keywords: CCAAT-enhancer binding protein β; Basic-leucine zipper domain; Transcription factor; Activation domain; DNA binding domain; *Xenopus laevis*; Development; Expression profile; Transcriptional activator; Mitogen activated protein kinase; DNA sequence; Electrophoretic mobility shift assays; Transient transfection assays; Embryogenesis; Temporal and spatial expression profile; Reverse transcription–polymerase chain reaction; Whole mount in situ hybridization; Western blot analysis; Blastopore lip; Endodermal cells

1. Introduction

Transcription factors belonging to the CCAAT-enhancer binding protein (C/EBP) family contain a highly conserved carboxyl-terminal basic-leucine zipper (bZIP) domain that consists of a basic region, involved in DNA recognition, and an adjacent helical structure, the leucine zipper, that mediates subunit dimerisation (Wedel and Ziegler-Heitbrock, 1995). In contrast, the N-termini of the proteins, which carry the regulatory and the *trans*-activation domains, are quite divergent. Six distinct C/EBP isoforms have been identified to date (C/EBPα to ζ) and the majority of these recognise similar DNA sequences, at least in vitro, activate gene transcription in vivo and form heterodimers in intra-familial combinations (Wedel and Ziegler-Heitbrock, 1995). Additionally, in the case of C/EBPα and C/EBPβ, polypeptides of different sizes and *trans*-activating capabilities can be produced from the same mRNA by alternative use of in-frame AUG codons because of a leaky ribosome scanning mechanism (Descombes and Schibler, 1991; Ossipow et al., 1993). For example, three isoforms, designated LAP*, LAP and LIP can be produced from the C/EBPβ mRNA, of which, LAP* and LAP are activators of gene transcription and LIP is a repressor (Descombes and Schibler, 1991). Thus, the number of C/EBP proteins that may be present in any given tissue/cell may be much higher than the number of C/EBP-encoding genes.

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Several roles have been ascribed to the C/EBP family, including the regulation of tissue-specific gene expression, cellular growth and differentiation and cytokine-induced gene expression during inflammation (Akira et al., 1990; Poli et al., 1990; Descombes et al., 1990; Cao et al., 1991; Katz et al., 1993; Ramji et al., 1993; Wedel and Ziegler-Heitbrock, 1995; MacDougald and Lane, 1995; Sterneck et al., 1997). Their expression profile and function in embryonic development, however, remains enigmatic. We report here the characterisation of the *Xenopus laevis* C/EBPβ (xC/EBPβ) gene, and, its developmental and tissue specific expression profile. The studies represent the first, to date, which have analysed the expression profile of the C/EBPβ gene during embryonic development.

2. Results and discussion

2.1. Cloning and characterisation of the xC/EBPβ gene

A genomic fragment containing the xC/EBPβ gene was isolated as described in Section 3. Sequence analysis revealed that the deduced 288-amino-acid coding sequence (EMBL accession number Y16591) shares the highest degree of sequence identity with the previously characterised human, rodent and avian C/EBPβ genes (Akira et al., 1990; Chang et al., 1990; Descombes et al., 1990; Poli et al., 1990; Cao et al., 1991; Williams et al., 1991; Katz et al., 1993) (overall identity of 39–41%; >80% in the bZIP domain). Additionally, several regions, which have been reported previously to be important for C/EBPβ function, are conserved in the *Xenopus* sequence, including two activation domains (positions 53–69 and 85–92) that have been mapped in the rat homologue (Williams et al., 1995); three methionine residues (amino acids 1, 23 and 144) (Descombes and Schibler, 1991); and a consensus site for mitogen activated protein kinase (PGTP; positions 177–180) (Nakajima et al., 1993).

2.2. xC/EBPβ activates transcription by binding to its recognition sequence

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of proteins produced by in vitro transcription and translation showed that xC/EBPβ mRNA could also give rise to the three polypeptides expected from alternative use of translation initiation codons (data not shown). Additionally, electrophoretic mobility shift assays (EMSA) using in vitro translated protein demonstrated specific binding of the polypeptides to a consensus C/EBP-recognition sequence (data not shown). In order to examine the *trans*-activation potential of xC/EBPβ, co-transfection experiments were carried out in human hepatoma Hep3B cell line using increasing concentrations of xC/EBPβ-expression vector and a C/EBP-binding site-reporter gene construct. As shown in Fig. 1, xC/EBPβ increased the reporter gene activity in a dose-dependent manner, thereby indicating that it acts as a transcriptional activator.

