Structural and functional characterization of the mouse p8 gene: promotion of transcription by the CAAT-enhancer binding protein α (C/EBPα) and C/EBPβ *trans*-acting factors involves a C/EBP *cis*-acting element and other regions of the promoter

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Rat p8 mRNA was discovered because of its strong activation in pancreas during the acute phase of pancreatitis. We report here structural and functional data on the mouse p8 gene. The mouse p8 polypeptide is 80 amino acids long and shows 91% and 75% identity with its rat and human counterparts respectively. The p8 gene is organized into three exons interrupted by two introns. Promoter regions involved in the regulation of p8 gene expression in NIH 3T3 cells were analysed. Chloramphenicol acetyltransferase (CAT) reporter assays with progressive deletions of the 5′ flanking region of the mouse p8 gene revealed four silencer elements located from nucleotides −5000 to −1472, −1471 to −671, −670 to −473, and −239 to −117 respectively. One positive element was identified between nucleotides −117 and −72. We identified a CAAT-enhancer binding protein (C/EBP) *cis*-acting element at position −111. Site-directed mutagenesis of this consensus site decreased promoter activity to 5% of that of the wild-type. An electrophoretic mobility-shift assay, using an oligonucleotide probe corresponding to the C/EBP consensus and nuclear extracts of NIH 3T3 cells transfected with C/EBPα or C/EBPβ expression vectors, generated specific DNA–protein complexes that were supershifted with specific antibodies against C/EBPα and C/EBPβ. Co-transfection with C/EBPα or C/EBPβ expression vectors and the p−116/+36p8-CAT construct increased the reporter gene activity in a dose-dependent fashion. Surprisingly, overexpression of C/EBPα or C/EBPβ still increased the promoter activity of both pC/EBP*mut*−116/+36p8-CAT (which contains the C/EBP mutated site) and the p−71/+36-CAT construct (which does not contain the C/EBP site). Collectively, these results show that C/EBPα and C/EBPβ *trans*-acting factors can promote transcription of the mouse p8 gene (i) by direct binding to the C/EBP consensus site, and (ii) by enhancing the activity of other *trans*-acting factors interacting with the p8 promoter.

Key words: 3T3 cells, CAT activity, gene expression.

INTRODUCTION

The p8 mRNA was cloned from a rat pancreatic cDNA library based on its overexpression in pancreatic acinar cells during the acute phase of pancreatitis [1]. It encodes an 80 amino acid polypeptide. Using the presumed initiation site, the deduced p8 primary structure cannot be aligned with any of the protein sequences present in public databases. However, p8 contains a potential bipartite signal for nuclear targeting, suggesting a nuclear location. p8-forced expression promotes cellular growth [1,2], whereas treatment of cells with p8 antisense oligonucleotides strongly decreases [^{3}H]thymidine incorporation into DNA (A. Garcia-Montero, S. Vasseur, G. Vidal Mallo and J. Lucio Iovanna, unpublished work), indicating a cellular-growth-related function. Interestingly, p8 is not the only growth-promoting factor activated in pancreas after experimentally induced pancreatitis. Expression of several well-characterized growth factors [i.e. insulin-like growth factor (IGF)-I and IGF-II, transforming growth factor β, epidermal growth factor (EGF), platelet-derived growth factor and hepatocyte growth factor] and their receptors {IGF-I receptor, EGF receptor subtype 1 (HER1), EGF receptor subtype *neu* (HER2), EGF receptor subtype Erb-3 (HER3) and c-met [3–8]}, and of some proto-oncogenes, such as c-*myc*, H-*ras*, Ki-*ras*, c-*Jun* and c-*fos* [9–12], is strongly activated. p8 and these growth-promoting factors are probably induced to promote pancreatic regeneration. Supporting this hypothesis, like p8, most of these genes are also expressed in developing and regenerating pancreas, but strongly down-regulated in the adult healthy pancreas [1].

Activation of p8 mRNA expression is not restricted to the pancreas with acute pancreatitis. In fact, p8 mRNA is strongly activated in brain after transient ischaemic injury (G. V. Mallo and J. L. Iovanna, unpublished work), indicating that p8 mRNA overexpression may be part of an ubiquitous defence programme against cellular injury or cellular stress. To understand the molecular basis of p8 expression, we cloned and sequenced the gene encoding the mouse p8 and characterized its promoter region. Using transient transfection studies with different fragments of the 5′ region of the mouse p8 gene, together with co-transfection of expression vectors and gel mobility-shift assays,

Abbreviations used: C/EBP, CAAT-enhancer binding protein; CMV, cytomegalovirus; IGF, insulin-like growth factor; EGF, epidermal growth factor; BAC, bacterial artificial chromosome; CAT, cloramphenicol acetyltransferase; EMSA, electrophoretic mobility-shift assays; DTT, dithiothreitol.

