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Characterization of the mouse CP27 promoter and NF-Y mediated gene regulation

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ABSTRACT

The *cp27* gene is a highly conserved and unique gene with important roles related to craniofacial organogenesis. The present study is a first analysis of the CP27 promoter and its regulation. Here, we have cloned the promoter of the mouse *cp27* gene, examined its transcriptional activity, and identified transcription factor binding sites in the proximal promoter region. Two major transcription start sites were mapped adjacent to exon 1. Promoter function analysis of the 5′ flanking region by progressive 5′ deletion mutations localized transcription repression elements between −1993 bp and −969 bp and several positive elements between −968 bp and the preferred transcription start site. EMSA and functional studies indicated two function-cooperative CCAAT boxes and identified the NF-Y transcription factor as the CCAAT activator controlling transactivation of the CP27 promoter. In addition, this study demonstrated that for its effective binding and function, NF-Y required not only the minimal DNA segment length identified by deletion studies, but also a defined nucleotide sequence in the distal 3′ flanking region of the CP27 proximal promoter CCAAT box. These results provide a basis for our understanding of the specific regulation of the *cp27* gene in the NF-Y-mediated gene transcription network.

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1. Introduction

Craniofacial development occurs in a complex signaling environment in which growth factors, transcription factors and structural genes of the extracellular matrix maintain signal-response cascades that ultimately result in the formation of the vertebrate head (Davidson, 1993; Slavkin and Diekwisch, 1996; Thesleff and Sharpe 1997). These signaling cascades involve a continuous communication between epithelial and mesenchymal components of adjacent tissues (Thesleff, 1995; Thesleff and Sharpe, 1997). One such gene that is expressed at several crucial sites in the epithelial–mesenchymal interface during craniofacial development is CP27 (Diekwisch et al., 1999; Diekwisch et al., 2002).

CP27 is a unique gene that is highly conserved in many species such as human, mouse, bovine, deer, goat, sheep, giraffe, and pig (Nobukuni et al., 1997; Diekwisch et al., 2002). Sequence analysis has also revealed significant homologues in zebrafish (*Danio rerio*) and yeast (*Saccharomyces cerevisiae*) (Diekwisch et al., 2002; unpublished observation). Originally, CP27 was cloned from an E11 early embryonic library (Nobukuni et al., 1997; Diekwisch and Marches, 1997; Diekwisch et al., 2002). Northern blot analysis of RNA from multiple mouse tissues demonstrated high levels of expression in developing mouse teeth, heart, lungs, and liver. Both the expression in presumably important sites related to organogenesis and the distinct changes in localization during development (Nobukuni et al., 1997; Diekwisch and Marches, 1997; Diekwisch et al., 2002) as well as gain- or loss-of-function studies (Diekwisch and Luan, 2002; Luan and Diekwisch, 2002) suggest that CP27 may play important roles during development.

To understand the expression of the *cp27* gene and elucidate the mechanisms that govern it, we have cloned the promoter region of the mouse *cp27* gene and characterized the cell-specific elements in the 5′ flanking region in embryonic fibroblasts. Using gel-shift and functional studies, we have identified NF-Y as a transactivator of the CP27 promoter that regulates *cp27* gene expression via multiple CCAAT boxes. Our results document for the first time the importance of the 5′ 2-kb flanking region in the expression of the mouse *cp27* gene and establish NF-Y as a transcriptional regulator of *cp27* gene expression.

2. Material and methods

2.1. Library screening and DNA sequencing

A mouse genomic lambda Fix II 129/SVJ library (Stratagene, La Jolla, CA) was screened with a full-length mouse CP27 cDNA, and five clones were identified. Using the *Eco*RI restriction enzyme, the DNA

Abbreviations: AMV, Avian myeloblastosis virus; AP-1, Activator protein transcription factor; CBF, Core binding factor; CDC25c, CDC25 phosphatase; C/EBP, CCAAT/enhancer binding protein; CP27, Craniofacial protein with a molecular weight of 27 kDa; Csx/Nkx 2.5, cardiac homeobox transcription factor; EMSA, Electrophoretic mobility shift assay; ETS, E-twenty-six transcription factor; FGF, Fibroblast growth factor; GATA, Transcription factor that binds to the GATA DNA sequence; HSP 70, Heat shock protein 70; NF-Y, Nuclear factor Y; NF-1, Nuclear factor 1; Oct, Octamer binding factor; POU, Pit-1, Oct-1, Unc-86 transcription factor family; RLM-RACE, RNA ligase mediated rapid amplification of cDNA ends; SOX, Sry-related high mobility group box transcription factor.