2.3. xC/EBPβ is expressed in several adult tissues and throughout embryogenesis

The tissue distribution of xC/EBPβ mRNA was determined by semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) using total RNA from several adult *Xenopus* tissues and specific primers designed against the xC/EBPβ gene or the constitutively expressed β-actin gene (control for template levels) (Fig. 2A). This showed that xC/EBPβ mRNA was present in all the tissues analysed. However, the relative abundance of transcripts in the different tissues (i.e. xC/EBPβ:β-actin ratio) varied in the order: kidney > lung > muscle/liver > adipose tissue > ovary/brain/heart.

The temporal expression profile of xC/EBPβ during embryogenesis was determined by similar RT–PCR except that primers against the constitutively expressed ornithine decarboxylase gene (ODC) were used as a control for the amount of cDNA template (Fig. 2B). Analysis of the relative xC/EBPβ:ODC ratios from three independent experimental series showed that low levels of transcripts, presumably maternal in origin, were present at the first embryonic stage analysed (stage 2), and remained relatively constant until mid-gastrula (stage 11). This was followed by a steady accumulation of xC/EBPβ mRNA, which continued throughout neurulation (stages 14 and 17), to reach a peak at early tailbud (stage 23) (overall induction of about 6-fold). A drastic reduction of xC/EBPβ expression

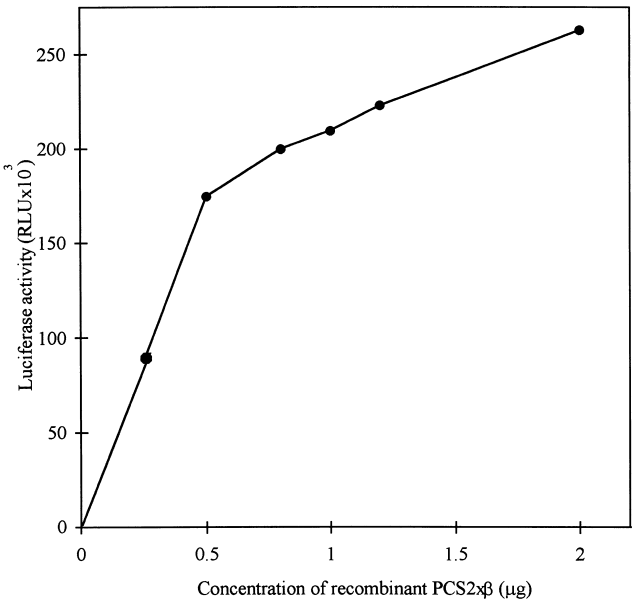

Fig. 1. Analysis of the ability of xC/EBPβ to activate gene transcription. Co-transfection experiments were performed in Hep3B cells as described in Section 3. The luciferase activity was normalised to the β-galactosidase activity, and the values are expressed as relative light units (RLU). Each value is the result of at least three independent experiments.

(approximately 25-fold) occurred at mid-tailbud (stage 26), and was followed by somewhat smaller increases during subsequent stages, with mRNA levels being approximately 1.5–2.5-fold higher than that present prior to gastrulation.

In order to examine whether the temporal expression of xC/EBPβ protein correlates with the mRNA expression profile, Western blot analysis was performed using total proteins from staged embryos and antisera against rat C/EBPβ. As shown in Fig. 2C, the antisera recognised two polypeptide species whose sizes were consistent with translation of the mRNA from the first two initiation codons (i.e. xLAP* and xLAP, respectively). Both polypeptides were present at about equal levels throughout embryogenesis except for stage 2 when only the xLAP* form was expressed, thereby indicating a potential involvement of translational mode of control at this stage. In longer autoradiographic exposures, a third species of about 20 kDa, derived from translation at the third initiation codon, could also be detected (data not shown). However, the signal was too weak to allow any firm conclusions on its expression profile during development. Overall, the changes in the temporal expression pattern of total C/EBPβ protein during embryogenesis followed closely the mRNA profile determined by RT–PCR analysis. Thus, the two peaks of mRNA expression at stages 23 and 36 were also apparent at the level of total C/EBPβ protein. This expression profile is, therefore, distinct from the *Xenopus* C/EBPα gene (Xu and Tata, 1992) which was present at low and relatively constant levels during early embryogenesis and induced drastically at the onset of metamorphosis.