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The nucleotide sequences reported in this paper appear in the GenBank database with the accession numbers AF131195 and AF131196.

we found that CAAT-enhancer binding protein α (C/EBPα) and C/EBPβ are involved in the regulation of p8 gene transcription in NIH 3T3 cells.

MATERIALS AND METHODS

Cloning of the mouse p8 mRNA

The sequence of the rat p8 cDNA was used to search for the mouse homologue in public DNA database using the BLASTN alignment program [13] and led to identification of an expressed sequence tag (GenBank accession number W42019). This clone (p8 mouse) was obtained from Genome Systems and completely sequenced by Genome Express (Grenoble, France).

Cloning of the mouse p8 gene

To clone the mouse p8 gene, we screened high-density filters containing genomic DNA fragments (from the mouse ES-129/SvJ strain) cloned into the bacterial artificial chromosome (BAC) vector (Genome Systems) using the p8 mouse cDNA insert as probe. The filters were hybridized with the corresponding ^{32}P-labelled probe for 16 h at 65 °C in 5 × SSPE (1 × SSPE is 180 mM NaCl/1 mM EDTA/10 mM NaH_2PO_4, pH 7.5), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02 % Ficoll 400/0.02 % poly(vinylpyrrolidone)/0.002 % BSA), 0.5 % SDS and 100 μg/ml single-stranded herring sperm DNA. The filters were then washed four times for 5 min at room temperature in 2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.2 % SDS, twice for 15 min at 50 °C in 0.2 × SSC/0.2 % SDS, and once for 30 min in 0.1 × SSC at 50 °C. Clones BACM-132(J12) and BACM-215(B18), which showed positive signals, were purchased from Genome Systems. DNA from these clones was purified and characterized by restriction-enzyme mapping. Several restriction fragments containing the p8 gene were ligated into the plasmid vector pBluescript and sequenced by Genome Express.

Chloramphenicol acetyltransferase (CAT) reporter gene constructs

All DNA constructs were generated by PCR using BACM-132(J12) as template. The accuracy of PCR was increased by using low dNTP concentration, 100 ng of DNA BAC as template and 30 cycles of DNA amplification [14]. Amplification was performed with the ExpandTM High Fidelity PCR System (Boehringer Mannheim) in a final volume of 50 μl containing 2 μM dNTP and 25 pmol of each primer. The reaction times were as follows: first cycle, denaturation at 94 °C for 2 min, annealing at 55 °C for 2 min and extension at 74 °C for 2 min; for the next 28 cycles, denaturation at 94 °C for 10 s, annealing at 55 °C for 2 min and extension at 72 °C for 2 min; for the last cycle, denaturation at 94 °C for 10 s, annealing at 55 °C for 2 min and extension at 72 °C for 10 min. Products were digested with *Hin*dIII and *Xba*I and subcloned into the promoterless vector pCAT-Basic (Promega) to generate plasmids p − 5000/ + 36p8-CAT, p − 1471/ + 36p8-CAT, p − 670/ + 36p8-CAT, p − 472/ + 36p8-CAT, p − 239/ + 36p8-CAT, p − 116/ + 36p8-CAT and p − 71/ + 36p8-CAT. Numbers in plasmid names refer to the position of first and last nucleotides of the insert in the p8 gene. A mutant plasmid was also constructed to monitor the function of a sequence in the p8 promoter corresponding to a putative C/EBP *cis* element. Plasmid pC/EBP*mut* − 117/ + 36p8-CAT, in which the C/EBP element (ATTGCATCAG) was changed to CGGTACGTCT, was generated using a PCR strategy as previously described [15]. The identity of the PCR-amplified DNA was confirmed by DNA sequencing (Genome Express), except for the p − 5000/ + 36p8-CAT construct which was only partially sequenced. Plasmid expressing C/EBPα (MSV/EBP-α) and C/EBPβ (MSV/EBP-β) (under the control of the mouse sarcoma virus promoter [16]) were kindly provided by Dr Steven L. McKnight (University of Texas Southwestern Medical Center, TX, U.S.A). In each experiment, cells were transfected with the promoterless vector pCAT-Basic as a negative control and with the pCMV/CAT construct as a positive control (CMV is cytomegalovirus). Plasmid DNA was purified with the Quiagen plasmid kit (Quiagen) and the DNA concentration was measured spectrophotometrically.

