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Identification of nucleolar and coiled-body phosphoprotein 1 (NOLC1) minimal promoter regulated by NF-κB and CREB

Xuesong Gao[1], Qi Wang[2], Wei Li[2], Biao Yang[2], Hao Song[2], Wei Ju[2], Shunai Liu[2] & Jun Cheng[1,2,]*

[1]Beijing Ditan Hospital, Peking University Health Science Center, Beijing 100083, [2]Beijing Ditan Hospital, Capital Medical University, Beijing 100015, China

Nucleolar and coiled-body phosphoprotein 1 (NOLC1) is a phosphoprotein that transiently associates with the mature nucleolar H/ACA and C/D box small nucleolar ribonucleoproteins (snoRNPs). Several lines of evidence indicate that NOLC1 plays an important role in the synthesis of rRNA and the biosynthesis of ribosomes. In the present study, we examined the transcriptional regulation mechanisms that govern the expression of NOLC1. We first performed functional dissection of the NOLC1 promoter. We demonstrated that transcription factors NF-κB and CREB could bind to the minimal NOLC1 promoter. This was demonstrated by electrophoretic mobility shift assays and chromatin immunoprecipitation. Mutagenesis and overexpression assays revealed that NF-κB and CREB positively regulated the NOLC1 promoter. These findings may provide new insight into the mechanisms that regulate NOLC1 expression. [BMB reports 2011; 44(1): 70-75]

INTRODUCTION

The nucleolar and coiled-body phosphoprotein 1 (NOLC1, also called Nopp140) family of proteins is characterized by a conserved C-terminal SRP40 domain. NOLC1 is a phosphoprotein composed of N- and C-terminal domains and a unique central repeated domain consisting of alternating acidic and basic amino acid clusters and localized in the nucleolus (1). The greatest steady state concentrations of vertebrate NOLC1 localize within the dense fibrillar component (DFC) of nucleoli (2).

NOLC1 was first identified as a nuclear localization signal-binding protein and is thought to be a chaperone for shuttling between the nucleolus and cytoplasm (2, 3). NOLC1 plays an essential role in the synthesis of rRNA and the biosynthesis of ribosomes. NOLC1 is not an integral part of small nucleolar ribonucleoproteins (snoRNPs) but interacts with box C/D and box H/ACA snoRNPs, guiding site-specific 2'-O-methylation and pseudouridylation of pre-rRNAs, respectively (4-6). Previous studies have shown that rDNA transcription and pre-rRNA processing are sequential and activated by newly synthesized rRNA. They coordinate and depend upon the interaction of upstream binding factor (UBF) and snoRNPs. The complexes, composed of NOLC1, chromatin and UBF, can collect nucleolar proteins to engage in activities during early ribosome biogenesis. The rRNA intermediate products are then transferred from the NOLC1-containing islets to the surrounding granular component compartment (GC) for further maturation (7, 8). NOLC1 rapidly shuttles between the nucleus and the cytoplasm, perhaps to facilitate the import of nucleolar ribosome assembly factors or the export of nucleolar products. Knockdown of NOLC1 blocks pre-rRNA synthesis (9), and NOLC1 may also play a role in the maintenance of nucleolar integrity. When an exogenous NOLC1 N-terminus is expressed, cell nucleoli structures become altered, mislocalize the endogenous Pol I and shut off cellular rRNA gene transcription. When NOLC1 is overexpressed, cells demonstrate an enlarged nucleolus (5).

The PKA-phosphorylated NOLC1s also have a transcription factor-like activity that activates the α-1 acid glycoprotein gene (AGP) by interacting with C/EBP-β (10, 11). Recently, NOLC1 has been demonstrated to participate in the regulation of nasopharyngeal carcinoma progression by binding to the promoter of the TP53 region and working synergistically with TP53 to regulate MDM2 expression (12). This result indicates that NOLC1 plays a role as an oncogene in tumorigenesis.

Given that NOLC1's function is complex, it is likely that control of NOLC1 occurs at multiple levels. However, the mechanisms regulating NOLC1 expression remain incompletely characterized. In this report, we cloned the sequentially truncated NOLC1 promoter and demonstrated that the region between −110 and +38 of the NOLC1 promoter is essential for its basal transcription in HepG2 cells. Importantly, we observed a critical role for the transcription factors NF-κB and cAMP response element-binding protein (CREB) in the regulation of NOLC1. These findings provide new insight into the regulation of NOLC1 expression.