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insert was cut and fragments were subcloned into the pBluescript vector (Stratagene). The resulting DNA sequence was determined with an ABI 373 automatic sequencer. One of the five genomic clones contained 2.1 kb of the 5′ flanking region of the *cp27* gene and was used for further analysis. The transcription factor binding sites within the 5′ flanking region were determined using MatInspector (www.genomatrix.de) and Signal Scan (www.bimas.cit.nih.gov/molbio/signal/).

2.2. Primer Extension Analysis

Primer extension was carried out using the Primer Extension System kit (Promega, Madison, WI). An antisense primer CP 82-61 (5′ GCTACCCACACGACTGCGCCAC 3′) was labeled with r-^{32}P using T4 polynucleotide kinase and annealed in AMV primer extension buffer at 58 °C for 40 min to 10 μg of total RNA from NIH 3T3 cells, which have been previously shown to express CP27 (Luan and Diekwisch, 2002) or tRNA. The primer was extended with AMV reverse transcriptase at 42 °C for 30 min. Resulting products were electrophoresed in an 8% denaturing urea polyacrylamide gel and autoradiographed. The sizes of the products were determined by ^{32}P-labeled ϕX 174 Hinf I DNA markers.

2.3. Ribonuclease protection assay (RPA)

The 5′ flanking region and partial exon 1 of the *cp27* gene were amplified via polymerase chain reaction using sense primer CP-261/-242 (5′ TATTAGCTTGTGAGCAAATT 3′) and antisense primer CP 82/61. The 343-bp fragment was then subcloned into the plasmid pCR II-TOPO (Invitrogen, Carlsbad, CA). Transcription was performed with T7 RNA polymerase and yielded α ^{32}P-labeled antisense RNA that was then used as a probe. The probe was annealed to 10 μg of total RNA from NIH 3T3 cells or yeast RNA at 42 °C for 16 h. Following digestion with RNase A and RNase T1 (Ambion, Austin, TX) according to manufacturer's instructions, the RNase-resistant radioactivity was size-fractionated in an 8% denaturing urea polyacrylamide gel and autoradiographed. The sizes of the protected fragments were determined by ^{32}P-labeled ϕX 174 Hinf I DNA markers.

2.4. 5′-Rapid amplification of cDNA ends (5′ RACE)

Rapid amplification of cDNA 5′ ends was performed using a RLM-RACE kit (Ambion). 10 μg of total RNA was treated with Calf intestine alkaline phosphatase to remove free 5′-phosphate. Tobacco acid pyrophosphatase was added to the reaction to remove the cap structure from full-length mRNA. A 45-base RNA adaptor oligonucleotide was ligated to the RNAs using T4 ligase. The first-strand cDNA was synthesized in a random-primed reverse transcription reaction. Amplification of the 5′ ends of CP27 transcripts was accomplished with two pairs of nested primers: a 5′ RACE outer primer 5′ GCTGATGGCGATGAATGAACACTG 3′ and a CP27 antisense outer primer 5′ TCTCTTCAGTCTCCTCGGCT 3′; a 5′ RACE inner primer 5′ CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG 3′ and a CP27 antisense inner primer 5′ GCTCCTCTTCATCTTCTTCACTGC 3′. The RACE products were subcloned into pCR II-TOPO and then sequenced.

