Transcriptional cooperation between NF- κ B p50 and CCAAT/enhancer binding protein β regulates *Nur77* transcription in Leydig cells

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Abstract

Expression of steroidogenic enzyme-encoding genes in testicular Leydig cells is complex and involves several transcription factors including the orphan nuclear receptor NUR77 (NR4A1) and the bZIP factor CCAAT/enhancer binding protein β (EBP β). How these transcription factors are integrated into a functional network, however, remains to be fully understood. Here, we report that the transcription factor C/EBP β can activate the *Nur77* promoter as revealed by transient transfections in MA-10 Leydig cells. Through 5' progressive deletions and site-directed mutagenesis, the C/EBP β -mediated activation of the *Nur77* promoter was found to be dependent on a novel species-conserved C/EBP element located at –110 bp. We also demonstrate using electromobility shift assay that C/EBP β specifically binds to this element. Furthermore, we report a functional cooperation between C/EBP β and the p50 subunit of NF- κ B that involves a previously uncharacterized κ B element located at –18 bp. Promoter analysis revealed that either the C/EBP or the κ B element was sufficient to sustain the C/EBP β -p50 cooperation thus suggesting that both factors physically interact. Altogether, our results provide new data regarding *Nur77* transcription in testicular Leydig cells in addition to providing new insights into the interplay between transcription factors involved in Leydig cell gene expression and function.

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Introduction

Testicular Leydig cells are the main androgen-producing cells of the mammalian testis. Androgens are essential for several critical processes such as virilization of the male fetus, initiation and maintenance of spermatogenesis, male sexual behavior, and male sex accessory gland development and function. In adults, the primary intra-testicular role of androgens (mainly testosterone) is to support sperm production. Leydig cell differentiation and function requires the well-orchestrated action of several hormones and signaling molecules produced by different endocrine and paracrine/autocrine cells (reviewed in Haider 2004). In response to these signals, expression of genes involved in testosterone biosynthesis is upregulated. Several transcription factors have been implicated in this process. These include steroidogenic factor 1 (SF1, NR5A1), GATA4, CCAAT/enhancer binding protein β (C/EBP β), sterol regulatory element binding protein (SREBF1), stimulatory protein 1 (SP1), cAMP response element binding protein (CREB1/CREM), members of the activating protein 1 (AP-1) family (c-FOS and c-JUN), dosage-sensitive sex reversal adrenal hypoplasia critical region on chromosome X gene 1 (DAX-1,

NR0B1; reviewed in Stocco *et al.* 2001, Manna *et al.* 2003 and Manna & Stocco 2005), distal-less homeobox 5 (DLX5; Nishida *et al.* 2008), and NUR77 (NR4A1, NGFI-B; Martin & Tremblay 2005, Martin *et al.* 2008). Several of these transcription factors have been shown to functionally cooperate on target genes. In addition, the temporal recruitment of transcription factors on target promoters, such as the steroidogenic acute regulatory protein (*Star*) promoter (Hiroi *et al.* 2004), is being elucidated thus providing a better understanding of the molecular events involved in translating a stimulus into a genomic response.

NUR77, also known as nerve growth factor induced-B (NGFI-B) and NR4A1, is a member of the NR4A family of orphan nuclear receptors, which also includes NURR1 (NR4A2) and NOR1 (NR4A3). These transcription factors are early response factors in that their expression/activity is usually rapidly induced by various stimuli in numerous tissues (reviewed in Eells *et al.* 2000). In testicular Leydig cells, NUR77 is the predominant NR4A member expressed and its expression was shown to be rapidly induced in response to LH/cAMP (Davis & Lau 1994, Song *et al.* 2001, Martin & Tremblay 2005, Martin *et al.* 2008). Despite the fact that NUR77 has been implicated in the

expression of several steroidogenic genes in Leydig cells, including rat *Cyp17a1* (Zhang & Mellon 1997), human *HSD3B2* (Martin & Tremblay 2005), mouse *Hsd3b1* (Hong *et al.* 2004), and mouse *Star* (Martin *et al.* 2008), surprisingly very little is known regarding the mechanisms regulating *Nur77* expression in these cells. So far, only members of the AP-1 and CREB1 families were shown to regulate *Nur77* promoter activity in Leydig cells in response to LH/cAMP (Inaoka *et al.* 2008).