*Corresponding author. Tel: 86-10-84322006; Fax: 86-10-84397196; E-mail: jun.cheng.ditan@gmail.com
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RESULTS

The highest expression of NOLC1 mRNA in HepG2 cells

To investigate differences in the expression of NOLC1 in different cell lines, we performed real-time PCR assay for NOLC1 gene expression in hepatic cell lines: HepG2, Huh-7, QGY-7701, SMMC-7721, BEL-7402 and two non-hepatic cell lines: HeLa and CaP. Expression of the NOLC1 gene was highest in the HepG2 cells (Fig. 1). Therefore, we elected to use the HepG2 cell line for further study.

Cloning and analysis of the NOLC1 promoter

We characterized the 5'-flanking region upstream of the NOLC1 gene using the NCBI database. There are no typical TATA boxes, but a high GC content and a number of transcription factor binding sites are presumed to exist. To identify and analyze the NOLC1 promoter, a 1,882-bp genomic DNA fragment was amplified from genomic DNA isolated from HepG2 cells. The putative NOLC1 promoter region was cloned upstream of the firefly luciferase gene into the reporter plasmid pGL4.10 Basic; this construct was named P1 and served as the template for further shortened NOLC1 promoter constructs. Luciferase assays were performed 24 hours after the transfection of HepG2 cells. Compared to pGL4.10 Basic, the luciferase activity of P1 increased significantly (about 100-fold), suggesting that the NOLC1 promoter was active in HepG2 cells. Next, our aim was to determine which regions of the promoter were important for transcriptional activity. To accomplish this task, we generated five additional constructs containing sequentially truncated promoter fragments (Fig. 2A). As shown in Fig. 2B, there was an increase in activity when the 5' end was shortened from P1 to P2, suggesting that the regulatory elements located from −1,844 to −996 might be acting as potential silencers. However, there was a loss of promoter activity with P6. Serial deletion analysis therefore suggested that P5, including a 148-bp fragment of the promoter (−110 to 38), contained the requisite sequences important for transcriptional activity.

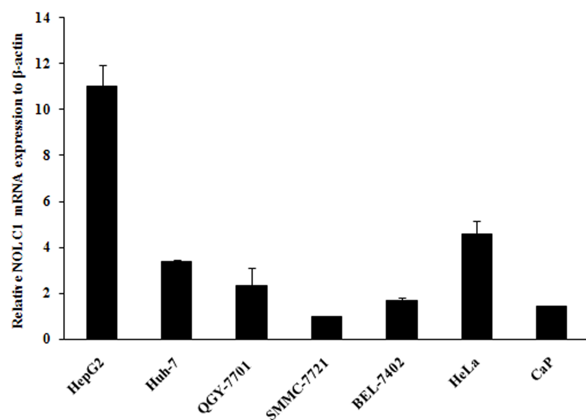

Fig. 1. Real-time PCR analysis of NOLC1 mRNA expression. NOLC1 mRNA expression was different in HepG2, Huh-7, QGY-7701, SMMC-7721, BEL-7402, HeLa and Cap cell lines. The highest level of NOLC1 gene expression was detected in HepG2 cells. β-actin was used as a control.

Identification of NF-κB and CREB binding sites

We searched Promoter Scan (http://www-bimas.cit.nih.gov/molbio/proscan/) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) to identify putative transcription factor binding sites. Based on our scans for consensus transcription factor binding motifs, two major cis-acting elements, with the highest scores for NF-κB and CREB, were present within the minimal promoter region (−110 to +38). The NF-κB binding site was found between −71 and −61, and the CREB binding site was found between −29 and −22. Sequences of the minimal promoter and transcription factor binding sites are provided in Fig. 2C.