Cell transfection and CAT assays

NIH 3T3 cells (3×10^5 cells per plate) were plated in 50-mm diameter culture plates 24 h before transfection. For each plate, 1 μg of the reporter vector of interest and 1 μg of pCMV/βgal were added to 6 μl of Fugene, according to the instructions of the supplier (Boehringer Mannheim). The mixture was added to Dulbecco's modified Eagle's medium containing 3 ml of 10 % (w/v) FCS. This method of transfection was chosen for our studies because, in contrast to the calcium phosphate transfection method, it does not result in p8 gene transcription in these cells (results shown). Co-transfection experiments were carried out with 0.7 μg of reporter plasmids, 0.7 μg of pCMV/βgal and 0.5, 1.0 or 1.5 μg of MSV/EBP-α or MSV/EBP-β. The amount of DNA was increased to 3 μg with a DNA carrier. After 24 h in culture, cell extracts were prepared using the reporter lysis buffer (Promega). CAT activity was determined using the phase-extraction procedure [17] and the β-galactosidase assay was performed essentially as described previously [15]. CAT activity was normalized to β-galactosidase activity. CAT and β-galactosidase activities were always within the linear range of the assay.

Nuclear extract preparation and electrophoretic mobility-shift assays (EMSA)

Nuclei were purified from the untreated NIH 3T3 cells, or 48 h after Fugene-mediated transfection with the MSV/EBP-α or MSV/EBP-β expression vectors, as described by Dignam et al. [18]. Nuclear extracts were prepared as described by Lavery and Schibler [19] using a 1 × NUN solution (0.3 M NaCl/1 % Nonidet P-40/25 mM Hepes, pH 7.9) and 1 mM dithiothreitol (DTT). Protein concentration was determined using the Bradford protein assay [20] and ranged from 8–10 mg/ml. The DNA probe for EMSA was prepared by labelling the single-stranded oligonucleotides 5′GGGCCAGATTGCATCAGACAG3′ and 5′CTGTCTGATGCAATCTGGCCC3′ with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase before annealing. The gel-shift mixture (30 μl) contained 0.33 M urea, 0.1 M NaCl, 0.3 % Nonidet P-40, 25 mM Hepes (pH 7.9), 1 mM DTT, 10 % (v/v) glycerol, 5 μg of acetylated BSA, 3 μg of poly[d(I-C)] and 50 c.p.s. of ^{32}P-labelled oligonucleotide. This mixture was incubated at room temperature 30 min before electrophoresis on 5 % polyacrylamide gel/0.5 × TBE (1 × TBE is 45 mM Tris/borate/1 mM EDTA). The mixture containing the antibodies against C/EBPα and C/EBPβ (Santa Cruz) as indicated was incubated at 4 °C while shaking for 45 min, and then at room temperature for 30 min after adding the probe.

RESULTS

Cloning and primary structure of the mouse p8 mRNA

An expressed sequence tag was identified by homology screening with the rat p8 sequence using the BLASTN alignment algorithm [13]. A single open reading frame was found in the corresponding

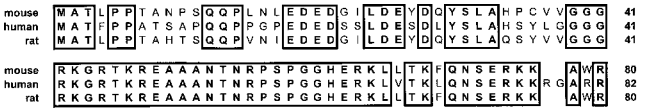

Figure 1 Sequence comparison of mouse, human and rat p8

Boxed areas correspond to amino acid identities.

mRNA sequence. The complete sequence comprised 626 nucleotides, exclusive of the poly(A) tail. A putative polyadenylation signal (AATAAA) was present 18 nt upstream of the poly(A) extension. The sequence reported here extends to 36 nt in the non-translated 5′ region. The p8 polypeptide is 80 amino acids long and shows an overall similarity of 91 % and 75 % to rat and human p8 respectively (Figure 1). To map the site of initiation of p8 transcription, a synthetic oligonucleotide (5′ATGTCGGACCGGGTAGGG3′) complementary to the mouse p8 mRNA was hybridized with total RNA from NIH 3T3 cells, and the heteroduplex was extended by reverse transcriptase. (Primer extension was performed and confirmed our presumed transcriptional initiation site.) The length of the major extended product was 141 nt, as determined by comparison with a sequence ladder in denaturing gel (results not shown). These results indicate that the p8 mouse clone contained the whole transcribed sequence.

