Molecular Cloning and Characterization of Human Homebox Gene Nkx3.1 Promoter

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Abstract Nkx3.1 is a prostate-specific homeobox gene related strongly to prostate development and prostate cancer. To study its regulation of transcription, 1.06 kb 5′ flanking region of Nkx3.1 gene and its 5′ deletion mutants (861, 617, 417 and 238 bp) were obtained by PCR and cloned into pGL3-basic, a promoter-less luciferase reporter vector, to examine their promoter activities driving the reporter gene transcription. pRL-TK, a Renilla luciferase reporter vector was used as internal control, and pGL3-control and pGL3-basic were used as positive and negative control respectively. The promoter activities were determined by dual-luciferase reporter assay 48 h after pGL3 constructs were cotransfected with pRL-TK into prostate cancer cell LNCaP. The results showed that dual-luciferase reporter assay (M1/M2) of pGL3-1.06 kb cotransfection with pRL-TK was 2.7, which was about 1.5-fold higher than that of pGL3-control cotransfection with pRL-TK and 50-fold higher than that of pGL3-basic cotransfection with pRL-TK. The results also showed that the relative activities (M1/M2) were 0.71, 0.84, 0.44 and 2.07 respectively for pGL3-861 bp, pGL3-617 bp, pGL3-417 bp, pGL3-238 bp, the last one still had 80% promoter activity compared with pGL3-1.06 kb, which showed that deletion from 1.06 kb to 238 bp had small effects on promoter activity. The conclusion was that the 238 bp fragment containing a TATA box and two CAAT boxes had strong promoter activity. However, the deletion from 1.06 kb to 861 bp reduced activity 3.8-fold while the deletion from 417 bp to 238 bp enhanced activity 4.7-fold, which indicated that these deleted sequences might contain some important positive or negative regulatory elements. It will be important to identify the elements within the Nkx3.1 promoter that contribute to regulation of the gene transcription in the future studies.

Key words homeobox gene Nkx3.1; promoter; deletion mutagenesis; LNCaP cell line

Nkx3.1 is an androgen regulated prostate-specific homeobox gene that is thought to play important roles in normal prostate development. In mice Nkx3.1 is exclusively expressed in prostate epithelium [1,2] and its targeted disruption leads to aberrations in prostate ductal morphogenesis and secretory protein production, and epithelial hyperplasia and dysplasia [3]. Notably Nkx3.1 mutant mice display the pathologic changes of prostatic intraepithelial neoplasia (PIN) [4] that is the presumed precursor to prostate cancer in human, which implies that loss of Nkx3.1 expression correlates with the initiation of prostate carcinogenesis. In human Nkx3.1 expression is generally restricted to the prostate and it is androgen regulated [5,6]. Since Nkx3.1 gene maps to the chromosomal region 8p21, a region with high loss of heterozygosity in human prostate cancer [7], the gene has been proposed to have tumor suppressor function. Loss of Nkx3.1 protein expression has been observed in about 40% of human prostate cancers and in about 20% of PIN lesions[8]. Furthermore, loss of Nkx3.1 protein expression correlates well with prostate tumor progression [8]. But no mutations in the Nkx3.1 gene have been found in prostate tumor specimens [7]. Its second allele is inactivated by some mechanisms other than mutations in the coding region. So
Materials and Methods

Materials

Human genomic DNA was extracted from human white blood cells. LNCap cell line was extracted from ATCC. Taq DNA pol, SacI, XhoI, KpnI, HindIII and pMD18-T vector were purchased from TaKaRa Biotech Co., Ltd. pGL3-control, pGL3-basic, lipofectimine™ 2000 reagent and dual-luciferase assay kit were purchased from Promega Co. RPMI 1640 media and fetal bovine serum were obtained from Gibco. The 1.06 kb flanking region of Nkx3.1 gene and its 5′ deletion mutants to determine their promoter activity. It will provide an insight into the regulatory mechanisms of Nkx3.1 gene expression in further study.