2.4. Spatial expression profile of xC/EBPβ during embryonic development

The spatial distribution of xC/EBPβ mRNA was examined by whole-mount in situ hybridisation using digoxigenin-labelled antisense or sense (control) probes derived from the N-terminal region of the gene, which is diverged among the different C/EBP family members (Wedel and Ziegler-Heitbrock, 1995). Because of the lower overall expression of xC/EBPβ mRNA during early embryonic development (Fig. 2B), the precise definition of the spatial distribution of transcripts was only possible at mid-gastrula (stage 11) and subsequent stages (Fig. 3). Transcripts at mid-gastrula were localised predominantly around the blastopore lip and in the cells of the yolk plug (Fig. 3A). At subsequent stages, including neurula, expression of xC/EBPβ was apparent along the whole dorsal side of the embryo. For example, transcripts were present in the forebrain and along the whole dorsal side at early tailbud (stage 22), including somitic mesoderm and the nervous system (Fig. 3B). At later embryonic stages, the expression of xC/EBPβ becomes almost ubiquitous. Thus, expression of the gene at stage 26 (mid-tailbud) was apparent in the craniofacial mesenchyme, hindbrain and spinal cord (data not shown). At late tailbud (stage 30), transcripts were abundant in the spinal cord, optic vesicles, branchial arches and somites (Fig. 3C,D). Early

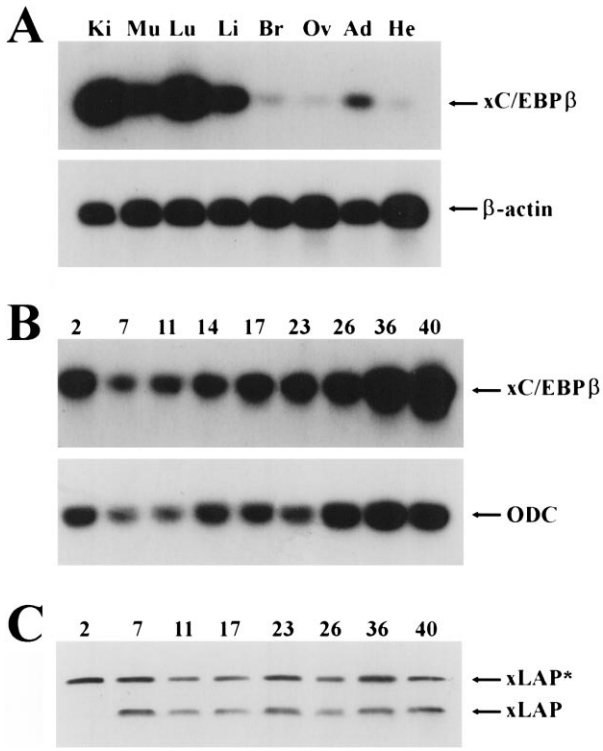

Fig. 2. Expression pattern of the xC/EBPβ gene. cDNA template was prepared against total RNA from either adult *Xenopus* kidney (Ki), muscle (Mu), lung (Lu), liver (Li), brain (Br), ovary (Ov), adipose tissue (Ad) and heart (He) (A) or from the following embryonic stages: 2 (cleavage), 7 (blastula), 11 (mid-gastrula), 14 (early neurula), 17 (mid-neurula), 23 (early tailbud), 26 (mid-tailbud), 36 (late tailbud) and 40 (early tadpole) (B). The cDNA was then used for PCR reactions in which primers against either the xC/EBPβ gene or the constitutively expressed β-actin or ODC gene (A and B, respectively) was present. The amplification products were subjected to Southern blot analysis, and their position is indicated by labelled arrows. (C). Western blot analysis was carried out on whole cell extracts from the embryonic stages indicated as described in Section 3. The blotted membranes were probed with rabbit antiserum to rat C/EBPβ (Poli et al., 1990; Ramji et al., 1993), and the antigen–antibody complexes were detected using the ECL detection system. The positions of the xLAP* and xLAP polypeptides, produced by alternate use of translation initiation codons, are indicated by labelled arrows. The description of changes in band intensity included in the text are based on the replicated outcome of laser densitometric quantification.

tadpoles (stage 38) maintain this expression profile and show additional strong expression of the gene underneath the eye and regions that give rise to the liver and the heart (Fig. 3E). In all cases, no signals were obtained using the sense probe (Fig. 3F–H), thereby confirming the specificity of the hybridisation signals.

In conclusion, we have isolated a genomic clone for the *Xenopus laevis* C/EBPβ gene, and determined its sequence organisation and both the tissue and developmental expression profile. The temporal and spatial expression profile of xC/EBPβ during embryogenesis suggests an important role for this gene during both early and late embryonic development (e.g. induction/patterning of mesodermal and neural tissues, organogenesis).