Structural organization of the mouse p8 gene

Two genomic clones were obtained from a BAC library using the p8 mouse cDNA as probe. The clones appeared similar, based on identical mapping by *Eco*RI, *Hin*dIII, *Sac*I, *Bam*HI and *Pst*I digestion of their DNAs and on a Southern blot assay using the p8 mouse cDNA probe (results not shown). Based on mapping and sequencing, the p8 gene structure was established. This structure includes three exons interrupted by two introns, plus the 5′ and 3′ untranslated regions (Figure 2). The sizes of exons I, II and III are 148, 144 and 334 nt respectively. The DNA sequence of all the exons agreed perfectly with that of the p8 cDNA. All the exon–intron boundary sequences conformed to the GT/AG rule [21]. The transcribed portion of the gene was distributed over 2.2 kilobases of genomic DNA.

Computer scanning of the 5′-flanking region with the transcription factor database from TRANSFAC (http://agave.humgen.upenn.edu/utess/tess) disclosed several putative binding sites for transcription factors (Figure 2). These include the C/EBP-binding site, Sp1 sites, Oct-1, AP-1, NFκB, IL6-RE, CREB and Myc. A TATA box equivalent (TATAAG) is located 43 bases upstream from the first sequenced nucleotide in the mouse p8 cDNA. In addition, a putative Z-DNA-forming region $(GT)_{20}$ is present at position −443. This region is followed by $(CTGT)_6$. Z-DNA regions are not randomly distributed on the genome, but are frequently located close to sites of transcription initiation, as is the case for the mouse p8 gene.

Functional importance of a promoter element located in the region between nt −116 and −72

A significant signal was observed when p8 mRNA expression was analysed by Northern blot in NIH 3T3 cells (Figure 3), indicating that these cells are a good model in which to study p8 promoter activity. To delineate the sequences essential for transcription in the mouse p8 gene, we constructed a series of mouse p8 promoter–CAT chimaeric plasmids in which various lengths of the 5′-flanking region of the mouse p8 gene were fused to the coding region of the bacterial CAT gene. These constructs were introduced into mouse NIH 3T3 cells and the transient expression of CAT activity was measured 24 h later. The promoterless CAT plasmid (negative control) was inactive, whereas the pCMV/CAT used as a positive control generated significant CAT activity (Table 1). Construct p−5000/+36p8-CAT showed similar activity to pCMV/CAT. Deletions of the upstream sequences, from nt −5000 to −1472, increased the transcription activity 2-fold, suggesting the presence of a negative element. A similar effect was observed when the regions from −1471 to −671 and from −670 to −473 respectively were deleted, suggesting the presence of at least two additional silencer elements. No change in promoter activity was observed when nt from position −472 to −240 were deleted. However, another strong increase in the gene reporter activity was observed when the fragment from position −239 to −117 was excluded, suggesting the presence of another negative element. Finally, the CAT activity decreased dramatically (> 100-fold) when the region between nt −116 and −72 was deleted, suggesting the presence of an important positive element within this region. Clearly, this study demonstrates that basal activity of the p8 gene promoter is localized within its proximal 116 bp.

C/EBPα and C/EBPβ activate p8 gene transcription in NIH 3T3 cells

To determine the molecular basis of the activation of transcription of the mouse p8 gene, which involves the (−116 to −71) region, we identified the *cis*-acting element localized in that fragment and characterized the *trans*-acting factors that interact with this positive regulatory region. A putative C/EBP *cis*-acting element (ATTGCATCAG) was found in position −111 to −101. To examine the implication of this *cis*-acting element, we constructed the pC/EBP*mut*−116/+36p8-CAT chimaeric plasmid, in which the putative C/EBP-binding element was mutated as described in the Materials and methods section. The promoter activity obtained with the pC/EBP*mut*−116/+36p8-CAT is decreased by 95 % compared with the wild-type (p−116/+36p8-CAT), as shown in Table 2. These results suggested that this *cis*-element is responsible for a large part of p8 gene expression in NIH 3T3 cells and that the region between position −100 and −72 has only minor activity. The binding of C/EBPα and C/EBPβ to the C/EBP site was then established by EMSA with a synthetic double-stranded oligonucleotide probe and nuclear extracts from untreated NIH 3T3 cells and the MSV/EBP-α- and MSV/EBP-β-transfected cells. In EMSA, this probe produced three retarded bands, which are similar for the untransfected cells and for the MSV/EBP-β-transfected cells, but have a stronger signal in cells overexpressing C/EBPβ (Figure 4). These bands were displaced by a 50-fold molar excess of unlabelled self-competitor, but not by the same excess of the mutated double-stranded oligonucleotide (5′GGGCCAGCGGTACGTCTACAG3′), attesting to the binding specificity (the mutated nucleotides are underlined). With nuclear extract from the MSV/EBP-α-transfected cells, a larger complex was obtained, composed of at least two specific retarded bands, as shown by the self and mutated competitor. Although these results indicate that C/EBPα and C/EBPβ recognized the probe, the recognition was indirect, precluding the precise identification of the proteins. To address this issue, we used specific antisera raised against C/EBPα and C/EBPβ. The anti-C/EBPβ yielded the same two supershifted bands in untreated and MSV/EBP-β-transfected cells, with a larger upper band in the gel for the MSV/EBP-β-transfected cells. It also yielded a supershifted