2.5. Promoter–reporter gene constructs

For this promoter study, a total of 15 promoter–reporter gene constructs were generated. The inserts for 14 of the 15 constructs were amplified by PCR with the screened genomic clone as a template using a common 3′ primer and selected 5′ primers (Tables 1 and 2). These primers also introduced a *Sac*I site at the 5′ end and a *Hin*dIII site at the 3′ end of the amplified fragments. The PCR fragments were gel-purified using Qiaquick PCR preps (QIAGEN, Valencia, CA), digested with *Sac*I and *Hin*dIII and subcloned into the pGL3-basic vector (Promega). Correct orientation of all inserts with respect to the pGL3 vector was verified by DNA sequencing. Only the pGL −1475/+48 plasmid was generated using a pGL3 vector into which a 1.5 kb *Bgl*II and *Hin*dIII fragment from pGL −1993/+48 was inserted. The constructs used for deletion mutation studies were pGL3-1993, pGL3-1475, pGL3-969, pGL3-720, pGL3-207, pGL3-93, and pGL3-17 (Fig. 3). The constructs pGL-93/-56M10-12, pGL3-1255, pGL3-1190, pGL−93/+48CATm, pGL-93-56M10-12, pGL−1255/+48CAT1m, pGL-1255/+48CAT5m, and pGL−1255/+48CAT1,5m were used for CCAAT box function studies. Plasmids carrying an “m” denominator were subjected to a mutation, and pGL−1255/+48CAT1,5m was subjected to a double mutation. All plasmid constructs contained part of the exon 1 noncoding region.

2.6. Transient Transfection and Dual Luciferase Assay

The mouse embryonic fibroblast cell line NIH 3T3 was used as the recipient cell line for transient transfection assays. NIH 3T3 cells (3×10^5 cells/well) were placed in 6-well plates and cultured for 24 h. For each transfection, cells were incubated with 1 μg of each promoter–reporter plasmid, 0.01 μg of pRL-TK (Promega), which was used as internal control for transfection efficiency, 4 μl of LipofectAMINE PLUS REAGENT (Invitrogen), and 2 μl of LipofectAMINE reagent (Invitrogen) in serum-free medium for 3 h. For co-transfection, 1 μg of the pGL−1475/+48 construct was introduced with 0.4 or 0.8 μg of expression vector pIRES-NFYA or with 0.8 μg of pIRES-NFYAm29, a domain negative NF-YA (courtesy of Dr. S. Chen, UTHSCSA). After removal of the DNA–PLUS-LipofectAMINE complex, cells were incubated in 2 ml of complemented medium for 48 h and then subjected to a dual luciferase assay according to the manufacturer's instructions (Promega). In this dual luciferase system, CP27 promoter fragments were linked to the firefly luciferase gene while the co-transfected renilla luciferase gene (pRL-TK) was driven by the SV40 promoter. The firefly and renilla luciferase activities were measured using TD-20/20 (Promega). Promoter activity measurements were a reflection of the ratio of firefly/renilla luciferase for each construct. For luciferase activity measurements, the means of luciferase activity measurements from five independent sets of experiments using a triplicate set of wells in each experiment were determined for each construct.

2.7. Nuclear extract preparation

NIH 3T3 cells (5×10^7) in 100 mm dishes were washed twice with cold phosphate-buffered saline (pH 7.4) and scraped off in 1 ml of lysis buffer (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DDT, 0.5 mM phenyl-methylsulphonyl fluoride (PMSF), 0.05%

Table 1
Primers used in the amplification of PCR fragments for promoter–reporter gene constructs.

Primer	Oligonucleotide sequence	Orientation
−1993/−1973	5′TACCGAGCTCGGCTAACCTGCTCAACTTTGG3′	sense
−1255/−1234	5′TACCGAGCTCTCTTAGGCTGATTCCCATTGC3′	sense
−1190/−1169	5′TACCGAGCTCGCATTGGTTGGTCCTCCCGAT3′	sense
−969/−947	5′TACCGAGCTCAGGTGATTTCTGAGGGACTAGGG3′	sense
−720/−699	5′TACCGAGCTCTCTAGCACTTTGTGTAGTGGC3′	sense
−207/−186	5′TACCGAGCTCTATTAGCTTGTGAGCAAATT3′	sense
−93/−73	5′TACCGAGCTCTGAGTGTAGACTGACCAATCGC3′	sense
−17/+4	5′TACCGAGCTCCCTCTAGGGCGGCCCTAGCT3′	sense
+48/+25	5′TCGCAAGCTTGCGAAGCTAGATATAGGGCGAGAC3′	antisense

Each primer was labeled by the location of the 5′ and the 3′ first nucleotide in the CP27 5′ flanking region. Each sense oligonucleotide was paired with the only antisense oligonucleotide for PCR amplification. A *Sac*I restriction enzyme site was added to the 5′ end of each sense oligonucleotide, while a *Hin*dIII site was added to the 5′ end of the antisense oligonucleotide. PCR products were then inserted into the vector pGL3-Basic to generate promoter–reporter gene constructs.

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