Specific binding of NF-κB and CREB transcription factors to the NOLC1 proximal promoter

To explore whether NF-κB and CREB could bind the NOLC1 promoter, electrophoretic mobility shift assays (EMSA) were performed. The first oligonucleotide chosen for EMSA contained sequences that matched a consensus NF-κB binding site. The nuclear extracts from the HepG2 cells showed strong binding to a wild-type probe containing an NF-κB binding site

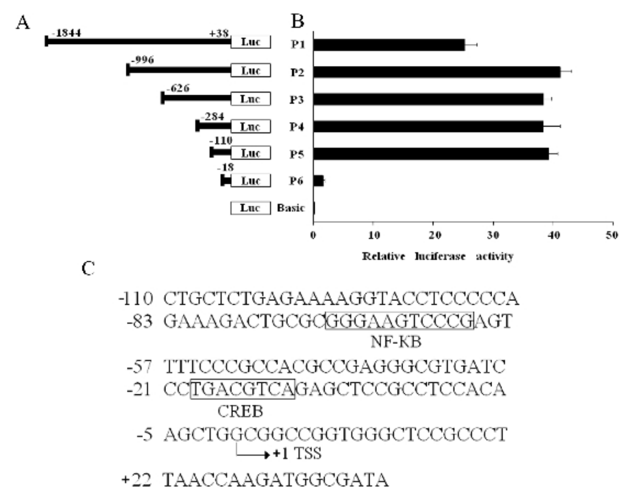

Fig. 2. Luciferase reporter assay to identify the minimal NOLC1 promoter. (A) Six luciferase reporter constructs containing promoter fragments with 5' deletions. (B) Relative luciferase activity derived from six NOLC1 promoter constructs. Promoter activity is normalized for variations in transfection efficiency using phRL-TK as an internal control. (C) Nucleotide sequence of the minimal NOLC1 promoter. The transcription start site (TSS) +1 is indicated. The region between −110 and +38 was analyzed with Promoter Scan and TFSEARCH. Two putative cis-elements, NF-κB and CREB, were discovered.

(lane 2 of Fig. 3A). We then performed competition experiments with unlabeled probes and mutant probes (lane 3 and 4 of Fig. 3A). The DNA-protein complex was specific, as demonstrated by the fact that it could be thoroughly out-competed by a 100-fold excess of unlabeled probe, whereas a mutant probe containing 3-bp substitutions (CTC instead of GGG) failed to compete. These results suggest that NF-κB is capable of binding to the NOLC1 minimal promoter.

The other oligonucleotide contained a CREB binding site. Fig. 3B demonstrates that the wild-type probe containing a CREB binding site bound to nuclear extracts (lane 2 of Fig. 3B). The DNA-CREB complex could be thoroughly out-competed by a 100-fold excess of unlabeled probe (lane 3 of Fig. 3B). However, a mutant probe containing 4-bp substitutions (GTAC instead of ACGT) failed to compete (lane 4 of Fig. 3B). These results suggest that CREB is capable of binding to the NOLC1 minimal promoter.

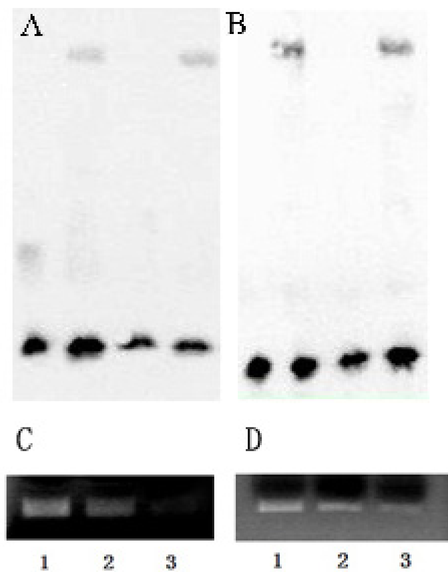

Fig. 3. Binding of NF-κB and CREB to the minimal NOLC1 promoter. EMSA was performed with 5 μg of HepG2 cell extracts. (A) Extracts were incubated with a biotin-labeled oligonucleotide containing NF-κB binding site. Lane 3 shows the competition by a 100-fold excess of unlabeled oligonucleotides. Lane 4 shows the competition by the unlabeled mutated oligonucleotides. (B) Extracts were incubated with a biotin-labeled oligonucleotide containing a CREB binding site. Lane 3 shows the competition by a 100-fold excess of unlabeled oligonucleotides. Lane 4 shows the competition by the unlabeled mutated oligonucleotides. (C) The binding of NF-κB to the minimal NOLC1 promoter was analyzed by a ChIP assay. Lane 1, Input represents PCR amplification of 1% of the genomic DNA without immunoprecipitation. Lane 2, PCR amplification of the genomic DNA immunoprecipitated by anti-NF-κB. Lane 3, PCR amplification of the genomic DNA immunoprecipitated by nomal IgG. (D) The binding of CREB to the minimal NOLC1 promoter was analyzed by a ChIP assay. Lane 1, Input represents PCR amplification of 1% of the genomic DNA without immunoprecipitation. Lane 2, PCR amplification of the genomic DNA immunoprecipitated by anti-CREB. Lane 3, PCR amplification of the genomic DNA immunoprecipitated by nomal IgG.