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Figure 2 Nucleotide sequence of the mouse p8 gene, including 1786 nucleotides of the 5′-flanking sequence and 509 nucleotides of the 3′-flanking sequence

Exon sequences are given in capital letters. Introns are indicated by lowercase letters. Numbering begins at the first nucleotide found in the mouse p8 cDNA. Putative C/EBP, Sp1, Oct-1, AP-1, IL-6RE, NFκB, c-Myc and CREB sequences are underlined. A putative Z-DNA-forming sequence is boxed.

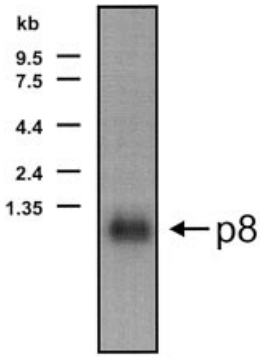

Figure 3 Expression of p8 mRNA in NIH 3T3 cells

Northern blot hybridization was carried out on RNA from NIH 3T3 cells using the mouse p8 cDNA insert as probe. The filter was exposed for 24 h at −80 °C. Molecular-mass markers in kb are shown on the left.

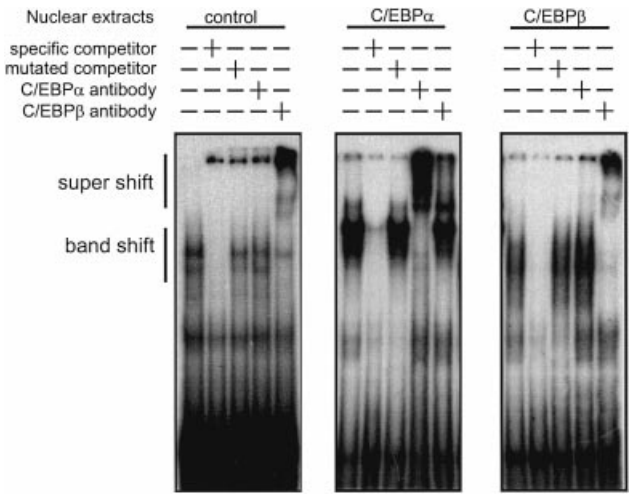

Figure 4 Ability of the C/EBPα and C/EBPβ *trans*-acting factors to bind to the C/EBP-binding site located at the proximal region of the p8 gene

Nuclei were isolated and nuclear extracts were prepared from control NIH 3T3 cells (left), C/EBPα-transfected cells (middle) and C/EBPβ-transfected cells (right). Gel-shift analysis was performed using 50 c.p.s. of ^{32}P-labelled double-stranded oligonucleotide, corresponding to the C/EBP-binding site from the p8 (5′GGGCCAGATTGCATCAGACAG3′) promoter, and 10 μg of nuclear proteins. DNA–protein complexes were separated on 5% polyacrylamide gel at 12 V/cm for 2.5 h after a pre-run at 5 V/cm for 1.5 h. Autoradiography was performed at −80 °C for 12 h. For competition assays, a 50-fold excess of unlabelled oligonucleotide, corresponding to the wild-type or mutated C/EBP-binding site (5′GGGCCAGCGGTACGTCTACAG3′) from the p8 promoter, was added to the preincubation mixture 15 min before adding the probe. Supershifting was performed by adding 4 μg of antibodies against C/EBPα or C/EBPβ to the preincubation mixture.

Table 1 Identification of regions responsible for promoter activity of the mouse p8 gene

NIH 3T3 cells were transiently transfected with each reporter plasmid containing progressive 5′ deletion of the promoter region, together with pCMV-βgal vector used as an internal control for the transfections. After transfection, the cells were incubated for 24 h and then harvested, lysed and assayed for both CAT and β-galactosidase activities. The pCMV/CAT and the promoterless CAT (pCAT-Basic) plasmids were used as positive and negative controls respectively. Results are represented as relative CAT activities obtained by dividing the normalized CAT activity by that with p−116/+36p8-CAT. Experiments were carried out in triplicate. The results are presented as means ± S.D.