The C/EBP family is composed of six members: C/EBPα, β, γ, δ, ε, and ζ (Ramji & Foka 2002). C/EBPβ is the predominant member present in Leydig cells and its expression is correlated with the differentiation status of these cells (Nalbant *et al.* 1998). C/EBPβ expression is upregulated in response to LH indicating a role for this factor in the hormonal regulation of Leydig cell gene expression (Nalbant *et al.* 1998). Consistent with this, C/EBPβ was found to activate *Star* transcription (Reinhart *et al.* 1999, Tremblay *et al.* 2002).

Because both NUR77 and C/EBPβ are expressed in Leydig cells and regulate steroidogenic gene transcription, we explored the possibility that C/EBPβ might regulate *Nur77* transcription in these cells. Here, we show that the *Nur77* promoter contains a functional C/EBP element at -110 bp that is specifically bound by C/EBPβ. We also found that C/EBPβ cooperates with the p50 subunit of NF-κB to further enhance *Nur77* promoter activity in Leydig cells. Thus, our results provide new insights into the mechanism of *Nur77* expression in Leydig cells.

Materials and methods

Plasmids

The rat -1013 bp *Nur77* (*Ngfi-B/Nr4a1*) promoter sequence was amplified by PCR from rat genomic DNA using primers described in Table 1. Deletions of the *Nur77* promoter to -747, -331, -276, -233, -121, and -65 bp were obtained by PCR (primers listed in Table 1) using the -1013 bp *Nur77* promoter as template. The -1013 bp *Nur77* reporter construct harboring a mutation inactivating the C/EBP element at -110 bp and the κB element at -18 bp were generated using the QuikChange XL mutagenesis kit (Stratagene, La Jolla, CA, USA) using the oligonucleotides reported in Table 1. All promoter constructs were cloned into a modified pXP1 luciferase reporter plasmid (Tremblay & Viger 1999) and verified by sequencing (Centre de Génomique de Québec, CHUQ Research Centre, Quebec City, Canada). Expression vector for C/EBPβ (Cao *et al.* 1991) was provided by Dr Steven McKnight (UT Southwestern Medical Center at Dallas, Dallas, TX, USA). The NF-κB p50 and p65 expression vectors (Liu *et al.* 2000) were provided by Dr Richard Pope (Northwestern University Feinberg School of Medicine, Chicago, IL, USA).

Cell culture and transfections

Mouse MA-10 Leydig cells (Ascoli 1981) were obtained from Dr Mario Ascoli (University of Iowa, Iowa City,

Table 1 Oligonucleotides^a used for generation of promoter constructs (deletions, mutagenesis) and for electromobility shift assays

<i>Nur77</i> promoter constructs (added cloning sites are italicized: <i>Kpn</i> I in reverse primer and <i>Bam</i> HI in forward primers)	
Common reverse primer	CTGGTACCGCGTGCCTCTGCAATCCTTC
Forward -1013 bp	CGGGATCCGCTACTACCTAGCTTAGTGACC
Forward -747 bp	CGGGATCCGCTAGTCAGCAGTGAAACTG
Forward -331 bp	CGGGATCCGCTCTGGAAGCTGCTATATTTAGCC
Forward -276 bp	CGGGATCCGATCAAACAATCCGCGCTC
Forward -233 bp	CGGGATCCGTCACGCGCGCAGACATTCCAG
Forward -121 bp	CGGGATCCTTGTATGGCCAAAGCTC
Forward -65 bp	CGGGATCCATGCGTCACGGAGCGCTTAAGAG
Mutagenesis C/EBPβ at -110 bp	Sense: CCCTTGTATGGCctAGCTCGCCGGGC
	Antisense: GCGCGAGCTaaGGCCATACAAGGG
	Sense: GGGTTCGCCAGGttAGCCCCAGTGCAG
	Antisense: CTGCACTGGGGCTaaCCTGGCGAACC
EMSA	
C/EBPβ element (bold) at -110 bp	Wild-type sense: CCCTTGT ATGGCCAAAGCT CGCCG
	Wild-type antisense: CGGCGAG CTTTGGCCATACAAGGG
	Mutated sense: CCCTTGT ATGGCct AGCTCGCCG
	Mutated antisense: CGGCGAG CTaaGGCCATACAAGGG
	Wild-type sense: TTCGCC AGGGGAGCCCCAGTGCAG
	Wild-type antisense: CTGCACT GGGGCTCCCC TGGCGAA
	Mutated sense: TTCGCC AGGttAGCCCCAGTGCAG
	Mutated antisense: CTGCACT GGGGCTaaCCT GGCGAA