To assess whether NF-κB and CREB bind the NOLC1 proximal promoter *in vivo*, we performed a chromatin immunoprecipitation (ChIP) experiment followed by the PCR assay with primers designed to amplify the region between −162 and 37. We found enrichment of NF-κB and CREB on P5 compared to controls precipitated with IgG (Fig. 3C, D). Taken together, these data reveal that NF-κB and CREB can specifically bind the NOLC1 promoter.

Functional assay of NF-κB and CREB for NOLC1 promoter activity

To ascertain whether the transcription factors NF-κB and CREB play a functional role in the activation of the NOLC1 promoter, we performed site-directed mutagenesis of the NF-κB and CREB binding sites within P5. We then analyzed the luciferase activity between the wild-type, NF-κB mutant and CREB mutant binding sites by transient transfection experiments. As shown in Fig. 4A, mutations of the NF-κB site had a dramatic effect on the promoter activity, as demonstrated by an 86% decrease in luciferase activity. Similarly, mutation of the CREB binding site caused a 55% decrease in luciferase activity. The mutation analyses indicate that abrogation of NF-κB or CREB binding sites is sufficient to disrupt NOLC1 promoter activity. In addition, we also conducted co-transfection analysis on the luciferase activity driven by P5 with NF-κB and CREB expression plasmids. As Fig. 4B displays, expression of NF-κB caused a 1.5-fold increase in luciferase activity in HepG2 cells as compared with pcDNA3.1 (−) plasmid. Similarly, expression of CREB also caused a 1.7-fold increase in luciferase activity in HepG2 cells (Fig. 4C). Taken together, these results strongly suggest that NF-κB or CREB is critical for maintaining NOLC1 transcriptional activity.

DISCUSSION

In the current study, we examined the mechanism of NOLC1 transcriptional regulation. First, we cloned a region of the NOLC1 promoter 1,844 bp upstream of the transcription start site. Then deleted promoters, constructed in a stepwise fashion, were analyzed for luciferase activity in HepG2 cells, which express NOLC1 at high levels. A minimal promoter sequence, spanning from −110 to +38, was sufficient to drive NOLC1 expression. Two major cis-elements for NF-κB and CREB were present in the minimal promoter. Further analysis demonstrated that NF-κB and CREB could bind to the NOLC1 minimal promoter. Thus, we assume that NF-κB and CREB may affect NOLC1 expression. *In vitro* mutagenesis and overexpression of NF-κB and CREB revealed that these transcription factors play an important role in maintaining the transcriptional activity of NOLC1. Many studies have shown that NF-κB is a key regulator of the expression of genes involved in

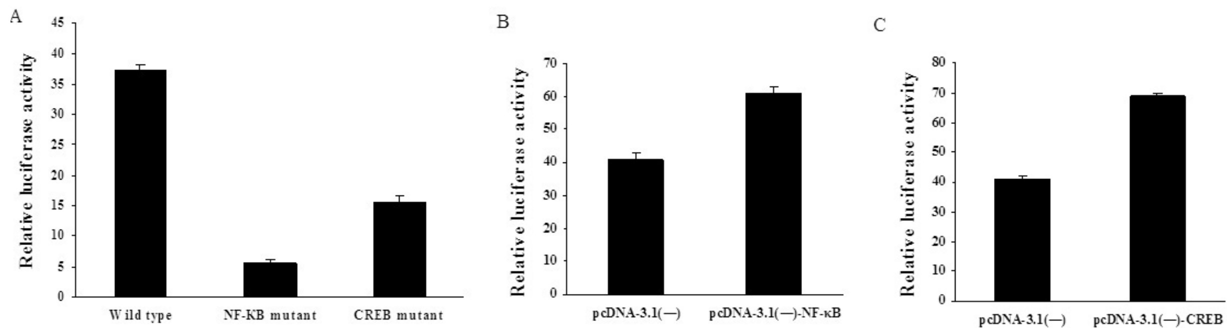

Fig. 4. Demonstration of NF-κB and CREB regulate the minimal promoter of NOLC1. (A) Mutation analysis of the minimal NOLC1 promoter. By site-directed mutagenesis, either the NF-κB or CREB binding site was abrogated. These altered promoters with single site mutations were tested by luciferase reporter assays and compared with the wild-type promoter. (B) NOLC1 minimal promoter (P5) was co-transfected with pcDNA3.1 (−)-NF-κB plasmid. (C) NOLC1 minimal promoter (P5) was co-transfected with pcDNA3.1 (−)-CREB plasmid. pcDNA3.1 (−) plasmid served as a negative control.