CAT constructs	Relative CAT activity
p−5000/+36p8-CAT	8.92 ± 1.02
p−1471/+36p8-CAT	16.52 ± 2.03
p−670/+36p8-CAT	28.49 ± 3.00
p−472/+36p8-CAT	54.92 ± 9.06
p−239/+36p8-CAT	61.97 ± 8.36
p−116/+36p8-CAT	100.00 ± 7.59
p−71/+36p8-CAT	0.60 ± 0.30
pCMV/CAT	9.32 ± 2.03
pCAT-Basic	0 ± 0

Table 2 p8 promoter requires the C/EBP consensus site for maximal activity

Each reporter plasmid p−116/+36p8-CAT, pC/EBP*mut*−116/+36p8-CAT (containing the mutated C/EBP binding site) and p−71/+36p8-CAT was transfected into NIH 3T3 cells with the pCMV-βgal vector used as an internal control for the transfections. After transfection, the cells were incubated for 24 h and then cell extracts were prepared and assayed for both CAT and β-galactosidase activities. pCMV/CAT and pCAT-Basic vectors were used as positive and negative controls respectively. Results are represented as relative CAT activities obtained by dividing the normalized CAT activity by that with p−116/+36p8-CAT. Experiments were carried out in triplicate. The results are presented as means ± S.D.

CAT constructs	Relative CAT activity
p-116/+36p8-CAT	100.00 ± 7.59
pC/EBP*mut*-116/+36p8-CAT	5.02 ± 2.30
p-71/+36p8-CAT	0.60 ± 0.30
pCMV/CAT	11.32 ± 2.11
pCAT-Basic	0 ± 0

Table 3 Transactivation by C/EBPα of p8 promoter–reporter gene constructs containing or not containing the C/EBP-binding site

p−116/+36p8-CAT, pC/EBP*mut*−116/+36p8-CAT and p−72/+36p8-CAT (0.7 μg of each) as reporter vectors were transfected into NIH 3T3 cells with the indicated amount of effector plasmid expressing C/EBPα and with 0.7 μg of pCMV-βgal as an internal control for the transfections. pBlueScript plasmid was used as a DNA carrier, instead of effector plasmid, for the controls (0 μg of effector plasmid) and was used to make the total amount of plasmid constant (3 μg) for each transfection. The cells were harvested, lysed and assayed 24 h after transfection for both CAT and β-galactosidase acitivities. For each reporter construct, results are presented as relative CAT activities obtained by dividing the normalized CAT activity by the corresponding control. Experiments were carried out in triplicate. The results are presented as means ± S.D.

CAT constructs	MSV/EBP-α (μg)	Relative CAT activity
P−116/+36p8-CAT	0	100.00 ± 8.44
	0.5	317.00 ± 26.00
	1.0	662.00 ± 51.00
	1.5	1480.00 ± 126.00
pC/EBP*mut*-116/+36p8-CAT	0	5.20 ± 0.98
	0.5	174.00 ± 12.20
	1.0	283.00 ± 15.20
	1.5	451.00 ± 38.00
p−71/+36p8-CAT	0	0.50 ± 0.09
	0.5	17.00 ± 1.20
	1.0	46.00 ± 6.00
	1.5	119.00 ± 18.00

band with nuclear extract from MSV/EBP-α-transfected cells, similar to the anti-C/EBPα-mediated downshifted band, indicating that a heterodimer can bind to this region and be shifted

Table 4 Transactivation by C/EBPβ of p8 promoter–reporter gene constructs containing or not containing the C/EBP-binding site

p−116/+36p8-CAT, pC/EBP*mut*-116/+36p8-CAT and p−72/+36p8-CAT (0.7 μg of each) as reporter vectors were transfected into NIH 3T3 cells with the indicated amount of effector plasmid expressing C/EBPβ and with 0.7 μg of pCMV-βgal as an internal control for the transfections. pBlueScript plasmid was used as a DNA carrier, instead of effector plasmid, for the controls (0 μg of effector plasmid) and was used to make the total amount of plasmid constant (3 μg) for each transfection. The cells were harvested, lysed and assayed 24 h after transfection for both CAT and β-galactosidase acitivities. For each reporter construct, results are presented as relative CAT activities obtained by dividing the normalized CAT activity by the corresponding control. Experiments were carried out in triplicate. The results are presented as means ± S.D.