^aSequences are from 5' to 3' ends.

IA, USA) and grown in Waymouth's MB752/1 medium supplemented with 1.2 g/l NaHCO₃, 15% horse serum, and 50 mg/l gentamicin and streptomycin sulfates, at 37 °C in 5% CO₂. MA-10 cells were transfected in 24-well plates by the calcium–phosphate coprecipitation method (Jordan *et al.* 1996). Briefly, MA-10 cells were plated at 120 000 cells/well prior to transfection. The next day, media was changed and cells were transfected with 0.5 μ g of *Nur77* promoter construct fused to the Firefly luciferase reporter gene, 0.5 μ g cytomegalovirus-driven expression vector (CMV-p50, CMV-p65, CMV-C/EBP β), 10 ng phRL-TK Renilla luciferase expression vector, used as an internal control for transfection efficiency, and pSP64 as carrier DNA up to 1.5 μ g/well. The same amount of DNA was used in all experiments (unless otherwise indicated in the figure legends). Two days later, MA-10 cells were harvested and luciferase activities measured using the Dual Luciferase Assay System (Promega Corp) and the EG&G Berthold LB 9507 luminometer (Berthold Technologies, Oak Ridge, TN, USA). Data reported represent the average of at least three experiments, each performed in duplicate using different DNA preparations.

Electromobility shift assays

Electromobility shift assays (EMSA) were performed using 10 μ g nuclear extracts from MA-10 Leydig cells along with ³²P-labeled double-stranded oligonucleotides (Table 1) corresponding to the C/EBP and κ B elements at –110 and –18 bp respectively. When overexpressed p50 or p65 were used, 20 μ g p50 and/or p65 expression vectors were transfected in MA-10 Leydig cells (1 $\times$ 10⁶ in 60 mm dish) using FuGENE HD (Roche Diagnostics) according to the manufacturer's recommendations. In the competition experiments, double-stranded oligonucleotides (Table 1) corresponding to wild-type and mutated versions of the C/EBP and κ B elements were used. For supershift/disruption experiments, 3 μ g commercially available anti-C/EBP β (C-19), anti-p50 (C-19), or anti-p65 (C-20) antiserum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were also added to the binding reaction.

Statistical analyses

Comparisons between two experimental groups were done using the one sample *t*-test. Comparisons between multiple groups for a given reporter (Figs 4 and 6) were done by one way ANOVA followed by the post-hoc Newman–Keuls multiple test. For all statistical analyses, *P* < 0.05 was considered significant. All statistical analyses were done using the SigmaStat software package (Systat Software Inc., San Jose, CA, USA).

Results

C/EBP β binds to and activates the *Nur77* promoter

Transient transfections in the MA-10 Leydig cell line was used to test whether C/EBP β could activate the rat *Nur77* promoter. As shown in Fig. 1A, an activation of up to 2.3-fold of the *Nur77* promoter by C/EBP β was observed. To locate the C/EBP β responsive element, a series of 5' deletion constructs of the *Nur77* promoter were generated and transfected in MA-10 Leydig cells. Deletion from –1013 bp to –121 bp had no significant effect on the C/EBP β -dependent activation

Figure 1 C/EBP β activates the rat *Nur77* promoter. (A) MA-10 Leydig cells were co-transfected with 500 ng of a –1013 to +45 bp rat *Nur77* promoter construct along with either an empty expression vector or increasing amounts (25, 50, 125, 250, and 500 ng) of an expression vector for C/EBP β . (B) Localization of the C/EBP β responsive element in the *Nur77* promoter. MA-10 Leydig cells were co-transfected with 500 ng of various 5' deletion constructs of the rat *Nur77* promoter (the 5'-end point of each construct is indicated on the left of the graph) along with 500 ng of either an empty expression vector or an expression vector for C/EBP β . Results are shown as fold activation $\pm$ s.e.m. over control (no C/EBP β) which was arbitrarily set to 1 (dotted line). An asterisk (*) indicates a statistically significant difference from control.