Amplification and subcloning of 1.06 kb 5′ flanking region of Nkx3.1 gene

Human genomic DNA was extracted from blood white cells by using the method of rapid isolation of mammalian DNA [9]. The primer pair PF (5′-CGCGAGCTCAAGGCAGGATCCTTGT-3′) with SacI site at 5′ end and PR (5′-GGCTCGAGCGCAACCGTTTAC-3′) with XhoI site at 5′ end were used to amplify the 5′ flanking region of Nkx3.1 gene from the extracted human genomic DNA. The primer pair PF and PR were designed using Primer3 software according to the sequence in Genbank (accession number AC012574). The PCR was conducted at 94°C for 5 min followed by 35 cycles at 94°C for 45 s, 60°C for 30 s, and 72°C for 1.5 min. PCR-amplified fragment was about 1.06 kb (+8 bp to −1032 bp of Nkx3.1 gene plus 18 bp of the two restriction sites for XhoI and SacI) that was subcloned into a T/A clone vector of pMD18-T to form T/A cloning recombinant.

Construction of luciferase reporter plasmid

The 1.06 kb fragment was excised from T/A cloning recombinant with XhoI and SacI and ligated into the equivalent site of pGL3-basic vector to form pGL3-1.06 kb construct. The resultant construct was confirmed by KpnI digestion and sequence analysis using the general primers Rp primer3 and Rp primer2.

Deletion mutagenesis of the 1.06 kb promoter

5′ deletion mutants of different sizes (238, 417, 617 and 861 bp) that all begin at +8 bp of Nkx3.1 gene were obtained from pGL3-1.06 kb by PCR using one primer PR spanning +8 bp and four different primers with SacI site at 5′ end, PFD1 (5′-CGCGAGCTCAAGGCAGGAAATTG-3′), PFD2 (5′-CGCGAGCTCAATTGGCTCTGACGTTCC-3′), PFD3 (5′-CGCGAGCTCACCACCTCTGCAACCGGA-3′), PFD4 (5′-CGCGAGCTCAACGGTGACCAAATCGT-3′).

All of the amplified fragments were separated by 1.5% agarose gel electrophoresis, excised and purified with QIAquick gel extraction kit. Then they were cut with XhoI and SacI and cloned into the equivalent site of pGL3-basic vector to construct 4 kinds of deletion mutants, pGL3-238 bp, pGL3-417 bp, pGL3-617 bp and pGL3-861 bp. All of them were sequenced.

Cell culture and transient transfection

LNCaP cells were grown at 37°C in 5% CO2 with RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and ampicillin 100 μg/ml and streptomycin 100 μg/ml. Within 60 h of passage and more than 90% confluent, LNCaP cells were transfected with lipofectamine™ 2000 in 24-well plates. Each well included 1.5×10⁶ cells, 1 mg pGL3, construct, 0.04 mg internal control vector pRL-TK, 2 μl lipofectamine™ 2000 and 500 μl RPMI 1640 media without serum and antibiotics. All cells were analyzed for dual-luciferase reporter gene expression 48 h after completion of the transfection procedure.

Dual-luciferase reporter assay

The activities of firefly luciferase in pGL3 and Renilla luciferase in pRL-TK were determined following the dual-luciferase reporter assay protocol recommended by Promega. The cells were rinsed with PBS after harvest and cell lysates were prepared by manually scraping the cells from culture plates in the presence of 1×PLB (passive lysis buffer). 20 μl of cell lysate was transferred into the luminometer tube containing 100 μl MLAR II, and firefly luciferase activity (Mf) was firstly measured then Renilla luciferase activity (Mr) was measured after adding 100 μl of Stop & Glo Reagent. The program of luminometer was a 2 s premeasurement delay followed by a 10 s measurement period for each assay.

Results

Amplification and Cloning of 1.06 kb 5′ flanking region of Nkx3.1 gene and its deletion mutants

Fig. 1(A) showed the 1.06 kb fragment amplified by PCR in electrophoresis. The fragments amplified by PCR were all cloned into pGL3-basic at the XhoI site and SacI site to form pGL3-1.06 kb, pGL3-861 bp, pGL3-617 bp, pGL3-417 bp and pGL3-238 bp. All of the pGL3 constructs

far, it is not well known that how the Nkx3.1 gene expression is regulated and what cis-acting elements and transacting factors are involved in the regulation. The importance and specificity of Nkx3.1 gene expression in prostate prompt us to study its promoter and regulation. We have firstly cloned the 1.06 kb

5′ flanking region of Nkx3.1 gene and its 5′ deletion mutants to determine their promoter activity. It will provide an insight into the regulatory mechanisms of Nkx3.1 gene expression in further study.

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were confirmed by restriction enzyme digestion [Fig. 1 (B)] and DNA sequencing (Fig. 2).