CAT constructs	MSV/EBP-β (μg)	Relative CAT activity
P−116/+36p8-CAT	0	100.00 ± 10.11
	0.5	153.00 ± 15.00
	1.0	191.00 ± 32.00
	1.5	205.00 ± 26.00
pC/EBP*mut*−116/+36p8-CAT	0	5.50 ± 0.79
	0.5	24.00 ± 7.00
	1.0	63.00 ± 11.00
	1.5	60.00 ± 8.00
p−71/+36p8-CAT	0	0.60 ± 0.10
	0.5	5.00 ± 1.10
	1.0	10.00 ± 3.00
	1.5	15.00 ± 2.60

by both antibodies. These results indicate that the C/EBPβ *trans*-acting factor, constitutively expressed or overexpressed in NIH 3T3 cells, is able to bind the p8 C/EBP consensus site. Anti-C/EBPα alone supershifted into three bands the complex obtained with nuclear extract from MSV/EBP-α-transfected cells. Therefore the C/EBPα *trans*-acting factor binds to the C/EBP consensus but is not contained in nuclear extract from untreated NIH 3T3 cells.

We also showed that C/EBPα and C/EBPβ were able to modulate transcription of the p8 gene by acting on the consensus C/EBP site. The functional interaction of C/EBPα and C/EBPβ with the p8 promoter was demonstrated by co-transfection experiments (Tables 3 and 4). Transfection of the wild-type p−116/+36p8-CAT construct together with different amounts of C/EBPα or C/EBPβ expression vectors (MSV/EBP-α and MSV/EBP-β) resulted in stimulation of the CAT activity. Co-transfection with 0.5, 1.0 and 1.5 μg of the C/EBPα expression vector increased the reporter activity about 3-, 6- and 14-fold respectively (Table 3). However, when the co-transfection experiments were performed using the C/EBPβ expression vector, increases were 50, 90 and 100% with 0.5, 1.0 and 1.5 μg respectively (Table 4).

The C/EBP *cis*-acting element is important, but not indispensable, for the C/EBPα and C/EBPβ *trans*-acting factors to activate p8 transcription in NIH 3T3 cells

It was interesting to test whether modulation of p8 transcription by C/EBPα and C/EBPβ was strictly dependent on the C/EBP *cis*-acting element. Surprisingly, when the pC/EBP*mut*-116/+36p8-CAT construct, containing the mutated C/EBP *cis*-acting element, was co-transfected with the C/EBPα expression vector, CAT activity was also increased in a dose-dependent manner. However, this effect was less than with the wild-type construct. Moreover, a small but significant effect was also observed by co-transfecting the C/EBPα expression vector with the p−71/+36p8-CAT construct. When the C/EBPβ expression vector was co-transfected with the pC/EBP*mut*−116/+36p8-CAT or the p−71/+36p8-CAT constructs, CAT reporter activity increased in a dose-dependent manner, although with a smaller amplitude than with the C/EBPα expression vector (see Tables 3 and 4). Taken together, these results strongly suggest that the C/EBP *cis*-acting element is important, but not indispensable for promotion of p8 gene transcription by the C/EBPα and C/EBPβ *trans*-acting factors. In addition, these results also suggest that C/EBPα is more active on the p8 promoter than is C/EBPβ.

DISCUSSION

As previously suggested by our group [1], p8 gene expression is regulated through a very complex system, since, whereas p8 mRNA is constitutively expressed in several tissues, in others it is only expressed in response to injury (i.e. pancreatitis in the pancreas, transient ischaemic episode in the brain and lipopolysaccarides in liver, kidney and pancreas) or during development. To understand the molecular basis of p8 gene expression we performed a detailed study of its promoter. It is well known that the transcriptional regulation of a particular gene is a complex process which usually involves interaction between multiple *cis*-acting regulatory elements and their cognate protein factors [22]. Our present results, based on dissection of the structural and functional organization of the 5′-flanking region of the mouse p8 gene, shed new light on the molecular mechanisms involved in the regulation of its expression. In this report, we show that the 5′-flanking sequence of the mouse p8 gene contains several distinct regulatory regions, including four with negative regulatory activity and a C/EBP *cis*-acting element located at position −111, which is critical for p8 positive expression.