(Fig. 1B). Further deletion to -65 bp, however, completely abrogated activation by C/EBP β thus indicating the presence of a C/EBP β -responsive motif between -121 and -65 bp (Fig. 1B). As shown in Fig. 2A, analysis of the $-121/-65$ bp sequence revealed the presence at -110 bp of a motif (ATGGC-CAAAG) that resembles the general consensus binding site for C/EBP members ((G/A)T(T/G)NNGNAA(G/T); Akira *et al.* 1990).

To assess whether this sequence is a bona fide C/EBP β binding element, EMSA were performed using nuclear extracts from unstimulated MA-10 Leydig cells. As shown in Fig. 2B, a single specific binding complex, which was not affected by cAMP treatment (data not shown), was observed. This binding was specifically competed by increasing doses of unlabeled wild-type oligonucleotides (Fig. 2B, lanes 3 and 4) but not by oligonucleotides harboring a mutation in the C/EBP element (Fig. 2B, lanes 5 and 6). Furthermore, this complex was super shifted by the addition of an anti-C/EBP β antiserum (Fig. 2B, lane 8). Thus, endogenous C/EBP β present in Leydig cells can specifically bind to the -110 bp C/EBP element.

The importance of the -110 bp C/EBP motif for the C/EBP β -dependent activation of the Nur77 promoter was next assessed by mutagenesis in the context of the -1013 bp promoter. As shown in Fig. 2C, mutation in the -110 bp C/EBP motif that disrupted C/EBP β binding (Fig. 2B) resulted in a 30% decrease in basal promoter activity. This mutated reporter was also unresponsive to C/EBP β indicating that the -110 bp element is a novel C/EBP β -responsive motif in the Nur77 promoter.

C/EBP β cooperates with the p50 subunit of NF- κ B on the Nur77 promoter

Because the Nur77 promoter was previously shown to contain a κ B element at -427 bp known to bind the NF- κ B transcription factor (Pei *et al.* 2005) and because NF- κ B and C/EBP β can cooperate in other systems (Sakitani *et al.* 1998, Montaner *et al.* 1999), we tested whether NF- κ B could activate and/or cooperate with C/EBP β on the Nur77 promoter in Leydig cells. Expression vectors encoding C/EBP β and each of the NF- κ B subunits (p50 and p65) were transiently transfected in MA-10 Leydig cells along with a -1013 bp rat Nur77 reporter. As shown in Fig. 3, NF- κ B (p50 and p65) had no significant effect on Nur77 promoter activity. However, a cooperation of about 7–8 fold was observed when the p50 subunit of NF- κ B was combined with C/EBP β (Fig. 3). No cooperation was observed between C/EBP β and p65. Furthermore, the C/EBP β -p50 cooperation was abrogated in the presence of p65. Thus, C/EBP β and p50 transcriptionally cooperate on the Nur77 promoter in Leydig cells.