inflammation, cell cycle regulation, apoptosis and tumorigenesis (13), and NF-κB up-regulation has been demonstrated in many cancer cells. It has been reported that suppressing NF-κB activation results in apoptosis of transformed hepatocytes and a failure to progress to hepatocellular carcinoma (HCC) (14). CREB is a ubiquitous transcription factor that activates the transcriptional activity of multiple promoters through its binding site and regulates many physiologically critical functions such as gluconeogenesis, lipid metabolism and cell proliferation in the liver (15, 16). It was recently demonstrated that CREB plays an important role in hepatocellular carcinoma progression by modulating tumor growth, angiogenesis and apoptosis (17). The induction of CREB target genes may be involved in the development of HCC. Our discovery of the involvement of the NF-κB and CREB-NOLC1 pathways in NOLC1 expression provides a previously undescribed mechanism for regulating NOLC1 expression; further details remain to be elucidated.

In conclusion, our experiments identified the minimal NOLC1 promoter and demonstrated that NOLC1 is regulated by NF-κB and CREB. Elucidation of the regulatory mechanisms that control NOLC1 expression will certainly contribute to a better understanding of NOLC1 functions.

MATERIALS AND METHODS

Cell culture and transient transfection

HepG2, Huh-7, QGY-7701, SMMC-7721, BEL-7402, HeLa and CaP cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified chamber at 37°C and 5% CO_2. Cells were seeded into 48-well plates, grown to a density of 90% confluence and transiently transfected using Lipofectamine 2,000 (Invitrogen, USA) according to the manufacturer's protocol.

RNA isolation and real-time PCR analysis

Total RNA was extracted from cultured cells using TRIzol reagent according to the manufacturer's instructions. A total of 0.1 μg of RNA from each sample was used to generate cDNA by reverse transcription with the One-step RT-PCR kit (Takara, Japan). A Taqman real-time quantitative PCR assay was performed on an ABI Prism 7500 by following the manufacturer's protocol. β-actin was used as reference for normalizing data. NOLC1 primers and probes were as follows: sense: 5'-GCATC ATCCCC ATTCCGAAGG-3'; antisense: 5′-ACCTGATTGGCTC GCTCTCC-3'; probe: 5′-CTCCGGCTGC ACCTCGCTTGGC-3'.

Genomic amplification and construction of expression plasmids

Genomic DNA was isolated from HepG2 cells using a Genomic DNA Purification kit (Promega, USA). A series of 5'-flanking DNA fragments upstream of the transcription initiation site of NOLC1 (P1 (−1,844 to +38), P2 (−996 to +38), P3 (−626 to +38), P4 (−284 to +38), P5 (−110 to +38), P6 (−18 to +38)) were inserted into *Nhe* I and *Hind* III restriction sites of a pGL4.10 Basic vector (Promega, USA). The PCR primers were as follows: sense: P1: 5'-AACTGCTGTTGTGCGT CT T-3'; P2: 5'-GCAGGATCATAGCTCATTG-3'; P3: 5'-AATGTGGCAGAA CTTGGA-3'; P4: 5'- GTATCAGAAAGGGCCTCC-3'; P5: 5'-CT GCTCTGAGAAAAGGTAC-3'; P6:sense: 5'-GCAG GATCATA GCTCATT G-3' and P6:antisense: 5'-CCA AGTTCTGCCACAT TC-3'. The NF-κB (p65) expression plasmid was generated by amplification of HepG2 cDNA and cloned into pcDNA3.1 (−) plasmid using restriction sites *Xho* I and *Hind* III. The CREB expression plasmid was generated using the restriction sites *EcoR* I and *BamH* I. The PCR primers were as follows: NF-κB (p65) sense: 5'-CTCGAGCCGGCCATGGACGAACTGTT-3'; antisense: 5'-AAGCTTC CCCTTAGGAGCTGATCTGAC-3'; CREB sense: 5′-GAATTCCGGAGGTGTAGTTTGACG-3'; antisense: 5'-GGA TCCTTAATCTG ATTTGTGGCAGT-3'.