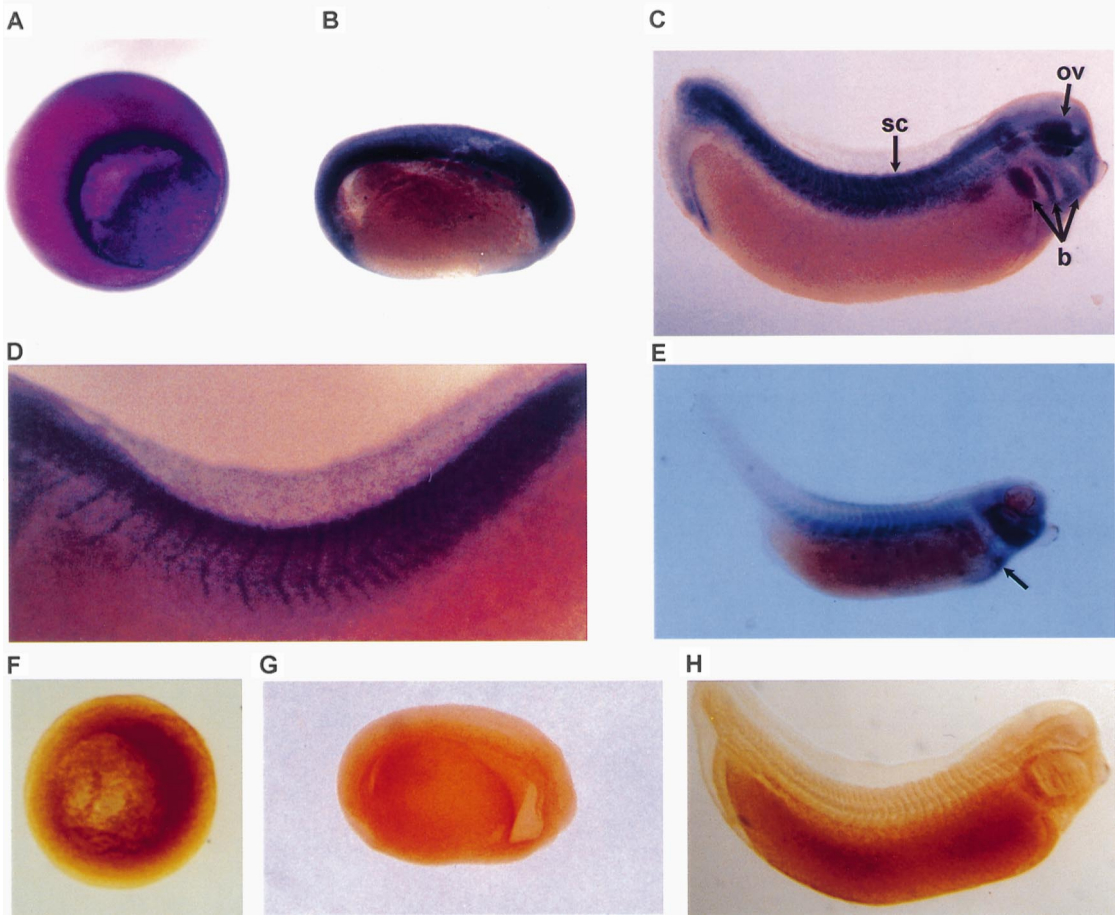

Fig. 3. The spatial pattern of xC/EBPβ expression during embryogenesis. Whole-mount in situ hybridisation to digoxigenin-labelled antisense (A–E) and sense probes (F–H), derived from the N-terminal region of the xC/EBPβ gene, was carried out as described in Section 3. (A) mid-gastrula embryo (stage 11) showing expression around the blastopore lip and in endodermal cells; (B) stage 22 embryo indicating expression in the fore brain and along the whole dorsal side; (C) stage 30 embryo indicating expression in the optic vesicle (ov), branchial arches (b), spinal cord (sc) and somites; (D) close view of a stage 30 embryo showing expression in the somites and the spinal cord; (E) stage 38 embryo, arrow indicates expression in the region that gives rise to the liver and heart; (F–H) representative control embryos probed with the sense probe, stages 11 (F), 22 (G) and 30 (I).