Figure 2 The C/EBP element at -110 bp is sufficient to confer C/EBP β responsiveness to the Nur77 promoter. (A) The sequence of the potential C/EBP element found at -110 bp in the Nur77 promoter is compared with the general consensus C/EBP binding site (Akira *et al.* 1990) where R=G or A, K=G or T, and N=A, C, G, or T. The mutated sequence (underlined) of the -110 bp C/EBP is also shown. (B) C/EBP β specifically binds to the -110 bp element. EMSA was used to determine the binding of the C/EBP β protein present in MA-10 Leydig cells. Binding was challenged by increasing doses (black triangles; molar excesses of $5\times$ and $25\times$) of unlabeled oligonucleotides corresponding to the wild-type -110 bp element (WT) and the -110 bp element mutated from ATGGCCAAAG to ATGGCCTTAG (Mut). The C/EBP β binding was super shifted by a C/EBP β antiserum (α C/EBP β). IgG: normal rabbit IgG. n.s.: non-specific. (C) MA-10 Leydig cells were co-transfected with 500 ng of either an empty expression vector (open bars) or an expression vector for C/EBP β (solid bars) along with 500 ng of either a wild-type -1013 bp to $+45$ bp Nur77 reporter or a reporter harboring a mutation (ATGGCCAAAG to ATGGCCTTAG) in the C/EBP element at -110 bp (represented by the black circle). The mutated element is indicated by a large X. Results are shown as % activity ($\pm$ s.e.m.) relative to the activity of the -1013 bp wild-type reporter in the absence of C/EBP β which was arbitrarily set to 100%. Different letters indicate a statistically significant difference.

Figure 3 C/EBP β and the p50 subunit of NF- κ B cooperate on the Nur77 promoter. MA-10 Leydig cells were co-transfected with 500 ng of a -1013 to +45 bp rat Nur77 promoter construct along with 500 ng of either an empty expression vector or expression vectors for C/EBP β and the two subunits of NF- κ B (p50 and p65) either alone or in combination as indicated (500 ng for each expression vector). Results are shown as fold activation $\pm$ S.E.M. over control (no transcription factor) that was arbitrarily set to 1 (dotted line). An asterisk (*) indicates a statistically significant difference from control.

Localization of the C/EBP β -p50 responsive elements

To locate the C/EBP β -p50 responsive element(s), 5' progressive deletion constructs of the rat Nur77 promoter were tested for C/EBP β -p50 responsiveness in MA-10 Leydig cells (Fig. 4). Deletion from -1013 to -331 bp that removes the previously characterized κ B element at -427 bp did not affect the C/EBP β -p50 cooperation. Further deletion to -65 bp that no longer contains the C/EBP element at -110 bp, abrogated the C/EBP β -dependent activation but the cooperation with p50 was retained (Fig. 4). Sequence analysis of the -65 bp fragment revealed the presence of another κ B element at -18 bp (GGGGAGCCCC) which might be sufficient to confer C/EBP β -p50 responsiveness (Fig. 5A). As shown by EMSA using nuclear extracts from MA-10 Leydig cells, a binding complex was observed (Fig. 5B, lane 2) that could be competed by wild-type (Fig. 5B, lanes 3 and 4) but not mutated (Fig. 5B, lanes 5 and 6) oligonucleotides. This complex was lost when an anti-p50 (Fig. 5B, lane 8) or anti-p65 (Fig. 5B, lane 9) antiserum was added to the binding reaction indicating that it is composed of both p50 and p65 subunits. This is further supported by the fact that

Figure 4 Mapping of the C/EBP β -p50 responsive element in the Nur77 promoter. MA-10 Leydig cells were co-transfected with various 5' deletion constructs of the rat Nur77 promoter (the 5' end point of each construct is indicated on the left of the graph) and either an empty expression vector (open bars) or expression vectors for NF- κ B p50 (grey bars), C/EBP β (hatched bars), or both (solid bars). The previously characterized κ B element (Pei *et al.* 2005) and the C/EBP element identified in this study are indicated by a grey diamond and a black circle respectively. Results are shown as fold activation over control ($\pm$ S.E.M.). Different letters within a given promoter construct indicate a statistically significant difference.

binding was increased with nuclear extracts from MA-10 cells transfected with expression vectors for both p50 and p65 (Fig. 5B, lane 12). The requirement of the C/EBP and κ B elements for the C/EBP β -p50 responsiveness was next analyzed by introducing mutations in each element in the context of the -1013 bp Nur77 reporter. As shown in Fig. 6, mutation of either the C/EBP (at -110 bp) or the κ B (at -18 bp) element individually did not affect the transcriptional cooperation between C/EBP β and p50. When both elements were mutated, however, the C/EBP β -p50 cooperation was abrogated despite the presence of an intact κ B motif at -427 bp. Altogether, these data indicate that the C/EBP β -p50 cooperation does not involve the κ B motif at -427 bp but rather relies on only one intact element for either C/EBP (-110 bp) or κ B (-18 bp) in the proximal Nur77 promoter.