Luciferase reporter assay

HepG2 cells were co-transfected with 0.4 μg of NOLC1 promoter plasmids and 13 ng of internal control phRL-TK plasmids using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. At 24 hours post-transfection, cells were harvested and lysed in 50 μl of passive lysis buffer. A fraction of protein was subjected to a dual-luciferase reporter assay system (Promega, USA). Firefly luciferase activity and Renilla luciferase activity were measured sequentially by a Veritas Microplate Luminometer (Turner BioSystems, USA). All transfections were performed in triplicate, and the promoter activities are expressed as the mean ± SD of three independent experiments. For NF-κB and CREB responses, 0.2 μg of NOLC1 promoter plasmids (P5), 13 ng of internal control phRL-TK plasmids and 0.2 μg of pcDNA3.1 (−)-NF-κB or pcDNA 3.1 (−)-CREB plasmids were co-transfected into HepG2 cells.

Electrophoretic mobility shift assay (EMSA)

For the gel shift assay, double-strand DNA oligos were synthesized with a biotin label at the 3' end (Invitrogen, Shanghai). Nuclear extracts were prepared using a Nuclear Extraction kit (Pierce, USA), and the protein content was measured using the BCA protein assay kit (Pierce, USA) following the manufacturer's protocol. Electrophoretic mobility shift assays were performed utilizing a LightShift chemiluminescence EMSA kit (Pierce, USA). The probes used included the following double-stranded oligonucleotides: NF-κB wild type 5'-CAGAAAGACTGCGCG GGAAGTCCCGAGTTTTCCCG -3'; mutant 5'-CAGAAAGACT GCGCCTCAAGTCCCGAGTTTTCCCG-3' (mutated bases shown as bold); CREB wild type 5'-GCGTGATCCCTGACGTCAGAG CTCCGCCTC-3'; mutant 5'-GCGTGATCCCTGGTACCAGAGC TCCGCCTC-3'. Nuclear extracts containing 5 μg of protein were incubated with the oligonucleotide probes for 20 minutes at 25°C. For the competition experiments, 100-fold excess of the unlabeled wide-type or mutant probe was pre-incubated with the nuclear extracts for 10 minutes before the labeled probes were added to the reaction. The DNA-protein complexes were separated using a 6.5% nondenaturing polyacrylamide gel.

Chromatin immunoprecipitation (ChIP)

HepG2 cells (1×10^6) were used for each ChIP. Chromatin isolation and ChIP assays were performed using an EZ-Zyme Chromatin prep kit and an EZ-ChIP kit (Millipore, USA). The chromatin solution was immunoprecipitation with 5 μg of anti-NF-κB, anti-CREB (Abcam, USA) or 5 μg of normal anti-IgG antibody and 20 μl of protein A agarose beads overnight at 4°C. Following multiple washes, the antibody-protein-DNA complex was eluted from the beads. After reverse cross-link incubation, the protein and RNA were removed by proteinase K and RNase, and a quantitative PCR assay was performed on the immunoprecipitated genomic DNA with primers specific for the NF-κB and CREB binding site upstream of the transcriptional start site. Primers were as follows: sense: TTGTCCACTC ATTACTCTG and antisense: ATCGCCATCTTGGTTAA G. The PCR products were separated on a 1% agarose gel.

Site-directed mutagenesis

P5 was utilized to perform site-directed mutagenesis of the putative NF-κB and CREB binding sites following the quick change site-directed mutagenesis protocol (Stratagene, USA). The oligonucleotides incorporating mutant bases were as follows: NF-κB: CAGAAAGACTGCGC CTCAAGTCCCGAGTTTT CCCG. CREB: GCGTGATCCCTGGTACCAGAGCTCCGCCTC. The PCR reaction was performed for 30 cycles (95°C for 30 s, 55°C for 30 s, 68°C for 10 min) following an initial denaturation at 95°C for 30 s. The mixture of input and amplified DNA was digested directly with *Dpn* I and then transformed into XL-10 Blue cells. The nucleotide sequences of the mutant were confirmed by sequencing.

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