3. Experimental procedures

3.1. Gene cloning and sequencing

A 167 bp product was amplified using cDNA from *Xenopus* total lung RNA using primers designed against the most conserved region of previously cloned C/EBPβ genes (see Section 3.2. for primer sequences). The RT–PCR product was then used to both screen a *Xenopus laevis* genomic library (Stratagene, Cambridge, UK) and Southern blot analysis to identify restriction enzyme fragment(s) in the positive clone that contained the coding sequence. A 1.6 kb fragment, obtained from the digestion of the recombinant phage DNA with *Pst*I, was subcloned into pUC18 (Gacesa and Ramji, 1994), and the nucleotide sequence of both strands determined using the Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing kit with 7-deaza-dGTP (Amersham, Buckinghamshire, UK) and the LI-COR automated DNA sequencer (Hybaid, Ashford, Middlesex, UK). In some cases, manual sequencing was performed by the dideoxy chain-termination method using the Sequenase Version 2.0 sequencing kit (US Biochemical, Cleveland, OH, USA).

3.2. RT–PCR analysis

Xenopus embryos were obtained by standard techniques and staged as described previously (Nieuwkoop and Faber, 1967). Total RNA was purified from adult tissues and embryos using the guanidinium isothiocyanate and phenol–proteinase-K method, respectively (Chomczynski and Sacchi, 1987; Wilson et al., 1986). For RT–PCR, the cDNA template was produced as described previously (Kousteni et al., 1997) and subjected to PCR under conditions which were in the exponential phase of amplification (i.e. 50 μl total reaction volume containing 2.5 units *Taq* DNA polymerase and PCR buffer (Promega, Southampton, UK) with 0.2 mM dNTPs, 100 pmol of each primer, 1 mM $MgCl_2$, 5%

(v/v) DMSO and 0.5 μl to 10 μl of original cDNA template; 20 cycles (β-actin for tissue RNA or ODC for embryo RNA) or 30 cycles (C/EBPs) of denaturation at 94°C for 2 min, annealing for 2 min at 58°C and extension at 72°C for 2 min). The sequences of the primers were 5′-GCGCGAGCGCAACAACATCT-3′ and 5′-TGCTTGAACAGTTCCGCAG-3′ for C/EBPβ, 5′-TGGAGAAGAGCTATGAGCTGCCTG-3′ and 5′-GTGCCACCAGACAGCACTGTGTTG-3′ for β-actin, and 5′-AATGGATTTCAGAGACCA-3′ and 5′-CCAAGGCTAAAGTTGCAG-3′ for ODC. The PCR products were subjected to Southern blot analysis as described previously (Kousteni et al., 1997) and the specificity of amplification was confirmed by sequencing of representative products following subcloning into the pGEM-T vector (Gacesa and Ramji, 1994). In addition, parallel control samples in which reverse transcriptase was omitted were also included, and were negative in each case.

3.3. Western blot analysis

Whole cell extracts from staged embryos were prepared as described by Taylor et al. (1991), and the concentration of the proteins determined using the Micro BCA protein assay reagent kit (Pierce). Western blots of samples (40 μg) were probed with rabbit antisera against rat C/EBPβ (Poli et al., 1990; Ramji et al., 1993) as the primary antibody, and horseradish peroxidase-conjugated anti-rabbit immunoglobulin as the secondary antibody. The immunoreactive polypeptides were visualised by the ECL detection system following the procedures recommended by the supplier (Amersham, Buckinghamshire, UK).

3.4. EMSA and transient transfection assays

The 1.6 kb genomic fragment was subcloned into the pCS2+ vector (Turner and Weintraub, 1994) downstream of the T3 RNA polymerase and CMV promoters (designated as pCS2xβ). In vitro transcription of *Bam*HI-linearized pCS2xβ and EMSA of in vitro translated proteins was performed essentially as described previously (Poli et al., 1990). Transient transfection of human hepatoma cell line Hep3B was carried out using lipofectin, according to the manufacturer's instructions (Qiagen, Crawley, UK). The DNA constructs added to the cells were recombinant pCS2xβ (0.2 μg to 2 μg), E_4 APluc (1 μg; four copies of the C/EBP E4 site from the IgH enhancer linked to the luciferase reporter gene) (Cooper et al., 1995) and CMV-β-galactosidase (0.5 μg; to provide an internal control for transfection efficiency). After 16 h, the cells were washed with PBS, and left in fresh culture medium for 24 h. The luciferase and β-galactosidase activity in cell extracts were then determined using commercially available kits (Promega, Southampton, UK). The luciferase activity was normalised to the β-galactosidase value, and each transfection was repeated at least three times.

3.5. Whole-mount in situ hybridisation

Whole-mount in situ hybridisation was carried out as described previously (van der Wees et al., 1996; Kousteni et al., 1997). The probe was synthesised using T7 or T3 RNA polymerase (antisense and sense strand, respectively) in the presence of digoxigenin-UTP using recombinant pCS2+ template in which a genomic fragment, containing sequences coding for the N-terminus of xC/EBPβ, was cloned.

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