Discussion

Although, the Nur77 promoter has been isolated from several species including mouse, rat, and human, so far, only a handful of transcription factors have been reported to regulate its activity (Eells *et al.* 2000). In testicular

Figure 5 Identification of a novel κB element in the Nur77 promoter. (A) The sequences of the two κB elements in the Nur77 promoter are compared with consensus sequences for the binding of NF-κB p65-p50 heterodimers and p50-p50 homodimers (Kunsch *et al.* 1992). The mutated sequence (underlined) of the -18 bp κB element is also shown. (B) NF-κB binds to the -18 bp element. EMSA was used to assess binding of the NF-κB subunits endogenously present in MA-10 Leydig cells or overexpressed p50, p65, and p50 + p65. Binding was challenged by increasing doses (black triangles; molar excesses of 5× and 25×) of unlabeled oligonucleotides corresponding to the wild-type -18 bp element (WT) and the -18 bp element mutated from GGGGAGCCCC to GGTTAGCCCC (Mut). IgG, normal goat IgG; n.s., non-specific.

Leydig cells more specifically, only two transcription factors have recently been shown to contribute to Nur77 transcription: AP-1 and CREB1 (Inaoka *et al.* 2008). In the present study, we found that C/EBPβ and the p50 subunit of NF-κB represent new regulators of Nur77 promoter activity in Leydig cells.

The C/EBPβ-dependent activation of the Nur77 promoter required a novel C/EBP element located in the proximal promoter region (-110 bp). By EMSA using nuclear extracts from MA-10 Leydig cells, we showed that this element is specifically bound by C/EBPβ. Consistent with the fact that C/EBPβ is the main C/EBP family member found in Leydig cells (Nalbant *et al.* 1998), an anti-C/EBPβ antiserum almost completely super shifted the binding complex in the EMSA experiments. As shown in Fig. 7, this C/EBP element at -110 bp has been evolutionarily conserved

Figure 6 The κB at -18 bp or the C/EBP element at -110 bp is sufficient for the cooperation between p50 and C/EBPβ on the Nur77 promoter. MA-10 Leydig cells were co-transfected with either an empty expression vector (open bars) or expression vectors for NF-κB p50 (grey bars), C/EBPβ (hatched bars), or both (solid bars) along with either a wild-type -1013 bp to +45 bp Nur77 reporter or reporters harboring point mutations in the C/EBP element at -110 bp (ATGGCCAAAG to ATGGCC7TAG), in the κB element at -18 bp (GGGGAGCCCC to GGTTAGCCCC), or in both elements. The κB (grey diamonds) and C/EBP (black circle) elements are indicated. The mutated elements are represented by a large X. Results are shown as fold activation over control (± s.e.m.). Different letters within a given promoter construct indicate a statistically significant difference.

since it is present in the Nur77 promoter from various species including rat, mouse, human, chimpanzee, monkey, cat, and dog thus suggesting an important regulatory function.

In addition to activating Nur77 transcription on its own, we also found that C/EBPβ could functionally cooperate with the p50 subunit of NF-κB to further stimulate the Nur77 promoter despite the fact that p50 on its own is a very weak activator of the Nur77 promoter. In fact, the p50-dependent activation was barely statistically significant on certain Nur77 reporters and not on others (Figs 4 and 6). The cooperation with C/EBPβ was only observed with the p50 subunit since p65, which was unable to activate the Nur77 promoter on its own, could not cooperate with C/EBPβ. Not only did the p65 NF-κB subunit fail to cooperate with C/EBPβ but in the presence of p65, the C/EBPβ-p50 cooperation was lost. A similar p65-dependent repression of a p50-C/EBPβ cooperation has been reported on the C-reactive protein promoter (Agrawal *et al.* 2001). A possible explanation could be that p65 may already be in sufficient levels in MA-10 Leydig cells to associate with exogenously expressed p50 and C/EBPβ to activate the Nur77 promoter. Overexpression of p65 might cause titration of other transcription factors or coactivators that are important for Nur77 promoter activity in MA-10 cells. Supporting this is the fact that p65 has been reported to repress transcription of several genes by a mechanism involving competition for