Sequence analysis revealed that the mouse p8 promoter contains various putative regulatory elements found in inducible genes (Figure 2). Although the actual function of these typical inducible elements in the mouse p8 5′-flanking region remains to be determined, the identification of these candidate regulatory elements provides an insight into the complex pattern of regulation of p8 expression. Transient transfection experiments revealed that several regulatory elements can modulate the minimal promoter activity of the mouse p8 gene. Four negative and one positive *cis*-acting regulatory element are present, and deletion of these elements results in alteration of promoter activity. The negative elements are located within regions between nt −5000 and −1472, −1471 and −671, −670 and −473, and −239 and −117 respectively. Conversely, when the region between nt −116 to −72 (construct p−71/+36p8-CAT) was deleted, CAT activity decreased to ~1% of that of the construct p−116/+36p8-CAT, indicating the presence of a strong positive *cis*-regulatory element within that region. A putative C/EBP *cis*-acting element was identified at position −111 and then analysed.

The two major proteins of the C/EBP family, C/EBPα and C/EBPβ, interact with an ATTGCATCAG site. These C/EBP proteins, constitutively expressed and inducible, can form homodimers or heterodimers through a basic leucine zipper domain and exhibit similar DNA-binding specificity. The C/EBP proteins are transcriptional factors known to mediate the regulation of several acute-phase protein genes (reviewed in [23]). In addition, these factors are strongly involved in normal tissue development, in cellular proliferation and in functional differentiation (reviewed in [24]). Functional analysis was performed by site-directed mutagenesis of the C/EBP site of the p8 promoter, followed by transient transfection of the construct. Results indicate that this sequence is responsible for most of the promoter activity, since its mutation reduced the reporter activity to ~5%

of that of the wild-type. Binding of the C/EBPα and C/EBPβ *trans*-acting factors to this sequence was confirmed by gel-shift and supershift assays. Moreover, transactivation analysis with a C/EBPα expression vector and the wild-type construct (which contains the intact C/EBP-binding site) showed that C/EBPα is able to function as a strong positive modulator in a dose-dependent manner, suggesting that intracellular levels of C/EBPα may modulate p8 promoter activity. A lesser effect was obtained in experiments using the C/EBPβ expression vector. This can be explained by the fact that C/EBPα and C/EBPβ have specific transactivation capacities. C/EBPα is, for example, a stronger transcriptional activator than C/EBPβ on other promoters [25]. On the other hand, in NIH 3T3 cells, C/EBPβ is the major species that binds to the C/EBP consensus site (Figure 4). Hence, it is possible that increasing amounts of C/EBPβ have only a minimal effect on p8 transcription in these cells.

Surprisingly, mutations in the C/EBP consensus site were unable to abolish completely the induction of basal CAT activity with C/EBPα or C/EBPβ. This result suggests that the C/EBP *trans*-acting factors can activate p8 promoter activity without direct DNA–protein interaction. That hypothesis is supported by the fact that, in co-transfection experiments using the p$-$71/$+$36p8-CAT constructs, a dose-dependent increase of CAT activity is also observed. However, CAT expression values obtained in the presence of the C/EBP-binding site were at least 2–3-fold stronger than those in which that site was mutated or deleted, indicating that DNA–C/EBP *trans*-acting factors interaction seems to be required to promote thorough p8 gene expression. It has recently been suggested [26] that transcriptional regulation may occur through co-operation of *trans*-acting factors, with regulated formation of multiprotein complexes that eventually bind to enhancers and promoters. The complexity of these processes is considered to result in an elaborate fail-safe mechanism for controlling gene expression [26]. In the present study we found that C/EBPα and C/EBPβ activate p8 gene expression by interacting with other, unidentified *trans*-acting factors on the p8 promoter. Our results are in fact not completely surprisingly, since, for example, the DNA-binding domain of the C/EBP was not required for its effect on phosphoenolpyruvate carboxykinase gene transcription (reviewed in [27]). These observations suggest that interaction between C/EBP *trans*-acting factors and other factors is critical to the full effect of C/EBPα and C/EBPβ on p8 gene transcription. It is also known that the C/EBP *trans*-acting factors can modulate transcriptional activity of a target gene by interacting directly with transcription factors such as NF-κB, Fos/Jun family, TATA box-binding protein, Sp1, v-myb and p300/CBP (reviewed in [24]). Alternatively, the C/EBP *trans*-acting factors could bind directly to a less conserved C/EBP *cis*-acting DNA sequence. However, we observed that the region between nucleotides -100 and $+36$ (without the classical C/EBP *cis*-acting sequence, but still responding to the C/EBP *trans*-acting factors) does not contain any active sequence, as judged by EMSA (results not shown). Taken altogether, these results support the view that the C/EBP nuclear factors transactivate the p8 promoter both by direct binding and by interacting with other *trans*-acting factors. That second mechanism offers an additional regulatory step in which promoter-specific C/EBP–protein(s) interaction yet to be discovered create an additional regulatory network that contributes to modulating and governing the inducible expression of p8.

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