Transient transfection and promoter activity assay of the cloned 1.06 kb fragment

The firefly luciferase expression driven by the 1.06 kb promoter of Nkx3.1 was examined to evaluate its promoter activity. To normalize all of the transfection experiment an internal control plasmid pRL-TK that contain Renilla luciferase was used to cotransfect with pGL3-1.06 kb into prostate cancer cell line LNCaP. The relative luciferase activity of experimental sample is presented by the ratio of the activities of firefly luciferase and Renilla luciferase (M1/M2). For the initial experiment two primary control transfections also were conducted. One control was a transfection with pGL3-basic (promoter-less). Another control transfection was performed with pGL3-control containing SV40 promoter and enhancer. Our result showed that dual-luciferase reporter assay (M1/M2) was 2.7, 48 hours after pGL3-1.06 kb were cotransfected with pRL-TK into LNCaP cells, which was about 1.5-fold higher than that of pGL3-control cotransfection with pRL-TK and 50-fold higher than that of pGL3-basic cotransfection with pRL-TK. The cloned 1.06 kb fragment of 5′ flanking region of Nkx3.1 gene presented a strong promoter activity.

Deletion mutagenesis analysis

To define the boundary of the functional promoter and its minimal sequence, four of 5′ deletion mutants of the 1.06 kb were produced by PCR and analyzed by dual-luciferase reporter assay. The results in Fig. 3 showed that the relative activities (M1/M2) were 2.7, 0.71, 0.84, 0.44 and 2.07 respectively for pGL3-1.06 kb, pGL3-861 bp, pGL3-617 bp, pGL3-417 bp and pGL3-238 bp, the last one still had 80% promoter activity compared with pGL3-1.06 kb, which showed that deletion from 1.06 kb to 238 bp had small effects on promoter activity. But the deletion from 1.06 kb to 238 bp reduced activity 3.8-fold while the deletion from 417 bp to 238 bp enhanced activity 4.7-fold.
Discussion

There is a strong association of Nkx3.1 with prostate development and prostate cancer, making this gene an attractive molecular target for further study. Little is known about the regulatory mechanisms of Nkx3.1 gene expression as well as relevant regulatory elements and factors. Our research on the Nkx3.1 gene has been initiated to define the promoter and to determine the degree to which this gene is regulated at the transcriptional level.

In this study, 1.06 kb 5′ flanking region of Nkx3.1 gene was amplified by PCR using human genomic DNA as the template. To evaluate its promoter activity, the 1.06 kb fragment was cloned into pGL3-basic vector that contains a firefly luciferase reporter gene. Our result has shown that pGL3-1.06 kb provided a much higher level of luciferase transcription in LNCaP cell line compared with promoterless pGL3-basic vector and also provided a significantly higher transcription level than did pGL3-control that contained a putative strong promoter of SV40, which indicated that cloned 1.06 kb 5′ flanking region of Nkx3.1 gene presented a strong promoter activity. To define the boundary of the functional promoter and its minimal sequence we constructed four deletion mutants to dissect the minimal promoter activity 4.7-fold, which implied that these deletions might contain some important positive or negative regulatory elements.

We also tested the effects of androgen and estrogen on 1.06 kb promoter activity. R1881, testosterone (10^{-6}–10^{-8} mol/L), 17β estradiol (10^{-7}–10^{-9} mol/L) and charcoal treated medium were used to treat the LNCaP cells that were cotransfected with pGL3-1.06 kb promoter and pRL-TK. They did not appear to affect the 1.06 kb promoter activity obviously (the results were not shown here). Androgen response element (ARE) and estrogen response element (ERE) were not found within this 1.06 kb fragment using software program (TRANSFAC). Maybe they are located farther upstream of Nkx3.1 gene. There is a TATA box at −30 bp and two putative CAAT boxes at −64 bp and −92 bp relative to the transcription start site. In addition, multiple consensus sequence elements have been identified in the 1.06 kb fragment using the software, such as Nkx2.5 consensus, SRY, MZF1, NF-Y and Sox-5. However, there is no evidence now indicating which, if any, of these sequence elements are functional. It will be important to further identify the cis-elements within the Nkx3.1 promoter that contribute to regulation of the gene transcription functionally. Since Nkx3.1 is specifically expressed in prostate epithelial cells, there may be a tissue-specific enhancer in the upstream regulatory regions or in the intron of the gene need to be identified.

References

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