	C/EBP –110 bp	κB –18 bp
Rat	TTGTATGGCCAAAGCTCG	GCCAGGGGAGCCCCAGT
Mouse	TTGTATGGCCAAAGCTCG	GCCAGGGGAGCCCCAGT
Cat	TTGTATGGCCATAGCTCG	GGCCGGGGAGCCCCAGT
Dog	TTGTATGGCCACAGCTCG	CGCCGGGGAGCCCCAGC
Monkey	TTGTATGGCCAAAGCTCG	GCCCAGGGAGTCCCAGT
Chimpanzee	TTGTATGGCCAAAGCTCG	GCCCAGGGAGTCCCAGT
Human	TTGTATGGCCAAAGCTCG	GGCCAGGGAGTCCCAGT
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Figure 7 The C/EBP element at –110 bp and the κB element at –18 bp are conserved across species. Sequence alignment of the C/EBP element at –110 bp and the κB at –18 bp in the *Nur77* promoter from rat, mouse, cat, dog, monkey, chimpanzee, and human. Conserved residues are indicated by asterisks.

a limited pool of coactivators (Caldenhoven *et al.* 1995, Ikeda *et al.* 2000).

A detailed promoter analysis of the *Nur77* promoter revealed that the C/EBPβ-p50 cooperation did not require a previously identified κB element at –427 bp found to be active in macrophages (Pei *et al.* 2005) but rather involved a novel species-conserved κB element at –18 bp (Fig. 7). Although, the sequence of this element (GGGGAGCCCC) is more closely related to the consensus binding site for NF-κB p50–p50 homodimer (GGGGATYCCC) than to the binding site for NF-κB p65–p50 heterodimer (GGGRNWTTC; Kunsch *et al.* 1992), we found that p50–p65 heterodimers preferentially bind to the –18 bp motif. Consequently, it remains possible that the C/EBPβ-p50 cooperation might involve dimerization between overexpressed p50 and endogenous p65. The C/EBPβ-p50 cooperation on the *Nur77* promoter was found to require only one intact element for either C/EBPβ (at –110 bp) or p50 (κB at –18 bp); the cooperation was abolished only when both elements were mutated. This strongly suggests that the C/EBPβ-p50 cooperation is the result of a direct interaction between the two factors in Leydig cells. Supporting this hypothesis is the important body of literature reporting direct protein–protein interaction between C/EBPβ and every subunit of NF-κB in various contexts (LeClair *et al.* 1992, Stein *et al.* 1993, Ruocco *et al.* 1996, Xia *et al.* 1997, Montaner *et al.* 1999, Agrawal *et al.* 2001). In terms of absolute numbers, however, the C/EBPβ-p50 cooperation was weaker (about 2.5-fold) on the –65 bp reporter (Fig. 4) that no longer contains the C/EBP element at –110 bp than on the –1013 bp construct with a mutated C/EBP element at –110 bp (about 6-fold, Fig. 6) despite the fact that both constructs have an intact –18 bp κB element. A likely cause for this difference in the intensity of the cooperation is the length of the promoter fragment (–1013 vs –65 bp). There are several possibilities that are not mutually exclusive that can explain this difference. For instance, it is possible that C/EBP-like element(s) further upstream might be utilized when the proximal C/EBP element at

–110 bp is mutated. It is also likely that other transcription factors binding upstream of –65 bp might contribute to the C/EBPβ-p50 cooperation. Some of these could include the AP-1 and CREB1 transcription factors that are known to regulate *Nur77* promoter activity in Leydig cells (Inaoka *et al.* 2008). In addition, both were shown to interact and transcriptionally cooperate with C/EBPβ in other systems (Zagariya *et al.* 1998, Flammer *et al.* 2006). These represent interesting research avenues to further our understanding on the mechanisms of *Nur77* transcription in testicular Leydig cells.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice their impartiality.

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